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Enhanced phosphorylation of the C-terminal domain of RNA polymerase II upon serum stimulation of quiescent cells: possible involvement of MAP kinases

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The largest subunit of RNA polymerase (RNAP) II contains at it C-terminus an unusual domain comprising tandem repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. This C-terminal domain (CTD) can undergo phosphorylation at multiple sites giving rise to a form of the enzyme designated RNAP IIO. The unphosphorylated form is designated RNAP IIA. The largest subunits of RNAPs IIO and IIA are designated IIo and IIa, respectively. In quiescent NIH 3T3 fibroblasts, subunits IIo and IIa are present in comparable amounts. Upon serum stimulation, the amount of subunit IIo increases markedly and remains elevated for several hours. The increase of subunit IIo also occurs in transcription-inhibited cells and, therefore, is not a consequence of serum-activated transcription. This observation suggests that serum stimulation activates a CTD kinase and/or inhibits a CTD phosphatase. This hypothesis is supported by the finding that serum stimulates phosphorylation of a β-galactosidase–CTD fusion protein expressed in these cells. Furthermore, an enhanced CTD kinase activity was discovered in lysates from serum-stimulated fibroblasts and was found to copurify with MAP kinases on a Mono Q column and to bind to anti-MAP kinase antibodies. The idea that MAP kinases phosphorylate the CTD *in vivo* is supported by the observation that subunit IIa, but not subunit IIb which lacks the CTD, is phosphorylated at multiple sites by purified MAP kinase. Consequently, the MAP kinases are a new class of CTD kinases which appear to be involved in the phosphorylation of RNAP II following serum stimulation. This phosphorylation may contribute to the transcriptional activation of serum-stimulated genes.

Key words: C-terminal domain phosphorylation/MAP kinase/RNA polymerase II/serum stimulation

Introduction

RNA polymerase (RNAP) II is a multi-subunit enzyme composed of a dozen different subunits (Sawadogo and Sentenac, 1990; Young, 1991). The largest subunit contains several regions with partial homology to the β′ subunit of *Escherichia coli* RNAP as well as a unique C-terminal domain (CTD). The CTD comprises multiple copies of the consensus repeat Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Although the primary sequence of the consensus repeat is highly conserved, the number of repeats varies from 26–27 in yeast to 52 in mammalian cells (Corden and Ingles, 1992). This domain is essential for cell viability and may play multiple roles in transcription (Corden, 1990; Peterson and Tjian, 1992).

Cells contain two forms of RNAP II, designated RNAPs IIA and IIO (Dahmus, 1981; Dubois *et al.*, 1994a). The CTD of the largest subunit of RNAP IIO is heavily phosphorylated (IIo subunit), primarily on serine and threonine although a subfraction of RNAP IIO is phosphorylated on tyrosine (Baskaran *et al.*, 1993; Greenleaf, 1993; Dahmus, 1994). In contrast, the CTD of the largest subunit of RNAP IIA is not phosphorylated (IIa subunit), although a subfraction contains O-linked GlcNAc (Kelly *et al.*, 1993). RNAPs IIA and IIO appear to have distinct functions in transcription (Corden, 1993; Dahmus, 1994). Indeed, RNAP IIA but not RNAP IIO stably interacts with the preinitiation complex formed on both the adenovirus 2 major late and murine dihydrofolate reductase promoters in the presence of a reconstituted HeLa cell transcription extract (Lu *et al.*, 1991; Chesnut *et al.*, 1992; Kang and Dahmus, 1993). Phosphorylation of the CTD is catalyzed by a CTD kinase, stably associated with the preinitiation complex, at about the same time as transcript initiation. This CTD kinase appears to be brought to the promoter as a component of basal transcription factor, TFIIH (BTF2, rat δ factor and yeast factor b) (Feaver *et al.*, 1991; Lu *et al.*, 1992; Serizawa *et al.*, 1992).

Previous studies have shown that heat-shock markedly affects the phosphorylation state of the CTD (Dubois *et al.*, 1994a). In heat-shocked HeLa cells, the relative amount of subunit IIa decreases whereas the amount of subunit IIo increases. This change in the distribution of IIa/IIo is not a consequence of alterations in the transcription pattern which occur during stress, it appears to involve a stress-activated CTD kinase (Legagneux *et al.*, 1990). In yeast, the relative amount of subunit IIa increases upon entry into the stationary phase (Choder and Young, 1993; M.Choder, personal communication). Our study is aimed at defining other physiological situations which lead to changes in the ratio of IIo:IIa in the hope of better understanding the factors that regulate the interconversion of RNAPs IIA and IIO.

Considerable work has been devoted to the stimulation of quiescent cells by growth factors. Like stress, such a stimulation results in well described changes in the transcription pattern and involves the activation of protein kinase/phosphatase cascades (Ruderman, 1993). Here we show that serum stimulation of quiescent NIH 3T3 mouse fibroblasts leads to an increase in the amount of phosphorylated RNAP II. The increase in RNAP IIO is

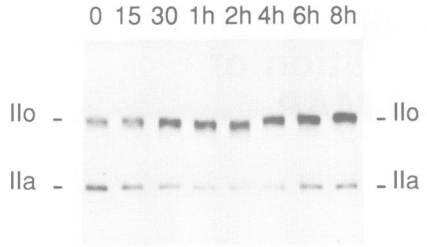

Fig. 1. Transient accumulation of the RNAP subunit IIo after the addition of serum to quiescent 3T3 cells. Whole-cell lysates prepared at 0, 15 or 30 min, 1, 2, 4, 6 or 8 h after serum stimulation were electrophoresed on 5% polyacrylamide–SDS gels and analyzed by Western blot using the POL 3/3 anti-RNAP II monoclonal antibody. Subunits IIa and IIo migrate with apparent molecular weights of 210 and 240 kDa, respectively.

probably a direct result of the activation of CTD kinase activity. Furthermore, the induced CTD kinase appears to be identical to the serum-activated MAP kinases. These results suggest that the enhanced phosphorylation of RNAP II due to serum stimulation might be a direct consequence of the activation of MAP kinases.

Results

Serum stimulation of quiescent NIH 3T3 fibroblasts increases phosphorylation of the RNAP II largest subunit

In NIH 3T3 cells, RNAP subunit IIa has an apparent molecular weight of 210 kDa when analyzed by SDS–PAGE. It is readily distinguishable from the phosphorylated IIo subunit which migrates as a 240 kDa protein. Both subunits IIa and IIo are visualized in Western blots of whole-cell lysates utilizing a monoclonal antibody which recognizes a conserved determinant contained in the largest subunit (Figure 1). RNAPs IIA and IIO are present in comparable amounts in quiescent fibroblasts as indicated by the equivalent intensities of the subunit IIa and IIo bands (Figure 1, time 0). However, upon serum stimulation there is a time-dependent decrease in the IIa band intensity and an increase in the IIo band intensity. These changes became apparent after 20 min of serum stimulation, and the increase of subunit IIo continued beyond 2 h. The IIa band intensity returned to almost the initial level after 6 h of stimulation. Thus, serum stimulation of quiescent cells results in an increase in the relative amount of phosphorylated RNAP II without an apparent change in the total amount of RNAP II present.

Serum-stimulated phosphorylation of RNAP II is not a consequence of changes in transcriptional activity

In an effort to establish whether or not these changes in RNAP II phosphorylation are dependent on transcription, subunits IIa and IIo were monitored in the presence of inhibitors of transcription. First, 5-6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was used as an RNAP II inhibitor (Tamm *et al.*, 1976). Treatment of quiescent NIH 3T3 cells with DRB results in a marked decrease in IIo band intensity (Figure 2A, time 0). A similar observation was made previously with exponentially growing cells (Dubois *et al.*, 1994a,b). Serum stimulation of DRB-treated cells results in an increased intensity of the IIo subunit which was clearly detectable within the first 10–20 min of stimulation, but did not increase much thereafter. It should be noted that a diffuse band also appears just below subunit IIo, indicative of a partially phosphorylated subunit (arrow).

