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Transcription-independent phosphorylation of the RNA polymerase II C-terminal domain (CTD) involves ERK kinases (MEK1/2)

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ABSTRACT

The largest subunit of the mammalian RNA polymerase II possesses a C-terminal domain (CTD) consisting of 52 repeats of the consensus sequence, Tyr[1]-Ser[2]-Pro[3]-Thr[4]-Ser[5]-Pro[6]-Ser[7]. Phosphorylation of the CTD is known to play a key role in gene expression. We now show that treatments such as osmotic and oxidative shocks or serum stimulation generate a new type of phosphorylated subunit, the IIm form. This IIm form might be generated *in vivo* by ERK-type MAP kinase phosphorylation as: (i) ERK1/2 are major CTD kinases found in cell extracts; (ii) the immunoreactivity of the IIm form against a panel of monoclonal antibodies indicates that the CTD is exclusively phosphorylated on Ser-5 in the repeats, like RNA polymerase II phosphorylated *in vitro* by an ERK1/2; and (iii) the IIm form does not appear when ERK activation is prevented by treating cells with low concentrations of highly specific inhibitors of MEK1/2. Since the IIm subunit is not affected by inhibition of transcription and is not bound to chromatin, it does not participate in transcription.

INTRODUCTION

The C-terminal domain (CTD) of the largest RNA polymerase II subunit comprises multiple repeats of a heptapeptide sequence (1). The CTD plays a key role in gene expression and its activity at different steps in the transcription cycle is regulated by multisite phosphorylation (2). The unphosphorylated form of RNA polymerase II, designated RNA polymerase IIA, assembles into a preinitiation complex on the promoter. Phosphorylation of the CTD occurs concomitant with initiation of transcription. Accordingly, elongation is catalyzed by the phosphorylated form of RNA polymerase II, designated RNA polymerase IIO. The largest subunits of RNA polymerases IIA and IIO are designated IIa and IIo, respectively. The unphosphorylated CTD mediates multiple protein–protein interactions involved in the assembly of the preinitiation complex whereas the phosphorylated CTD facilitates the assembly of the various enzymatic complexes involved in the processing of the primary transcript (3–7). To reinitiate transcription, the CTD should be dephosphorylated.

Equivalent amounts of IIa and IIo forms are found in growing mammalian cells (8). However, the IIa/IIo ratio is rapidly modified upon treatment with transcription inhibitors (8), heat-shock (9) and serum stimulation (10). Taken together, these observations indicate that RNA polymerase II undergoes a continuous phosphorylation/dephosphorylation cycle. Numerous cyclin-dependent kinases (CDK) phosphorylate the CTD *in vitro*: CDK1 (11) and CTDK-I (12) were first identified. Most interestingly, three CTD kinases are subunits of complexes involved in transcription: CDK7 and its partner, cyclin H, are subunits of the general transcription factor TFIIH (13); CDK8 and its partner cyclin C are subunits of the mediator/NAT (negative regulator of activated transcription) complex in RNA polymerase II holoenzyme (14–16); and CDK9 and its partners cyclins T1 and T2, are subunits of P-TEFb, a positive transcription elongation factor (17). The CTD is dephosphorylated in a few minutes upon inactivation of the KIN28 gene product, the yeast CDK7 homolog (18,19). The CTD is rapidly dephosphorylated in mammalian cells treated with CDK9 inhibitors including 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (8,20). Hence, CDK7 and CDK9 are likely to contribute to the overall CTD phosphorylation *in vivo*. DNA-PK (21) and MAP kinases of the ERK type (10) are also efficient CTD kinases *in vitro*. However, much less is known about the contribution of each of these kinases to phosphorylation of the CTD *in vivo*. ERK-type MAP kinases are major signalling kinases controlling gene expression. Phosphorylation of RNA polymerase II correlates with the ERK-type MAP kinases, ERK1/2, activity in serum-stimulated mouse fibroblasts (10) and during meiosis in *Xenopus* (22). The present study attempts to establish that ERK1/2 phosphorylate RNA polymerase II in living cells. Advantage is taken of highly specific inhibitors of MEK1/2, the ERK1/2 activator (23,24) and a panel of monoclonal antibodies recognizing distinct epitopes on the CTD (25).

