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Phosphorylation of the RNA Polymerase II Largest Subunit during *Xenopus laevis* Oocyte Maturation

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Xenopus laevis **oogenesis is characterized by an active transcription which ceases abruptly upon maturation. To survey changes in the characteristics of the transcriptional machinery which might contribute to this transcriptional arrest, the phosphorylation status of the RNA polymerase II largest subunit (RPB1 subunit) was analyzed during oocyte maturation. We found that the RPB1 subunit accumulates in large quantities from previtellogenic early diplotene oocytes up to fully grown oocytes. The C-terminal domain (CTD) of the RPB1 subunit was essentially hypophosphorylated in growing oocytes from Dumont stage IV to stage VI. Upon maturation, the proportion of hyperphosphorylated RPB1 subunits increased dramatically and abruptly. The hyperphosphorylated RPB1 subunits were dephosphorylated within 1 h after fertilization or heat shock of the matured oocytes. Extracts from metaphase II-arrested oocytes showed a much stronger CTD kinase activity than extracts from prophase stage VI oocytes. Most of this kinase activity was attributed to the activated Xp42 mitogen-activated protein (MAP) kinase, a MAP kinase of the ERK type. Making use of artificial maturation of the stage VI oocyte through microinjection of a recombinant stable cyclin B1, we observed a parallel activation of Xp42 MAP kinase and phosphorylation of RPB1. Both events required protein synthesis, which demonstrated that activation of p34^{cdc2} kinase was insufficient to phosphorylate RPB1 ex vivo and was consistent with a contribution of the Xp42 MAP kinase to RPB1 subunit phosphorylation. These results further support the possibility that the largest RNA polymerase II subunit is a substrate of the ERK-type MAP kinases during oocyte maturation, as previously proposed during stress or growth factor stimulation of mammalian cells.**

In *Xenopus laevis*, as in many species, transcription is arrested in the matured gametes. During oogenesis, proteins and RNAs accumulate to support early embryogenesis. Although the fully grown oocyte contains as much RNA polymerase II (RNAPII) activity as 10^5 individual somatic cells (43), little is known about its properties at maturation, after the germinal vesicle breakdown. From this perspective, we decided to investigate the phosphorylation of the RNAPII largest subunit (RPB1) in the developing and maturing oocytes. The RNAPII core enzyme is an assembly of 12 subunits (29, 45). Extensive studies have assigned an important role to the phosphorylation of the C-terminal domain (CTD) of RPB1 in regulating the initiation of transcription (11, 18). The hypophosphorylated RPB1 (IIa subunit) binds to the TATA box binding protein (TBP) within a preinitiation complex of transcription, this interaction being abolished by phosphorylation of the CTD (28, 52). The steady-state distribution between the hypophosphorylated IIa and the hyperphosphorylated IIo forms of RPB1 results from the antagonistic activity of CTD kinases and CTD phosphatases. A CTD phosphatase has recently been purified and characterized, but few data are available concerning the dephosphorylation step (5). In contrast, many protein kinases have been found to phosphorylate the CTD in vitro. The p34^{cdc2} kinase was the first CTD kinase to be identified (6, 7). Later, the CTD was also shown to be a substrate for other cyclin-dependent kinases (cdk), such as the yeast Ctk1 (32, 48); the yeast Kin28 (19) and its vertebrate homolog, cdk7 (MO15) (44, 46, 47); and the yeast Srb11 (33) and its vertebrate homolog, cdk8 (50). The Kin28 and cdk7 kinases are, respectively, subunits of the yeast and mammalian general transcription factor TFIIH (reviewed in references 26 and 49) and are required for mRNA transcription (8, 44, 53). Furthermore, the Kin28 kinase is essential for RPB1 phosphorylation in vivo in exponentially growing yeast cells (53, 54). The DNA-dependent protein kinase (17), the c-Abl tyrosine kinase (3), and the mitogen-activated protein (MAP) kinases (14) were also found to phosphorylate the CTD in vitro. The MAP kinases may contribute to RPB1 phosphorylation in vivo in serum-stimulated (14) and heat-shocked (55) cells. Oocyte maturation is triggered by a hormonal signal which switches on a developmental program leading the fully grown oocyte in prophase to enter meiosis and to arrest in metaphase II. This process is controlled by an intricate network of protein kinase cascades (reviewed in references 34 and 37). Two potential CTD kinases are activated and play a central role in meiosis: the p34^{cdc2} kinase and the Xp42 MAP kinase.