The effect of actinomycin D was also examined. Actinomycin D inhibits transcription at concentrations >0.1 μg/ml. As shown in Figure 2C, 0.5 μg/ml actinomycin D causes an increase in the amount of subunit IIo relative to subunit IIa as described previously (Dubois *et al.*, 1994a,b). The addition of serum to actinomycin-treated cells further enhanced the ratio of IIo:IIa, as is apparent from the decrease in subunit IIa intensity (Figure 2B). Thus, serum stimulation results in an increase in the level of RNAP II phosphorylation even in transcription-inhibited cells. An increase in the level of RNAP IIO could result from either an increase in CTD kinase activity and/or a decrease in CTD phosphatase activity.

Serum stimulation of quiescent cells results in an increased phosphorylation of a CTD–β-galactosidase fusion protein

If indeed the enhanced phosphorylation of the largest RNAP II subunit is independent of transcription, it should occur independent of the ability of RNAP II to interact with the template. Phosphorylation of the largest RNAP II subunit is known to be confined to the CTD (Cadena and Dahmus, 1987). Furthermore, the CTD-containing fusion protein, βYCTD (β-galactosidase fused to the yeast CTD), has been shown to be a good model substrate for CTD kinases (Lee and Greenleaf, 1989). Therefore, an examination of the ability of serum to induce the *in vivo* phosphorylation of the βYCTD protein provides a test of the idea that serum-induced phosphorylation is independent of transcription.

A eukaryotic expression vector was engineered and stably transfected into 3T3 cells as described in Materials and methods. A βYCTD protein-expressing clone was isolated giving rise to the βYC6F cell line. It is unlikely that the βYCTD protein interacts with chromatin; it was readily extracted in the low salt non-denaturing buffer B (data not shown). In whole-cell lysates from quiescent cells, three major bands were recognized by anti-β-galactosidase antibodies (Figure 3A, time 0). These bands were not found when lysates of the parental 3T3 cells were probed (data not shown). The upper band ('a' at time 0) has an apparent M_r of 155 kDa which corresponds to that expected for the full-length unphosphorylated βYCTD fusion protein. Furthermore, this band has a migration identical to that of a minor band detected by the same antibody in lysates from *E.coli* expressing the βYCTD protein (Figure 3B, lane 4) and to the major band recognized by an anti-CTD monoclonal antibody in the same lysates (lane 5). As already reported, the full-length βYCTD protein is produced in low yield in comparison with truncated β-galactosidase fragments, but it is readily identified in lysates from *E.coli* as a 155 kDa band when unphosphorylated (Lee and Greenleaf, 1989). Consequently, the major bands recognized by the anti-β-galactosidase antibodies in βYC6F cell lysates are likely to correspond to incomplete β-galactosidase fragments lacking the yeast CTD portion and the 'a' band corresponds to the unphosphorylated βYCTD protein. Several faint

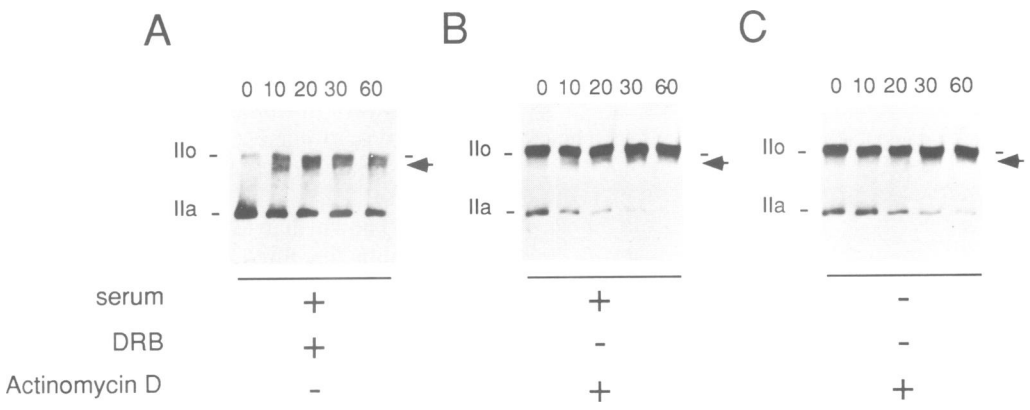

Fig. 2. Accumulation of subunit IIo after addition of serum to transcription-arrested quiescent 3T3 cells. Quiescent cells were preincubated for 30 min with DRB (100 μM) (**A**) or actinomycin D (0.5 μg/ml) (**B** and **C**); the end of the preincubation was taken as time 0. At time 0, serum (20%) was added (A and B) or not (C) to the transcription-arrested cells which were lysed at various times thereafter. Times are indicated in minutes. The arrows indicate the position of a phosphorylated subunit of slightly greater mobility than the usual IIo subunit. Whole-cell lysates were analyzed by Western blot as described in the legend to Figure 1.

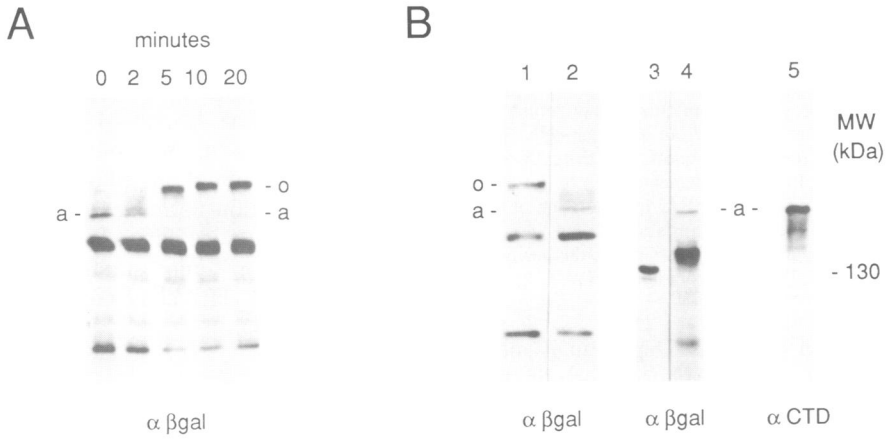

Fig. 3. Phosphorylation of the βYCTD protein upon stimulation of quiescent βYC6F cells. In (**A**) cells expressing the βYCTD protein were lysed at the indicated times (in minutes) after serum addition and whole-cell lysates analyzed by Western blot using an anti-β-galactosidase monoclonal antibody (α βgal). (**B**) The characterization of the βYCTD protein expressed in serum-stimulated βYC6F cells (lanes 1 and 2) or in *E.coli* (lanes 3–5). Serum-stimulated βYC6F cells were lysed in buffer A and 60 μl of clarified lysates were incubated for a further 30 min at 30°C in the absence (lane 1) or presence of 260 U of bacterial alkaline phosphatase (lane 2). β-Galactosidase (lane 3) or βYCTD protein (lanes 4 and 5) made in *E.coli* were purified by affinity chromatography. 'a' and 'o' indicate the unphosphorylated and the phosphorylated full-length βYCTD proteins, respectively. Lysates and samples were analyzed by Western blot using an anti-β-galactosidase monoclonal antibody (α βgal) (lanes 1–4) or the 8WG16 anti-CTD monoclonal antibody (α CTD) (lane 5).

bands with an M_r between 155 and 165 kDa are also apparent.