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MATERIALS AND METHODS

Cells

Monolayers of NIH 3T3 cells were propagated on tissue culture dishes or tubes in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DMEM; Gibco-BRL). For serum stimulation, subconfluent cells (24 h after plating) were placed in serum-free medium for 16 h. The resulting quiescent cells were serum-stimulated by addition of fetal calf serum (20%) to the medium. DRB (Sigma), actinomycin D (Sigma), PD098059 (Alexis) and U0126 (Promega) were dissolved in dimethylsulfoxide.

Non-denaturing cell lysis and fractionation

Cells submitted to stress or not were washed twice with cold phosphate-buffer saline and lysed on ice in GP buffer (20 mM sodium glycerophosphate, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM vanadate, 0.5% Nonidet P-40, 10% glycerol, 0.1% β-mercapto-ethanol and adjusted to pH 7.5). The cell extract was fractionated into a supernatant and a pellet by centrifugation at 15 000 *g* for 10 min at 4°C.

In vitro phosphorylation of the CTD from purified RNA polymerase II

Purified RNA polymerase II (10^{-2} U) (26) was incubated at 30°C in the presence of activated murine GST-p42 MAP kinase (ERK2) (Upstate Biotechnology) (0.8 µg) in GP buffer containing 5 mM ATP. Reactions were arrested by addition of 2× Laemmli sample buffer and analyzed by western blotting.

Western blots and antibodies

Whole cell extracts, supernatants and pellets in GP buffer were supplemented with Laemmli sample buffer and heated for 6 min at 95°C. Heated samples were loaded on sodium dodecyl sulfate (SDS) polyacrylamide gels. After electrophoretic transfer to Hybond-P membranes (Amersham), the blots were probed with monoclonal antibodies against either ERK2 MAP kinase (Santa Cruz) or RNA polymerase II largest subunit. The POL 3/3 monoclonal antibody (gift from Dr Ekkerhard Bautz) recognizes the RNA polymerase II largest subunit at an evolutionarily conserved epitope located outside the CTD (27). Epitopes in the CTD were recognized by the monoclonal antibodies, CC3 (gift from Dr Michel Vincent) (28), B3 (gift from Dr Ronald Berezney) (29), H5 and H14 (gift from Dr Stephen Warren) (30), MARA3 (gift from Dr Bart Sefton). V6 and V15 monoclonal antibodies were obtained after immunization of Biozzi mice with the synthetic peptide (YSPTSPS)3 coupled to ovalbumin. The immunoreactive bands were visualized using anti-mouse IgG horseradish peroxidase conjugates (Promega) and enhanced chemiluminescence (Pierce).

RESULTS

Phosphorylation of the CTD in cells submitted to an osmotic stress

To investigate the role of ERK in the phosphorylation of RNA polymerase II, cells were submitted to an osmotic stress that had been reported to activate ERK1/2 kinases (31). In NIH 3T3 cells, addition of sorbitol to the culture medium resulted in the appearance of the slow migrating ERK2 band indicative of its activation (Fig. 1). To investigate the phosphorylation of the CTD, a whole cell lysate from NIH 3T3 cells was analyzed by western blotting with the POL3/3 monoclonal antibody. This antibody binds to an epitope of the RNA polymerase II largest subunit, localized outside the CTD (27). In control cells, two major bands are detected in equivalent amounts that migrate above the 200 kDa marker (Fig. 1, lane 0). The fastest one and the slowest one correspond respectively to the IIa (hypophosphorylated) and IIo (hyperphosphorylated) forms of RNA polymerase II largest subunit (8,32). Addition of sorbitol to the culture medium resulted in the appearance of a new band migrating between the IIa and the IIo forms (Fig. 1). The mobility of this band remained faster than that of the IIo form even after 60 min. In the following paragraphs, the new band will be designated as IIm.