In this work, the phosphorylation of the RPB1 subunit was investigated during *Xenopus* oocyte growth, maturation, and fertilization. The proportion of hyperphosphorylated RPB1 subunit decreased along with an increase in oocyte size. Upon oocyte maturation, this proportion increased abruptly in par-

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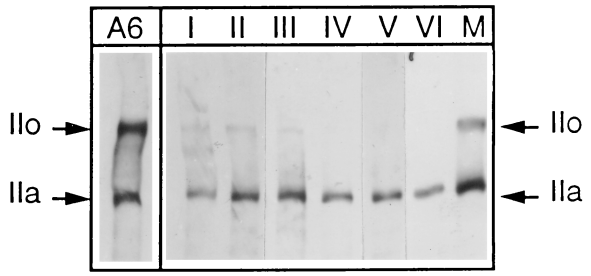

FIG. 1. Phosphorylation of the RNAPII largest subunit (RPB1) in developing *Xenopus* oocytes. Whole-cell lysates in Laemmli buffer were electrophoresed in a 6% polyacrylamide gel. The RPB1 subunit was detected by Western blotting with the POL3/3 antibody and chemiluminescence. The volumes of oocyte lysates were adjusted to give comparable RPB1 signal intensities in each lane, as described in Materials and Methods. The positions corresponding to migration of the multiphosphorylated IIo subunit and hypophosphorylated IIa subunit of the RNAPII are indicated by arrows. Oocytes from a single female were staged (I to VI) according to the Dumont classification; metaphase II-arrested unfertilized eggs (M) were laid by a distinct female.

allel with the appearance of a CTD kinase activity in native extracts. Most of this kinase activity could be attributed to the Xp42 MAP kinase.

MATERIALS AND METHODS

Cell culture. A6 cells derived from *Xenopus* kidney (41) were cultured in Leibovitz's L-15 medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), 100 U of penicillin G per ml, and 100 μg of streptomycin per ml at 25°C.

Oocyte and egg collection and microinjection. Oocytes were prepared as described previously (1). Briefly, ovarian tissue was surgically removed from an adult female frog anesthetized on ice, the tissue was minced, and follicle cells were digested away from the oocyte for 2 h at 25°C with 1 mg of collagenase (Sigma) per ml in OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 5 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-NaOH [pH 7.8]) before extensive rinsing in OR2 medium supplemented with 10 μg of penicillin and streptomycin per ml. Oocytes were staged according to the method of Dumont (16). Fully grown oocytes (stage VI) were referred to as prophase oocytes, and mature oocytes were referred to as metaphase II oocytes, a state normally found in unfertilized eggs. Ex vivo maturation of selected stage VI oocytes was performed either by addition of progesterone (Sigma) (1 μg/ml) in OR2 medium or by cytoplasmic microinjection at the equator level of 30 nl (30 ng/oocyte) of bacterially expressed glutathione *S*-transferase fused to sea urchin cyclin B1 with its 13 N-terminal amino acids deleted (Δ13-cyclin B1) (36). Microinjections were performed with a Drummond microinjector under a dissectory microscope (Leica). After 6 h in the presence of progesterone, a white spot appeared on the animal hemisphere, indicative of a successful maturation. Microinjected stage VI oocytes were maintained in OR2 medium and collected 3 h after injection (24). When indicated, cycloheximide (200 μg/ml) was added before and maintained after microinjection to prevent protein synthesis.