As shown in Figure 3A, within 5 min of serum stimulation of quiescent fibroblasts expressing the βYCTD protein, the 155 kDa band ('a') disappeared while the intensity of the 165 kDa band ('o') increased. The 165 kDa probably corresponds to the 155 kDa protein phosphorylated on the CTD, since in extracts from serum-stimulated cells treated with alkaline phosphatase the 165 kDa band was converted to the 155 kDa band (Figure 3B, compare lane 1 with lane 2). These results suggest that serum stimulation results in the rapid activation of a transcription-independent CTD kinase.

Increased CTD kinase activities in lysates from serum-stimulated cells

In an effort to understand the mechanism responsible for the enhanced CTD phosphorylation in serum-stimulated cells, cell lysates were examined for a serum-inducible CTD kinase activity. Initially, crude low salt cell lysates were incubated with [γ-^{32}P]ATP and the labeled proteins were analyzed by autoradiography after SDS−PAGE (Figure 4). Little difference was observed between quiescent and serum-stimulated cells. However, upon addition of the CTD-containing fusion protein βYCTD, produced in *E.coli* and partially purified on a β-galactosidase affinity column, enhanced CTD kinase activity was apparent in extracts from serum-stimulated cells. The addition of βYCTD resulted in the incorporation of ^{32}P into the full-length 155 kDa βYCTD in the presence of extracts from serum-stimulated cells, but no incorporation in the presence of extracts from quiescent cells (Figure 4). A diffuse band of ^{32}P incorporation was also observed below the βYCTD protein band, most likely due to phosphorylation of incomplete or partially degraded βYCTD molecules (also detected with the anti-CTD antibody as shown in Figure 3B, lane 5). A similar observation was made by Lee and Greenleaf (1989, 1991) using the same substrate and the CTK1 kinase. Since purified β-galactosidase is not labeled under these conditions (data

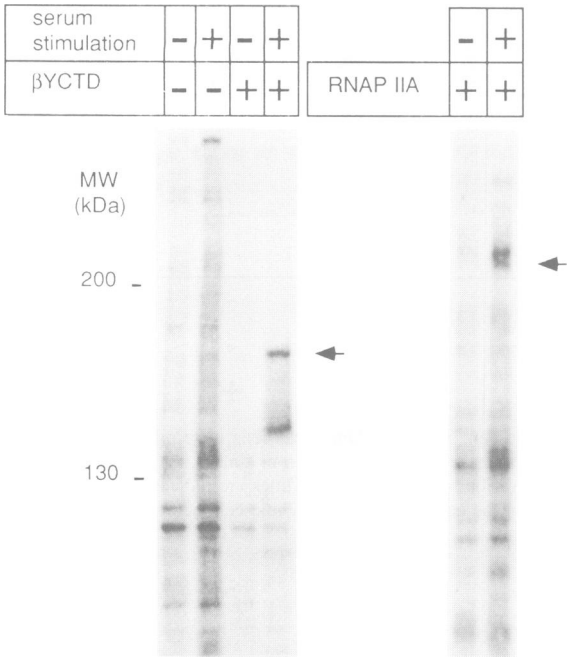

Fig. 4. Assay for CTD kinases in cytosols from quiescent or serum-stimulated 3T3 cells. Cytosols were prepared from quiescent cells (−) or 30 min after the addition of serum (+). Aliquots from the cytosols were incubated at 30°C with [γ-^{32}P]ATP supplemented (+) or not (−) with either βYCTD protein or purified RNAP IIA. Incorporation of ^{32}P into proteins was detected after electrophoresis in 5% polyacrylamide gels and autoradiography. Arrows indicate the position of phosphorylated full-length βYCTD protein or subunit IIa.

not shown), the labeling of the βYCTD protein is most probably the consequence of phosphorylation of the CTD moiety.

Upon the addition of RNAP IIA to these same lysates, increased labeling of a band corresponding in mobility to subunit IIa was observed (Figure 4). Such labeling was not observed in lysates from quiescent cells. Serum stimulation of quiescent cells, therefore, appears to activate a CTD kinase able to phosphorylate the βYCTD protein and the largest subunit of RNAP II.

Serum stimulation results in a rapid transient activation of CTD kinase activity which correlates in time with phosphorylation of MAP kinases

To characterize the CTD kinase activity stimulated by serum factors, cell lysates were prepared after various times of serum stimulation and kinase activity determined in the presence of either βYCTD protein or RNAP IIA. Incorporation of ^{32}P was clearly observed in βYCTD protein with lysates of cells stimulated by serum for 5 min (Figure 5A), while little incorporation could be detected with lysates from quiescent cells. A gradual decrease of βYCTD labeling occurred when the cells were stimulated for >20 min. Similarly, when RNAP IIA was added to lysates from serum-stimulated cells, an increased incorporation of ^{32}P was clearly observed in a protein comigrating with subunit IIa. Consequently, CTD kinase activity increased within minutes after serum stimulation and decreased gradually after 20 min.

MAP kinases are major protein kinases rapidly activated upon serum stimulation of quiescent cells (Blenis, 1993; Nishida and Gotoh, 1993). The CTD consensus repeat contains two MAP kinase motifs (bold): Tyr-**Ser-Pro**-Thr-**Ser-Pro**-Ser. Furthermore, purified MAP kinases have been shown to phosphorylate a peptide made of four repetitions of this motif (Clark-Lewis *et al.*, 1991). Therefore, it was of interest to establish the correlation between serum-stimulated MAP kinases and the activation of CTD kinase(s).

It is known that MAP kinases are activated by phosphorylation on threonine and tyrosine residues (Maller, 1991). This phosphorylation results in a slight decrease in electrophoretic mobility which can be observed readily on Western blots. In lysates from quiescent cells, anti-MAP kinase antibodies recognize two major bands at 42 and 44 kDa that probably correspond to the p42^{mapk} and p44^{mapk} proteins (ERK2 and ERK1 gene products), respectively (Figure 5B). After 5 min of serum stimulation, these bands disappeared and were replaced by two bands with reduced electrophoretic mobilities. Such a mobility shift is the consequence of phosphorylation and hence is an indicator of activation. The nearly complete mobility shift within 5 min indicates that activation occurs within minutes after serum stimulation, whereas inactivation is initiated at ~20 min as indicated by both the gradual appearance of the 42 and 44 kDa higher mobility bands and a reduction in the amount of the phosphorylated species. Such a rapid phosphorylation followed by a slow dephosphorylation correlates with the serum stimulation of CTD kinase activity described above. Thus, the time course for serum stimulation of CTD kinases in quiescent NIH 3T3 cells correlates with MAP kinase phosphorylation.

Serum-stimulated protein kinases that phosphorylate βYCTD and RNA polymerase IIA copurify with MAP kinases

To characterize further the CTD kinases, cytosolic lysates were prepared from quiescent and serum-stimulated cells and fractionated by anion-exchange chromatography. Protein kinase activity in the fractions was analyzed using RNAP IIA, βYCTD or myelin basic protein (MBP) as exogenous substrates.