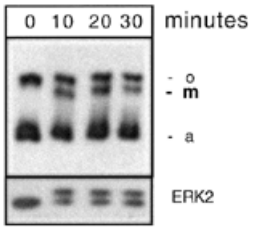

Figure 1. The IIm form of RNA polymerase II largest subunit is generated in NIH 3T3 cells submitted to an osmotic shock. Sorbitol (300 mM) was added to the culture medium for various times indicated in minutes. Whole cell extracts were analyzed by western blotting using POL3/3 antibody (upper panel) or anti-ERK2 antibodies (lower panel). The position of the hypophosphorylated IIa form, the hyperphosphorylated IIm and IIo forms of the largest subunit are indicated.

The IIm form of RNA polymerase II largest subunit is not engaged in transcription

Transcription involves a succession of CTD phosphorylation and dephosphorylation steps that might have been altered by the osmotic stress. To question the involvement of the IIm form in transcription, the cells were lysed in a low salt non-denaturing buffer and fractionated by centrifugation. RNA polymerase molecules engaged in transcription at the time of lysis are thought to remain bound to the nuclei pelleted in conditions that correspond to those used in run-on transcription assays (33). A significant proportion of the IIa form was released in the soluble phase whereas most of the IIo form remained bound to the pellet, which contained the nuclear material (Fig. 2). In contrast, the IIm form generated in osmotically-shocked cells was completely released in the soluble phase.

To further disconnect the appearance of the IIm form from transcription, the effect of sorbitol treatment on CTD phosphorylation was investigated in cells that had been pretreated with transcription inhibitors. Treatment with DRB alone resulted in the accumulation of the dephosphorylated IIa form at the expense of the IIo form as described previously (8). When sorbitol was added to the DRB-treated cells, the IIm form was still generated (Fig. 3). Its intensity and migration characteristics were similar to that in control untreated cells (Fig. 1). Treatment with actinomycin D promoted an accumulation of the IIo form (8,20). Addition of sorbitol to the actinomycin-treated cells generated again a IIm form at the expense of the remaining IIa

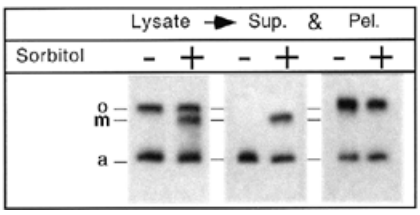

Figure 2. The IIm form of RNA polymerase II largest subunit is readily extracted in a low salt buffer. NIH 3T3 cells treated (+) or not (–) with sorbitol (300 mM) during 30 min were lysed in GP buffer and the lysates were fractionated by centrifugation into a supernatant (Sup.) and a pellet (Pel.). The RNA polymerase II largest subunit was detected by western blotting using the POL3/3 monoclonal antibody.

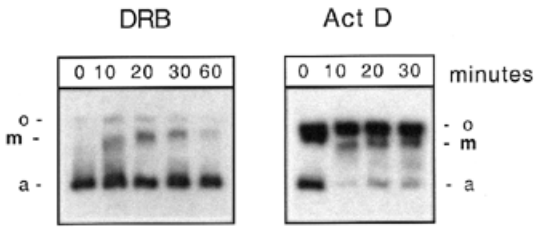

Figure 3. The IIm form of RNA polymerase II largest subunit is also generated in the presence of transcription inhibitors. NIH 3T3 cells were pretreated with DRB (100 µM) or actinomycin D (ActD, 20 µg/ml) for 30 min; sorbitol (300 mM) was then added to the medium. The duration of sorbitol treatment is indicated in minutes. The RNA polymerase II largest subunit in whole cell extracts was detected by western blotting with the POL3/3 monoclonal antibody.

form (Fig. 3). Thus, an osmotic stress generates the IIm form, irrespective of the transcriptional activity of the cell. The IIm largest subunits are unlikely to be engaged in transcription.