X. laevis metaphase II-arrested eggs were prepared as described previously (2). Briefly, human chorionic gonadotropin (Sigma) (1,000 U) was injected into the dorsal lymph sac of adult female frogs. Eggs laid over a 12-h period were collected in 0.1 M NaCl and dejellied in 2% cysteine–0.1 M NaCl-NaOH (pH 7.9) for 10 min, washed extensively in 0.1 M NaCl, and used immediately. Fertilization was performed in vitro with a fresh suspension of sperm as described previously (2).

Western blots. Cells, oocytes, and eggs were lysed in Laemmli buffer (60 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 1% 2-mercaptoethanol, 0.002% bromophenol blue) and subsequently heated for 10 min at 90°C. The whole-cell lysates were electrophoresed in 6% polyacrylamide–SDS gels (SDS-polyacrylamide gel electrophoresis [PAGE]) for RPB1 detection and in 10% polyacrylamide–SDS gels for Xp42 MAP kinase detection, as previously described (14). The monoclonal antibody POL3/3 recognizes the RPB1 subunit at an evolutionarily conserved epitope located outside the CTD and was kindly provided by E. K. Bautz (30). The rabbit anti-MAP kinase (anti-ERK1) antiserum came from Santa Cruz Biotechnology. The antigen-antibody complexes were visualized with an anti-mouse antibody coupled to horseradish peroxidase (Promega) followed by enhanced chemiluminescence (ECL system; Amersham). Quantification was performed with a radioiodinated anti-mouse antibody (Amersham) followed by phosphorimaging (Fujix BAS 1000).

For Fig. 1, pools of 15 stage I, 10 stage II, 10 stage III, 10 stage IV, 10 stage V, 10 stage VI, and 10 metaphase II-arrested oocytes were lysed, respectively, in 110, 75, 375, 375, 750, 750, and 750 μl of Laemmli buffer. The volumes of lysate loaded per lane correspond to 8 stage I oocytes, 2.7 stage II oocytes, 1.06 stage III oocyte, 0.26 stage IV oocyte, 0.2 stage V oocyte, 0.2 stage VI oocyte, and 0.2 egg.

Native extracts and FPLC Mono Q chromatography. For kinase assays, 10 prophase or 10 metaphase II-arrested oocytes were homogenized at 4°C in 1 ml of EB buffer (40 mM sodium glycerophosphate [pH 7.3], 10 mM EGTA, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM 2-mercaptoethanol) and centrifuged at 100,000 × *g* for 45 min. Four hundred microliters of the resulting clear supernatant was recovered, aliquoted, and stored at −80°C. The protein concentration in these native extracts was 0.28 mg/ml (Bradford assay). For fast protein liquid chromatography (FPLC), metaphase II-arrested oocytes (3.7-ml dry volume) were washed at 4°C in EB buffer. The buffer was removed, and the oocytes were centrifuged at 4°C for 30 min at 10,000 × *g*. The clear interphase under the yellow layer of lipids was collected and centrifuged at 4°C for 1 h at 100,000 × *g*. The resulting clarified supernatant (2.2 ml) was recovered, aliquoted, and stored at −80°C. The protein concentration in this extract was 25 mg/ml (Bradford assay). A total of 0.6 ml of this extract was applied to an HR 5/5 Mono Q column (Pharmacia). The column was developed at a flow rate of 1 ml/min, first with a 30-ml linear NaCl gradient (0 to 300 mM) in EB buffer and afterwards with an 8-ml linear NaCl gradient (300 to 1,000 mM) in EB buffer. Fractions of 0.5 ml were collected and stored at −80°C.