The distribution of CTD kinase activity in Mono Q fractions utilizing RNAP IIA as substrate is shown in Figure 6A. Incorporation of ^{32}P into subunit IIa was quantitated with a PhosphorImager and is shown in Figure 6C. CTD kinase activity eluted in two peaks at ~165 (fractions 60 and 61) and ~220 mM NaCl (fractions 71 and 72). CTD kinase activity was not detected in fractions eluting at <300 mM NaCl when the column was loaded with a lysate from quiescent cells (data not shown). The total CTD kinase activity which eluted from the Mono Q column within both peaks was found to be twice the total activity of the crude extract. Such a discrepancy might be explained by the presence of inhibitors such as competing phosphatase activities in the crude extracts. Nevertheless, this observation suggests that the majority of serum-induced CTD kinase activity present in the crude extracts elutes from Mono Q at low ionic strength. Similar results were obtained when the βYCTD protein was used as a substrate. The amount of ^{32}P incorporation into the full-length βYCTD protein was determined with a PhosphorImager and is shown in Figure 6D. The peaks of kinase

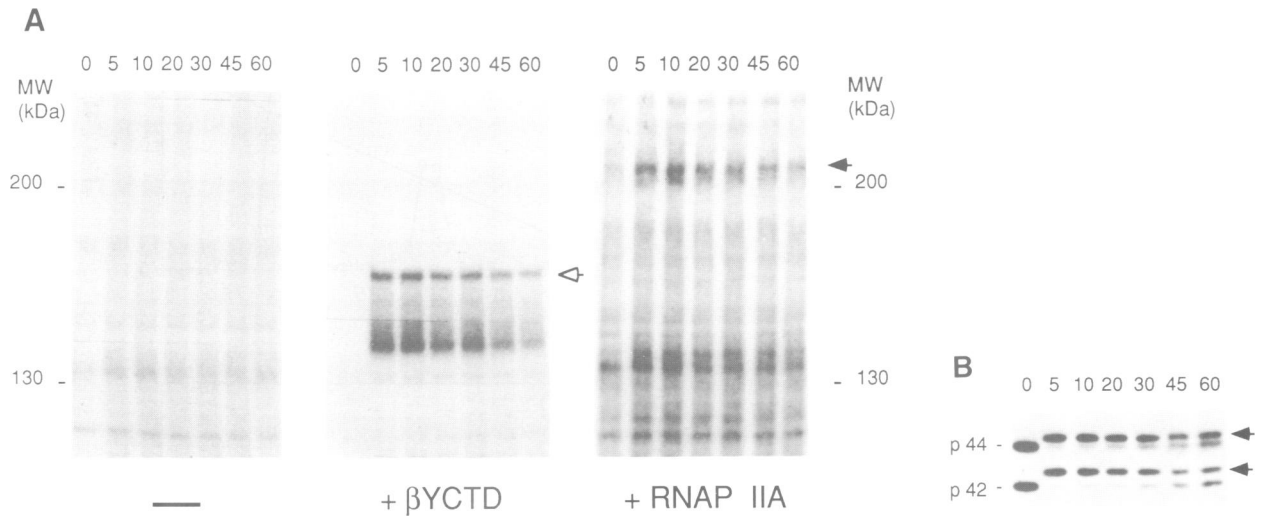

Fig. 5. Time course for serum stimulation of CTD kinase and MAP kinase activities. Quiescent 3T3 cells (0) were stimulated by serum for 5–60 min. (**A**) Protein kinase activity was followed in corresponding cytosols utilizing βYCTD or RNAP IIA as substrate in the presence of [γ-^{32}P]ATP. The open arrow indicates the position of βYCTD and the closed arrow indicates the position of RNAP subunit IIa. (**B**) MAP kinase phosphorylation after serum stimulation of 3T3 cells. Whole-cell lysates from quiescent (0) or serum-stimulated cells (5–60 min) were analyzed by Western blot using anti-MAPK antibodies after electrophoresis on 10% polyacrylamide–SDS gels. The arrows indicate the position of phosphorylated p42^{mapk} and p44^{mapk}.

activity utilizing βYCTD and RNAP II are coincident (compare Figure 6C with D).

To establish a correlation between the serum-activated kinases responsible for the phosphorylation of the CTD-containing proteins and MAP kinases, Mono Q fractions were tested for the presence of MAP kinases by Western blot. When cytosolic lysates from quiescent cells were chromatographed on Mono Q, p42^{mapk} eluted between fractions 51 and 58 with a maximum concentration in fraction 54, whereas p44^{mapk} eluted between fractions 59 and 64 with a peak in fractions 61 and 62 (Figure 6B). Note that the non-phosphorylated forms of p42^{mapk} and p44^{mapk} migrate as single peaks. When lysates from serum-stimulated cells were used, a large portion of both p42^{mapk} and p44^{mapk} proteins eluted at higher ionic strengths, presumably the consequence of phosphorylation. Although a small amount of unphosphorylated p42^{mapk} (fractions 51–56) eluted early as found previously, the bulk of phosphorylated p42^{mapk} eluted in two peaks at higher ionic strength. The first peak (fractions 53–56) overlapped with the unphosphorylated p42^{mapk} peak, whereas the second peak (fractions 57–63) contained only phosphorylated p42^{mapk} with a maximum concentration in fractions 60 and 61. In the case of p44^{mapk}, the first peak was observed between fractions 60 and 64 and contained a mixture of both the unphosphorylated and phosphorylated proteins (fractions 62 and 63). The second peak (fractions 70–73) contained only the phosphorylated protein.

Fractions containing active MAP kinases were identified by their ability to phosphorylate MBP (Figure 6E). Two major peaks of MBP kinase activity eluted from the Mono Q column loaded with the serum-stimulated cell lysate. These peaks corresponded to the fractions which had maximal CTD kinase activity as determined by the phosphorylation of RNAP II or the βYCTD protein (compare Figure 6C and D with E). The first peak of kinase activity correlates with phosphorylated p42^{mapk} protein (fractions 58–62), whereas the second peak of kinase activity correlates with phosphorylated p44^{mapk} protein (fractions 70–73). A small peak of MBP kinase activity was also detected in fractions 55 and 56 and might be due to the presence in these fractions of a small amount of phosphorylated p42^{mapk} protein. A minor peak of CTD kinase activity was detected in these same fractions. Such MBP kinase activities were not found in the elution profile after loading a lysate from quiescent cell lysates (data not shown). These results establish that the serum-stimulated CTD kinase and MAP kinase activities coelute from a Mono Q column as two major peaks which correspond to phosphorylated p42^{mapk} and p44^{mapk}.

Serum-stimulated CTD kinases are immunoprecipitated with anti-MAPK antibodies

The copurification of MAP kinases and CTD kinases suggests that these kinases might be identical. To test this idea further, the ability of MAP kinase-specific antibodies to immunoprecipitate the serum-stimulated CTD kinase(s) from cell lysates was examined. As shown in Figure 7A, a CTD kinase able to phosphorylate the βYCTD protein was adsorbed on protein A when serum-stimulated lysates were incubated with an anti-MAPK antiserum. In lysates from quiescent cells, no detectable CTD kinase activity was retained on the protein A beads in the presence of the same anti-MAPK antiserum. Furthermore, no activity was retained in either lysate in the presence of the 12CA5 monoclonal (α Tag) and rabbit anti-mouse IgG antiserum (α mouse).