Pretreatment of cells with MEK1/2 inhibitors prevents the appearance of the IIm form in osmotically shocked and serum-stimulated cells

A correlation between ERK1/2 activation and RNA polymerase II phosphorylation has been observed in several biological systems (10,22) and here with osmotic stress. Indeed, treatment of cells with sorbitol resulted in a decreased mobility of ERK2, indicating the phosphorylation of the ERK1/2 by ERK kinases (MEK1/2) (Fig. 4A). To test the involvement of ERK1/2 in CTD phosphorylation the cells were pretreated with the highly specific inhibitors of the MEK1/2, PD098059 (23) and U0126 (24). When cells were pretreated with inhibitors, the slow migrating ERK2 band decreased and disappeared above a concentration of 10 µM PD098059 and 1 µM U0126. As little as 3 µM PD098059 and 1 µM U0126 suppressed the IIm form. Thus, the latter compound was more efficient in both suppressing formation of the IIm form and preventing ERK2 phosphorylation/activation. A continuum of intermediates between the IIa and IIo bands did not disappear with high inhibitor concentrations and might correspond to forms already present in unstimulated cells.

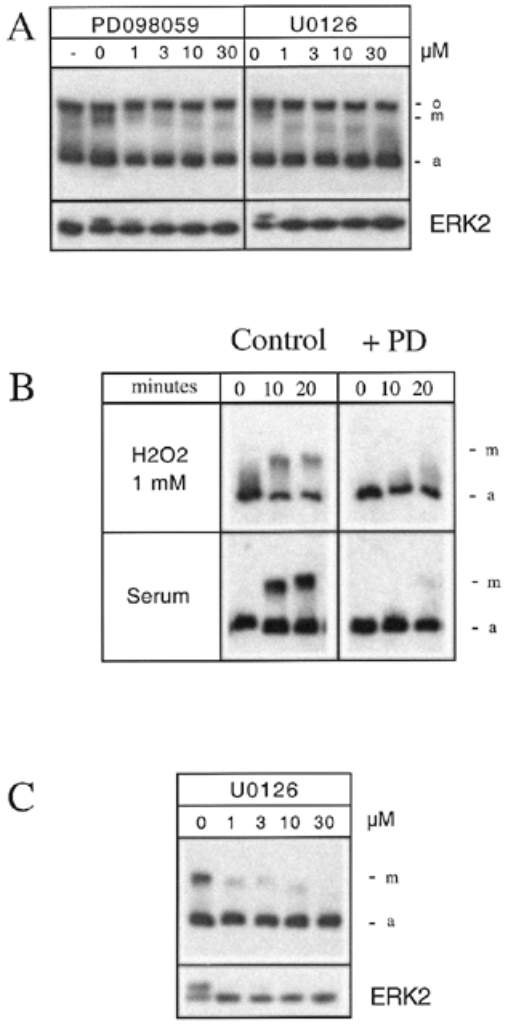

Figure 4. Pretreatment of cells with MEK1/2 inhibitors prevents ERK2 activation and the appearance of the IIm form. (**A**) NIH 3T3 cells were pretreated with various concentrations of PD098059 or U0126 for 30 min before addition of sorbitol (300 mM) for 30 min. Whole cell extracts were analyzed by western blotting using the POL3/3 monoclonal antibody (upper panel) or an ERK2-specific monoclonal antibody (lower panel). (**B**) PD098059 (100 µM) was either added (+PD) or not added (Control) 30 min before the addition of H_2O_2 (1 mM) to exponentially growing cells (upper panel) or before the addition of serum (20%) to serum-starved cells (lower panel). Centrifugation-clarified supernatants of GP extracts prepared after 0, 10 or 20 min treatments were analyzed by western blotting using POL3/3 antibody. (**C**) Serum-starved cells were treated with various concentrations of U0126 for 30 min before the addition of 20% serum for 15 min. Centrifugation-clarified supernatants of GP extracts were analyzed by western blotting using POL3/3 antibody (upper panel) or anti-ERK2 antibodies (lower panel).