To demonstrate that MAP kinase itself and not an associated kinase was a CTD kinase, the immunoprecipitation assay was repeated using epitope-tagged recombinant MAP kinases which carry or not an inactivating point mutation. First, NIH 3T3 fibroblasts were transfected with pCMV/HA, an expression vector coding for an epitope-tagged hamster p44^{mapk} (Meloche *et al.*, 1992). The tag and hence the modified MAP kinase are recognized

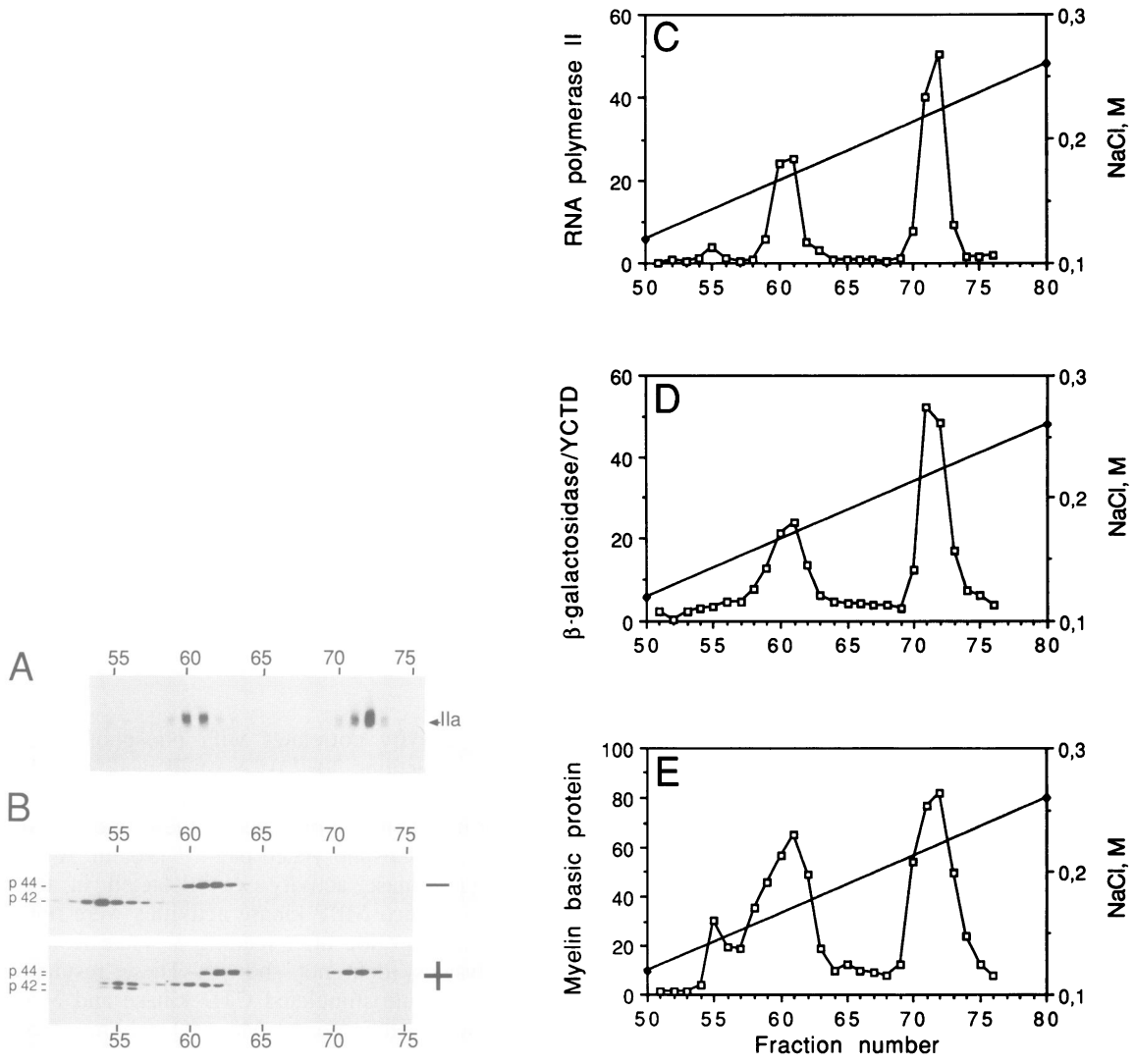

Fig. 6. Chromatography of CTD kinase(s) and MAP kinases on Mono Q. Cytosolic extracts of quiescent cells or cells stimulated for 15 min by serum were fractionated by chromatography on Mono Q as described in Materials and methods. Aliquots of Mono Q fractions were incubated with purified RNAP IIA, βYCTD protein or MBP and [γ-^{32}P]ATP. (**A**) The incorporation of ^{32}P into subunit IIa in the presence of Mono Q fractions from a column loaded with cytosols from serum-stimulated cells. (**B**) Western blots for p42^{mapk} (p42) and p44^{mapk} (p44) in fractions eluted from the Mono Q column loaded with cytosols from either quiescent (−) or serum-stimulated (+) cells, respectively. (**C**) A quantitation of the phosphate incorporated into RNAP subunit IIa in the presence of Mono Q fractions from a column loaded with cytosol from serum-stimulated cells, whereas (**D**) shows the incorporation into βYCTD protein. (**E**) A quantitation of MAP kinase activity in these same fractions utilizing MBP as substrate. The radioactive labeling of the substrates (arbitrary units) was plotted against fraction number. The oblique line represents the NaCl concentration gradient. No significant labeling was observed in the presence of aliquots of fractions <54 or >76.

by the monoclonal α Tag. As shown in Figure 7A, this monoclonal antibody immunoprecipitated a CTD kinase from lysates of serum-stimulated transfected cells but not from lysates of quiescent transfected cells. However, this antibody did not retain any CTD kinase activity from lysates of untransfected cells. Next, the cells were transfected with pCMV/HA-T192A which codes for a hamster p44^{mapk} variant (TA) carrying a point mutation (threonine 192 was replaced by an alanine). This variant has no MAP kinase activity (Pagès *et al.*, 1993). Indeed, in this case no CTD kinase activity was immunoadsorbed on protein A with the monoclonal α Tag (Figure 7A). Lysates from cells transfected with either wild-type or mutant expression vector contained similar amounts of recombinant protein (Figure 7B). These experiments provide strong support for the idea that the serum-stimulated MAP kinases function as CTD kinases, and hence the enhanced phosphorylation of RNAP II that results from serum stimulation is a direct consequence of the activation of MAP kinases.

A purified active MAP kinase phosphorylates the βYCTD protein and the CTD of RNAP II

The CTD kinase assays described above (Figures 4 and 5), in which an ATP concentration of 0.1 mM was used, were optimized for sensitivity. In these assays, the phosphorylated substrates, βYCTD protein and RNAP largest subunit, showed the same electrophoretic migration as their unphosphorylated precursors. This is most probably a consequence of low levels of phosphorylation in the presence of limiting nucleotide substrate. In contrast, phosphorylation of the CTD in the cell occurs on multiple sites and results in a marked reduction in the electrophoretic mobility of the largest RNAP II subunit. A similar reduction in the electrophoretic mobility of the βYCTD

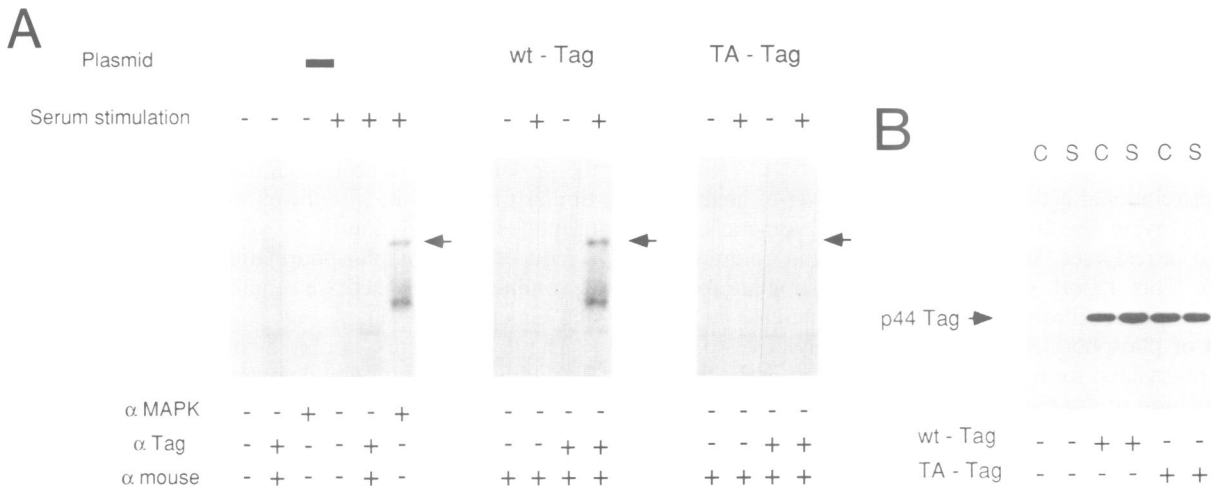

Fig. 7. Immunochemical characterization of the serum-stimulated CTD kinase. (**A**) The immunoprecipitation of CTD kinase with an anti-MAP kinase antiserum (α MAPK) and with a monoclonal antibody directed against an epitope-tagged MAP kinase (α Tag). The serum-stimulated CTD kinase from lysates of untransfected 3T3 cells (−) was immunoprecipitated with the anti-ERK1 (α MAPK) antiserum. The 12CA5 monoclonal antibody (α Tag) was used as a control in the left panel. The serum-stimulated CTD kinase from lysates of cells expressing either an active [transfection with the pCMV/HA plasmid (wt-Tag)] or an inactive epitope-tagged recombinant MAP kinase [transfection with the pCMV/HA-T192A plasmid (TA-Tag)] was immunoprecipitated with the 12CA5 anti-Tag monoclonal antibody. The arrow indicates the position of full-length phosphorylated βYCTD protein. (**B**) The presence of epitope-tagged proteins in cell lysates as determined by Western blot using the 12CA5 anti-Tag monoclonal antibody. C and S refer to control and serum-induced samples, respectively.

protein was shown above (Figure 3; see also Lee and Greenleaf, 1989). Therefore, it was of interest to determine if purified MAP kinase could catalyze this same mobility shift in the RNAP II largest subunit and the βYCTD protein. The βYCTD protein was incubated at 30°C with purified p44^{mpk} from sea star in the presence of physiological concentrations of ATP (4 mM) and analyzed by Western blot with a monoclonal anti-CTD antibody. A gradual decrease in electrophoretic mobility of βYCTD protein was observed as a function of time which plateaued after 60 min of incubation (Figure 8, lanes 1–3). This change in electrophoretic mobility was similar to that observed as a consequence of *in vivo* phosphorylation (compare lane 3 with lane 5). A similar experiment was performed with Mono Q fractions and the mobility shift was observed only with fractions containing active MAP/CTD kinase activities (data not shown).

The ability of MAP kinase to phosphorylate the largest subunit of purified RNAP IIA (lanes 9–11) and RNAP IIB (lanes 6–8) was also examined. Reactions were probed with a monoclonal antibody directed against a conserved region of the largest subunit located outside the CTD. A decrease in the electrophoretic mobility of the IIa subunit was observed after 20 min in the presence of 4 mM ATP. In contrast, incubation of RNAP IIB with MAP kinase and ATP did not affect the electrophoretic mobility of subunit IIb (lanes 6–8). The observation that subunit IIb, which lacks the CTD, is not phosphorylated by MAP kinase indicates that phosphorylation of subunit IIa is confined to the CTD.

The electrophoretic mobility of subunit IIo phosphorylated *in vitro* by MAP kinase is greater than that of *in vivo*-phosphorylated subunit IIo (compare lane 11 with lane 12). Although the MAP kinase remained fully active after 60 min, only a small additional decrease in electrophoretic mobility was observed upon continued incubation (data not shown). The position of *in vitro* MAP-phosphorylated subunit IIo corresponds in mobility to a diffuse band

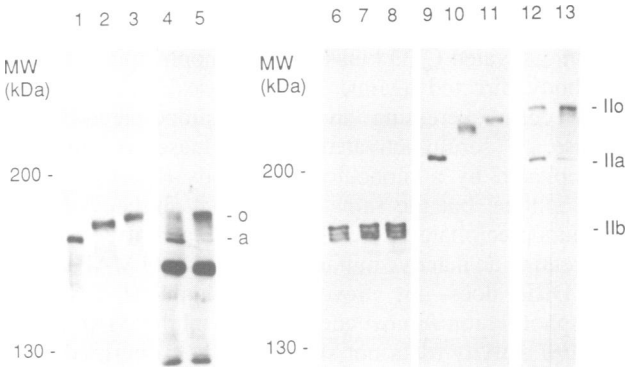

Fig. 8. Phosphorylation of βYCTD protein and RNAP IIA with purified MAP kinase. Affinity-purified βYCTD protein was incubated with purified active sea star p44^{mpk} and 4 mM ATP at 30°C for 0, 20 or 60 min for lanes 1–3, respectively. The *in vitro*-phosphorylated proteins were detected by Western blot using the 8WG16 monoclonal antibody directed against the CTD. Lysates from quiescent (lane 4) or serum-stimulated (20 min) βYC6F cells (lane 5) expressing the βYCTD protein were coelectrophoresed and analyzed by Western blot with an anti-β-galactosidase monoclonal antibody. Bands corresponding to the phosphorylated ('o') and unphosphorylated ('a') full-length βYCTD proteins are indicated. RNAP IIB (lanes 6–8) and RNAP IIA (lanes 9–11) were incubated with purified p44^{mpk} and 4 mM ATP at 30°C for 0, 20 or 60 min, respectively. The *in vitro*-phosphorylated RNAP largest subunits were detected by Western blot using the POL 3/3 monoclonal antibody. Lysates from quiescent (lane 12) or serum-stimulated (40 min) cells (lane 13) were coelectrophoresed and analyzed with the POL 3/3 monoclonal antibody; bands corresponding to the subunit lacking the CTD (IIb) and to the unphosphorylated (IIa) and phosphorylated (IIo) subunits are indicated. The positions of myosin heavy chain (200 kDa) and β-galactosidase (130 kDa) are indicated on the left.

appearing just below subunit IIo upon serum stimulation (compare lane 11 with lane 13). The presence of such species migrating slightly faster than the IIo subunit was clearly visible in the lysates from serum-stimulated cells exposed to transcription inhibitors (Figure 2). Thus, purified p44^{mpk} phosphorylates the CTD of the largest

RNAP II subunit at multiple sites and gives rise to a subunit with properties similar to that generated *in vivo* upon serum stimulation.

Discussion

The transcriptional activation of a specific set of genes is a primary event involved in the release of somatic cells from G_0 arrest (see Wick *et al.*, 1994 and references therein). This report shows that serum stimulation of quiescent mouse fibroblasts results in an increase in the amount of phosphorylated RNAP II (IIO) relative to the unphosphorylated form (IIA). This increase resulted from the activation of a transcription-independent CTD kinase. The release of somatic cells from G_0 arrest by serum or growth factors involves the activation of MAP kinases (Ruderman, 1993). MAP kinases are central components of a cascade of protein kinases involved in a wide variety of signal transduction pathways (Blenis, 1993; Crews and Erikson, 1993; Davis, 1993; Neiman, 1993; Nishida and Gotoh, 1993). The following observations suggest that the serum-activated MAP kinases might phosphorylate *in vivo* the RNAP II largest subunit: (i) the activation of CTD kinase correlates with the activation of MAP kinase; (ii) serum-activated CTD kinases are found in cell lysates and copurify with the serum-activated MAP kinases; (iii) a serum-activated CTD kinase is immunoprecipitated by an antibody directed against MAP kinases; (iv) in lysates from cells expressing an active epitope-tagged MAP kinase, a serum-activated CTD kinase is immunoprecipitated by a monoclonal antibody directed against this epitope, but the same monoclonal antibody did not immunoprecipitate a CTD kinase activity in cells expressing an inactive mutant epitope-tagged MAP kinase; (v) DRB does not prevent serum-induced RNAP II phosphorylation *in vivo* and does not inhibit MAP kinase *in vitro* activity (data not shown); and (vi) purified MAP kinase phosphorylates the CTD of RNAP subunit IIa at multiple sites resulting in the characteristic shift in electrophoretic mobility.