Interestingly, the appearance of such a phosphorylated form was not restricted to osmotic stress. A soluble IIm form was also generated upon serum stimulation or upon an oxidative stress provoked by exposure to hydrogen peroxide (Fig. 4B). The appearance of this form was prevented by PD098059. When the IIm form was generated upon serum stimulation, its formation was also inhibited by as little as 1 µM of U0126 (Fig. 4C). Therefore, MEK1/2 activity is required to generate the IIm form of the RNA polymerase II largest subunit.

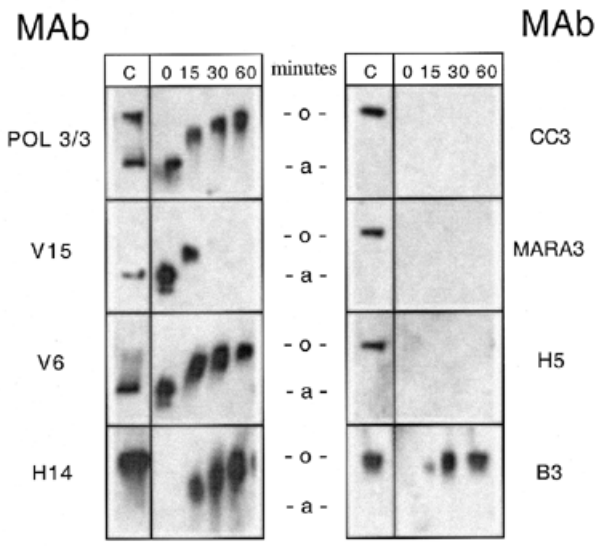

Figure 5. Immunoreactivity of RNA polymerase II largest subunit phosphorylated by ERK type MAP kinase. Purified RNA polymerase II was incubated with activated murine GST-ERK2 and ATP. The reaction mixture was sampled during a time course indicated in minutes and analyzed by western blotting using the indicated monoclonal antibodies (MAb). In lanes C, a whole-cell lysate from unstressed NIH 3T3 cells was loaded and used as a control to localize the IIa and IIo forms. The localization of the different forms was determined by reprobing all membranes with the POL3/3 antibody (data not shown).

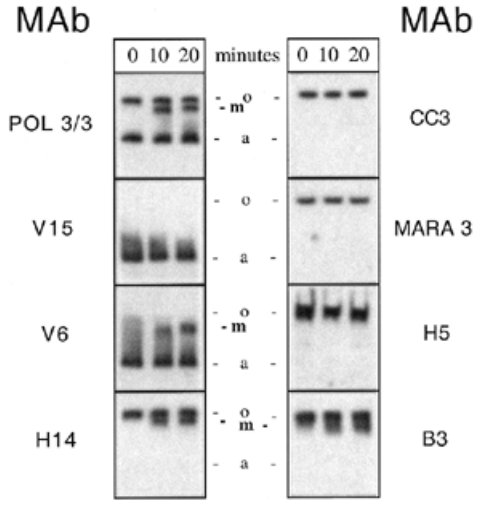

Figure 6. Effect of osmotic shock on immunoreactivity of RNA polymerase II largest subunit. NIH 3T3 cells were exposed or not to sorbitol (300 mM) for 10 or 20 min. The RNA polymerase II largest subunit in whole cell extracts was detected by western blotting using the indicated monoclonal antibodies (MAb). The localization of the IIa, IIm and IIo forms was determined by reprobing all membranes with the POL3/3 antibody (data not shown).