MAP kinases can now be added to the list of numerous CTD kinases already identified and purified to varying degrees from eukaryotic cells (for a review see Dahmus, 1994). CTD kinases have been characterized by their ability to catalyze phosphorylation of synthetic peptides corresponding to the consensus repeat, CTD-containing fusion proteins or the largest subunit of RNAP II. Most CTD kinases appear to be serine/threonine kinases, although tyrosine phosphorylation of the CTD has been demonstrated recently (Baskaran *et al.*, 1993). Although it remains to be established if these kinases phosphorylate the CTD *in vivo*, CTK1 kinase from yeast (Lee and Greenleaf, 1991) has been shown to play a role in the *in vivo* phosphorylation of RNAP II. It has not yet been possible to establish the level of RNAP II phosphorylation in the absence of MAP kinase. However, the finding that the activation of MAP kinase leads to increased phosphorylation of a β-galactosidase−CTD fusion protein, in addition to the results summarized above, supports the idea that MAP kinases phosphorylate RNAP II *in vivo*.

The purified $p44^{mpk}$ does not appear to fully phosphorylate subunit IIa as indicated by the increased electrophoretic mobility relative to subunit IIo present in quiescent cells. However, since the relationship between mobility shift and stoichiometry of phosphorylation is complex, mobility differences are not a reliable indicator of different levels of phosphorylation. High-resolution electrophoresis of lysates from stimulated cells revealed the presence of a largest subunit species with a migration similar to that of the *in vitro*-phosphorylated subunit. It is of interest that deletion of the yeast CTK1 gene results in a reduced level of phosphorylation of yeast RNAP II as determined by the increased mobility of subunit IIo (Lee and Greenleaf, 1991).

The CTD appears to play a role in the recruitment of RNAP to the preinitiation complex, whereas phosphorylation of the CTD is thought to be involved in promoter clearance (for a review see Dahmus, 1994). This is supported by the observation that RNAP IIA preferentially assembles into preinitiation complexes, relative to RNAP IIO, on both the adenovirus 2 major late and murine dihydrofolate reductase promoters. In contrast, transcript elongation is catalyzed by RNAP IIO. Consequently, phosphorylation of the CTD by a promoter-associated protein kinase(s) appears to be involved directly in mediating the interaction of RNAP II with the preinitiation complex. The enhanced phosphorylation of RNAP II assembled into preinitiation complexes should facilitate promoter clearance and hence stimulate transcription. Conversely, the enhanced phosphorylation of free RNAP II would decrease the amount of RNAP IIA available for association with the promoter and could, in principle, inhibit transcription. Consequently, the observation that serum stimulation results in both the enhanced phosphorylation of RNAP II and transcriptional activation appears inconsistent with the model proposed above. However, it is impossible to know if the increased phosphorylation of RNAP II following serum stimulation is sufficient to reduce the amount of RNAP IIA to a point where it is limiting the rate of preinitiation complex formation. Furthermore, it is likely that the CTD functions at additional steps in the transcription process, including elongation and termination (Allison *et al.*, 1988; Corden, 1990; Dahmus, 1994). Indeed, drugs that interfere with CTD dephosphorylation affect transcript elongation (Fraser *et al.*, 1978; Zandomeni *et al.*, 1986; Chodosh *et al.*, 1989; Meulia *et al.*, 1993; Dubois *et al.*, 1994b). Consequently, phosphorylation of the CTD at different steps may be catalyzed by specific CTD kinases that differentially affect RNAP II activity. This idea is consistent with the multiplicity of CTD kinases that have been reported. Therefore, the primary effect of increased RNAP II phosphorylation may be to stimulate transcript elongation.

The regulation of transcript elongation may emerge as an important control mechanism for stress- and mitogen-inducible gene expression (Rougvie and Lis, 1990; O'Brien and Lis, 1991; Krumm *et al.*, 1993). In the non-induced state, RNAP IIA arrests or pauses on the heat-shock genes after initiating transcription (Giardina and Lis, 1993; Weeks *et al.*, 1993). In the induced state, passage of the paused polymerase into the elongation stage coincides with phosphorylation of the CTD (O'Brien *et al.*, 1994). Similar observations were also made with non-heat-shock genes where the polymerase pauses at the 5′ end. Heat-shock stress also activates CTD kinases which copurify

with the stress-activated MAP kinases (Legagneux *et al.*, 1990; Dubois and Bensaude, 1993; Dubois *et al.*, 1994a; unpublished data). Therefore, activation of one or more CTD kinases might contribute to the rapid phosphorylation of paused RNAP II thereby participating in the activation of transcript elongation on inducible genes.

The CTD has also been shown to mediate the action of acidic transcriptional activators (Scafe *et al.*, 1990). The addition of negative charges to the activation domain of the GAL4 transcriptional activator can partially compensate for a reduction in the number of heptad repeats within the CTD (Allison and Ingles, 1989). Conversely, the deletion of negative charges in the activation domain of GAL4 can be partially compensated for by an increase in the number of heptad repeats to the CTD. The CTD itself may function as a transcriptional activator as indicated by the observation that it activates transcription when fused to the GAL4 DNA binding domain (Seipel *et al.*, 1993). As suggested, the CTD could act as a 'portable' acidic domain for transcriptional activation.

Phosphorylation of the transcriptional activators is involved in the regulation of serum-activated gene expression. Phosphorylation of factor $p62^{TCF}$/Elk-1 by MAP kinases stimulates ternary complex formation at the c-fos promoter (Gille *et al.*, 1992; Treisman, 1992; Janknecht *et al.*, 1993). Phosphorylation of c-jun on serines 63 and 73 occurs during serum stimulation of quiescent cells and stimulates c-jun activity. These serines might be phosphorylated by MAP kinases (Woodgett, 1991; Chou *et al.*, 1992; Kamada *et al.*, 1994) and by JNK1, a stress-inducible kinase related to MAP kinase (Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994). The major site of phosphorylation of c-myc *in vivo* is also a site for MAP kinase phosphorylation *in vitro* and is associated with enhanced transactivation of gene expression (Gupta *et al.*, 1993). Thus, increased phosphorylation of transcription factors and of the CTD by MAP kinases might act synergistically to increase the transcriptional activity of serum-inducible genes.

The ability of heat-shock and serum stimulation to influence the conversion of RNAP IIA to IIO is striking. However, additional studies are necessary to establish if MAP kinases preferentially phosphorylate RNAP II at a discrete step in the transcription cycle and to establish the direct physiological consequences of that phosphorylation.

Materials and methods

Plasmids

Plasmid pCH-βYCTD was constructed by ligation of the 6.3 kb *Eco*RI fragment of plasmid pCH110 (Hall *et al.*, 1983) with the 1.1 kb *Eco*RI fragment of pβYC (Lee and Greenleaf, 1989) to express a β-galactosidase−yeast CTD (βYCTD) fusion protein in mammalian cells. Plasmid pCH110 contains the eukaryotic promoter and the β-galactosidase moiety, whereas plasmid pβYC contains the yeast CTD. Plasmids with the correct insert orientation were selected. Plasmid pSVTKneo (Nicolas and Berg, 1983) confers resistance to neomycin.

Cells

Mouse NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS). Cells were rendered quiescent by maintaining subconfluent cultures for 16 h in serum-free medium. Quiescent cells were serum-stimulated by the addition of FCS (20% final concentration).

The βYC 6F cell line expressed the βYCTD fusion protein. It was derived from NIH 3T3 mouse fibroblasts cotransfected with pCH-βYCTD and pSVTKneo by the standard calcium phosphate procedure and selected first in G418 medium. The growing colonies of transfected fibroblasts were next stained for β-galactosidase expression (Sanes *et al.*, 1986). After amplification of a colony giving positive blue cells, two sequential elutriations in the presence of fluorescein di-β-D-galactopyranoside were performed to establish a cell line that stably and homogeneously shows β-galactosidase activity (Nolan *et al.*, 1988).

Partial purification of βYCTD protein

The βYCTD protein was affinity purified (Ullmann, 1984). A culture of *E.coli* transformed with plasmid pβYC was grown to 0.8 OD and induced with 1 mM isopropylthio-β-D-galactoside for 3 h (Lee and Greenleaf, 1989). The bacterial pellet from 1 l of culture was sonicated in 5 ml of buffer A (20 mM Tris, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 1 mM 2-mercaptoethanol). The lysate was centrifuged at 10 000 *g* for 10 min and applied to a 1 ml column of *p*-aminobenzyl 1-thio-β-D-galactopyranoside-agarose (Sigma) equilibrated with the same buffer. After extensive washing of the column (10 ml of buffer A), the βYCTD protein was eluted with 1 ml of sodium borate (100 mM, pH 10) containing 1 mM 2-mercaptoethanol. The eluted fractions were dialyzed against buffer A without Triton X-100.

Western blot analysis

Cells were rapidly washed with ice-chilled PBS and lysed in Laemmli buffer (60 mM Tris−HCl, pH 6.8, 2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.002% bromophenol blue). These whole-cell lysates were heated for 10 min at 90°C and electrophoresed in 5% polyacrylamide−SDS gels. Proteins in the gel were electrotransferred onto nitrocellulose membranes (0.45 μm) (Schleicher & Schuell). The membranes were blocked in Tris-buffered saline (20 mM Tris−HCl, pH 7.6, 137 mM NaCl, 0.2% Tween 20) containing 5% non-fat dry milk and incubated with a 1/5000 dilution of the first antibody in blocking solution for 1 h. They were then washed in Tris-buffered saline and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (1/7500) in blocking solution for 1 h. The antigen−antibody complexes were visualized using chemiluminescence (Amersham ECL system).

The monoclonal antibody POL 3/3 recognizes the RNAP II largest subunit at an evolutionarily conserved epitope located outside the CTD and was kindly provided by E.K.Bautz (Krämer *et al.*, 1980). The monoclonal antibody 8WG16 recognizes specifically the CTD and was kindly provided by N.Thompson (Thompson *et al.*, 1989). The monoclonal antibody 12CA5 (Babco, Emeryville, CA) recognizes an epitope from the influenza hemagglutinin HA1 protein (HAP) used to tag the recombinant hamster $p44^{mapk}$ protein (Meloche *et al.*, 1992; Pagès *et al.*, 1993). Both the rabbit anti-MAP kinase antiserum (Wang *et al.*, 1992) and the rabbit anti-ERK1 antiserum (Santa Cruz Biotechnology) recognize both the $p42^{mapk}$ and $p44^{mapk}$ proteins (Dubois and Bensaude, 1993). Anti β-galactosidase monoclonal antibody, horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG were obtained from Promega.

Preparation of cytosolic lysates

NIH 3T3 cells were rendered quiescent after 16 h of serum starvation and stimulated with FCS (20% final concentration). The cells were rinsed twice with chilled PBS and lysed in buffer B (20 mM sodium glycerophosphate, pH 7.3, 1 mM EGTA, 1 mM EDTA, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.5% Nonidet P40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM Na_3VO_4), homogenized with a Dounce homogenizer and centrifuged at 10 000 *g* for 10 min at 4°C. The clarified supernatants (10 mg of protein/ml) were aliquoted and stored at −80°C.

FPLC Mono Q chromatography

A total of 5 ml of clarified cytosolic lysates obtained from 10^8 cells were filtered and applied to an HR 5/5 Mono Q column in buffer C (20 mM sodium glycerophosphate, pH 7.3, 1 mM EGTA, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 1 mM Na_3VO_4) at a flow rate of 1 ml/min. The column was developed with a 30 ml linear 0–300 mM NaCl gradient in buffer C and 0.5 ml fractions were collected. The fractions were aliquoted and stored at −80°C.

Protein kinase assays

Aliquots (4 μl) of cytosolic lysates or of fractions eluted from the Mono Q column were mixed on ice with 10 μl of reagent mix made of buffer C containing non-radioactive ATP at a final concentration of 0.1 mM, 0.5 μCi of [γ-^{32}P]ATP (Amersham Corp.) and the appropriate exogenous

substrate: MBP (2.5 μg; Sigma), RNAPs IIA or IIB (9×10^{-3} U; Kim and Dahmus, 1988) or βYCTD protein (10 μg of affinity-purified protein mixture). RNAP IIB is identical to RNAP IIA except that the largest subunit (IIb) lacks the CTD (Cadena and Dahmus, 1987). Reactions were incubated for 30 min at 30°C and were arrested by the addition of 15 μl of 2× Laemmli buffer. MBP was resolved in 15% polyacrylamide−SDS gels and migrated as a 23 kDa protein. The βYCTD protein and the largest subunit of RNAP II were resolved in 5% polyacrylamide−SDS gels. The gels were fixed, dried and autoradiographed or analyzed with a PhosphorImager (Molecular Dynamics).

To observe the electrophoretic mobility shift due to multisite phosphorylation of the CTD, 3 μl of purified RNAP IIA or IIB (6.6×10^{-3} U) or 14 μg of affinity-purified βYCTD protein mixture were incubated at 30°C with 54 ng of active sea star $p44^{mpk}$ purified to homogeneity (Upstate Biotechnology Inc.) and brought to 40 μl with buffer C containing ATP (4 mM final concentration). Aliquots (6 μl) were removed at the indicated times, electrophoresed and probed by Western blot with POL 3/3 or 8WG16 monoclonal antibodies.

Immune complex kinase assay

At 1 day after subculture, NIH 3T3 cells were transfected with either plasmid pCMV/HA or pCMV/HA-T192A (Meloche *et al.*, 1992; Pagès *et al.*, 1993) and serum-starved on the following day. After 16 h of serum starvation, FCS (20% final concentration) was added for 15 min and cells were lysed in buffer B. 1 μl of the appropriate antibody (12CA5) or antiserum (anti-ERK1) was added to 100 μl of ice-chilled cytosolic lysate obtained from 10^6 cells. After 30 min of incubation, lysates were added to 40 μl of pelleted protein A−Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) and kept on ice for 30 min with gentle homogenization every 5 min. To adsorb the 12CA5 monoclonal antibody, protein A−Sepharose beads (600 μl) were first coated by overnight incubation with 100 μl of rabbit anti-mouse serum (RAM/7S from Nordic Immunology) in buffer B followed by an extensive wash. After having reacted with the lysates, the protein A−Sepharose beads were washed three times with 1 ml buffer B. Beads (20 μl) were then incubated with βYCTD protein in the presence of ATP as described in the protein kinase assay. After 20 min of incubation at 30°C with gentle homogenization every 5 min, the reaction was stopped by the addition of 20 μl Laemmli buffer.

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