The IIm form and the CTD phosphorylated by ERK2 have the same immunoreactivity

ERK1/2 are major CTD kinases found in cell extracts (10). To strengthen the suggestion that ERK1/2 phosphorylate the CTD *in vivo*, the antigenicity of the IIm form was compared with that of the polymerase phosphorylated *in vitro* by ERK2. Given the repetitive nature of the CTD and the number of amino acids with hydroxylated side-chains in the repeats, the identification of the residues phosphorylated *in vivo* is a formidable task. However, a set of monoclonal antibodies has recently been characterized and shown to recognize distinct epitopes on the CTD (25,28). The various CTD kinases phosphorylate distinct amino acid residues in the CTD (34) and generate different epitopes (28). When purified RNA polymerase IIA (corresponding to hypophosphorylated IIa largest subunit) was incubated with purified ERK2 in the presence of ATP, the electrophoretic mobility of the largest subunit decreased gradually with phosphorylation time and approached that of the phosphorylated IIo form after 60 min of reaction as revealed by western blotting using the POL 3/3 antibody (Fig. 5). Phosphorylation rapidly suppressed the V15 immunoreactivity but had little influence on the V6 immunoreactivity. The H5, B3, MARA3, H14 and CC3 phosphoepitopes are all generated *in vitro* by phosphorylation of the CTD with $p34^{cdc2}$ (25). However, H14 and B3 were the sole antibodies to bind the CTD phosphorylated by MAP kinase, though H14 antigenicity required shorter phosphorylation times than B3 (corresponding to lesser phosphate incorporation) (Fig. 5).

Next, whole-cell lysates from control or osmotic shocked cells were analyzed by western blotting using the anti-CTD monoclonal antibodies. The H14, B3, H5, MARA3 and CC3 antibodies did not bind to the unphosphorylated IIa subunit, but they all reacted with a phosphorylated IIo form present in unstressed cells (Fig. 6). In contrast, the V6 and V15 antibodies both reacted with the unphosphorylated IIa form but not with the phosphorylated IIo form present in unstressed cells. Among the whole set, B3, H14 and V6 were the only antibodies to bind a form that co-migrated with the IIm form.

To summarize, the immunoreactivity of the IIm form was fully consistent with its generation by ERK phosphorylation (Table 1).

Table 1. Summary of the antigenic recognition of RNA polymerase II (RNAPII) largest subunit

MAbs	POL3/3	V15	V6	H14	B3	H5	CC3	MARA3
IIa	+	+	+	–	–	–	–	–
IIo	+	–	–	+	+	+	+	+
IIm	+	–	+	+	+	–	–	–
ERK2	+	–	+	+	+	–	–	–
CDK1/cdc2	+	ND	ND	+	+	+	+	+
CDK7	+	ND	ND	ND	ND	ND	+	ND
Antigenic phosphoserine	/	No	No	5	5	2	2	2 + 5

Data for IIa, IIo and IIm forms are taken from Figure 6. Data for the largest subunit phosphorylated *in vitro* by ERK2 are taken from Figure 5, $p34^{cdc2}$ from Patturajan and coworkers (25), phosphorylated by CDK7 from Dubois and coworkers (28). Characterization of antigenic phosphoserines is from Patturajan and coworkers (25). ND, not determined.

DISCUSSION

Here we report that in NIH 3T3 cells submitted to osmotic stress, oxidative stress or serum stimulation, a new form of the RNA polymerase II largest subunit is generated. Activation of an ERK-type MAP kinase is required to induce the appearance of this form, which possesses the same antigenic determinants as the largest subunit phosphorylated *in vitro* by ERK. CTD phosphorylation by ERK is gradual. Similarly, the migration of the new form slows down gradually during cell stimulation. Hence, this new form is designated IIm as it is associated with MAP kinase activity.

Patturajan and coworkers have initiated the characterization of the phosphoepitopes recognized by some of the antibodies used in this work using repeats of the CTD consensus sequence Tyr^1-Ser^2-Pro^3-Thr^4-Ser^5-Pro^6-Ser^7, fused to glutathione *S*-transferase (25). Phosphorylation with purified $p34^{cdc2}$ generates the H5, H14, B3, MARA3 and CC3 antigenicity. In contrast, ERK2 only generates H14 and B3 recognition (Table 1). From this result, it is concluded that ERK2 phosphorylates the CTD less extensively than $p34^{cdc2}$. Using fusion proteins in which specific serines had been mutated, Patturajan and coworkers showed that on the one hand, Ser-2 phosphorylation is essential for CC3 and H5 recognition but not for H14, MARA3 and B3. On the other hand, Ser-5 phosphorylation is essential for H14 but not for MARA3, CC3 and H5 recognition (25). Furthermore, B3 reactivity is enhanced when only Ser-5 is phosphorylated (Ser-2 replaced by alanine). The antigenicity of the IIm form and of the MAP kinase phosphorylated CTD indicates that they are phosphorylated exclusively on Ser-5 in contrast to the IIo form, which is phosphorylated on both Ser-2 and Ser-5.

$p34^{cdc2}$ (35) and hSRB10/CDK8-containing complexes (16) phosphorylate both Ser-2 and Ser-5. The TFIIH-associated CDK7 phosphorylates Ser-5 on a synthetic peptide (34). However, TFIIH is likely also to phosphorylate Ser-2 on the degenerated repeats of the mammalian CTD as it generates the CC3 epitope on the C-terminal half of the CTD (28). The ERK type MAP kinase preferentially phosphorylates Ser-5 on synthetic peptides (34). We now show that in contrast to the above-mentioned CDKs, the ERK-type MAP kinase phosphorylates the CTD exclusively on Ser-5. This characteristic is shared by the IIm form.

ERK1/2 MAP kinases are major CTD kinases that are activated in serum-stimulated (10), osmotically stressed (this work) and meiotic cells (22). By coincidence, an enhanced CTD phosphorylation occurs. In contrast to ERK1/2 (10), P-TEFb phosphorylation of the CTD is extremely DRB-sensitive *in vitro* (36) and *in vivo* (8,20). Taken together with the characteristic antigenicity of the IIm form and the blocking effect of the MEK1/2 inhibitors, our data suggest that ERK type MAP kinases phosphorylate the CTD *in vivo* and contribute to generating a previously undescribed form of RNA polymerase II largest subunit, the IIm form. A Ras-dependent CTD phosphorylation has been reported in cardiac myocytes (37). As Ras is an upstream activator of the ERK1/2 kinases, the resulting phosphorylated subunit, which was soluble in RIPA buffer, might correspond to the IIm form.

The IIm form is unlikely to belong to transcribing RNA polymerase II complexes. Indeed, the IIm form is not tightly bound to chromatin as it is readily extracted in a low salt buffer and its appearance following a stress (this work) or serum stimulation (10) is not affected by inhibition of transcription with DRB or actinomycin D. A small proportion of RNA polymerase II complexes seems to be targeted by ERK type kinases in murine or *Xenopus* (not shown) cultured cells. In contrast, the CTD phosphorylation that might be ascribable to ERK-type kinases is a major event during *Xenopus* meiosis (22). During meiosis, the transcription is completely arrested and the nuclear structure is disrupted, thereby increasing the probability for an ERK/RNA polymerase II encounter. Thus, CTD phosphorylation might have a function disconnected from transcription.

Interestingly enough, Hirose and Manley recently reported that RNA polymerase II is an essential cofactor in transcription-independent polyadenylation and splicing assays (38,39). The CTD is required for such an activity, which seems to be enhanced by phosphorylation. Furthermore, mRNA capping enzymes are recruited by the phosphorylated CTD (6) and stimulation in guanylyltransferase activity increases with the number of Ser-5-phosphorylated heptads (but not Ser-2) (40). There are scarce reports of transcription-independent mRNA modifications in somatic cells; an increased polyadenylation of vasopressin mRNAs has been described in osmotically stressed neurons of the hypothalamus (41). However, transcription-independent mRNA modifications are well documented during oocyte maturation: polyadenylation of specific mRNAs such as Mos is increased (42,43); and guanine-7-methyl transferase activity, the second step in mRNA capping, has been found to increase during *Xenopus* meiosis (44). The polyadenylation of Mos mRNA has been shown to be stimulated by ERK activity during *Xenopus* meiosis (45). Hence, it might be speculated that transcription-independent phosphorylation of RNA polymerase II by ERK-type kinases could be involved in regulating post-transcriptional modifications of mRNAs.

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