Protein kinase assays. Aliquots (5 μl) of native extracts or fractions eluted from the Mono Q column were mixed on ice with 10 μl of a reagent mix in EB buffer containing ATP (0.1 mM), 0.5 μCi of [γ-^{32}P]ATP (3,000 Ci/mM [Amersham Corp.]), and the appropriate exogenous substrate: purified human RNAPII (kindly provided by M. E. Dahmus) (27), histone H1 (Sigma) (10 μg), myelin basic protein (MBP) (Sigma) (5 μg), and the Ctd-4 peptide (5 μg) [(Ser-Pro-Thr-Ser-Pro-Ser-Tyr)$_4$, synthesized by O. Siffert at the Institut Pasteur—Paris]. Phosphorylations were allowed to proceed for 30 min at 30°C and were arrested by the addition of 15 μl of 2× Laemmli buffer. The phosphorylated Ctd-4 peptide and MBP migrated like 23-kDa proteins, whereas histone H1 migrated like a 33-kDa protein in 15% polyacrylamide–SDS gels. The gels were fixed, dried, and autoradiographed or analyzed with a PhosphorImager (Fujix BAS 1000).

Adsorption on P13-agarose beads. Agarose beads bound to yeast p13^{suc1} (Upstate Biotechnology Incorporated) were equilibrated overnight in EB buffer containing bovine serum albumin (fraction V [Sigma]) (0.5 mg/ml) and washed in EB buffer. Unbound agarose beads were used in a parallel control experiment. Clarified oocyte extracts in EB buffer (8 μl) were diluted in 40 μl of EB buffer and added to the pelleted beads (30 μl). After a 1-h incubation on ice, the beads were centrifuged for 1 min at 5,000 × *g*. Supernatants (48 μl/assay) and the beads resuspended in EB buffer were assayed for protein kinase activities as described above.

Immunoadsorption of Xp42 MAP kinase. For immunoadsorption, 80 μl of pelleted protein A-agarose beads (Pharmacia) was preincubated for 1 h at 4°C with 20 μl of anti-*Xenopus* MAP kinase antiserum R or with 20 μl of rabbit anti-mouse antiserum (Nordic) for controls. Anti-*Xenopus* MAP kinase antiserum R was produced by immunizing a rabbit with recombinant *Xenopus* MAP kinase. The production and characterization of this antiserum will be described in detail elsewhere (26a). Ten microliters of fraction 36 eluted from the Mono Q column diluted in 300 μl of EB buffer was next incubated with 80 μl of pelleted antibody-coated beads for 1 h at 4°C and centrifuged for 1 min at 5,000 × *g*. Fifteen microliters of the resulting supernatant or 15 μl of the pelleted beads was assayed for Ctd-4 kinase activity as described above.

RESULTS

The RPB1 subunit phosphorylation is tightly regulated during *X. laevis* oogenesis. To investigate the phosphorylation of RPB1 during oogenesis, *Xenopus* oocytes from stage I to stage VI (16) were analyzed by Western blotting with the POL3/3 antibody. In SDS-PAGE, the RPB1 subunits hyperphosphorylated (IIo) or hypophosphorylated (IIa) on the CTD migrate like 240- and 214-kDa proteins, respectively (12, 15). For quantification, the POL3/3 antibody was detected with a radioiodinated anti-mouse antibody. The total amount of RPB1 molecules present in each oocyte was roughly proportional to the oocyte volume (Table 1). The exact amount of RPB1 subunits found in stage VI oocytes depended on the animal from which the oocytes originated and was found to be roughly equivalent to the amount of RPB1 found in 4×10^4 to 8×10^4 A6 cells. These values are consistent with those from previous reports (43).

Equivalent amounts of both hypophosphorylated (IIa) and hyperphosphorylated (IIo) forms were found in exponentially growing somatic *Xenopus* cells (A6 cells) (Fig. 1). The hypo-

TABLE 1. Quantification of RPB1 subunit in oocytes[a]

Expt. no.[b]	Stage	Oocyte vol (mm^3)	Amt of RPB1 (IIa + IIo)/cell (arbitrary units)
1	I	0.015	8
2	II	0.05	28
3	III	0.11	67
4	IV	0.525	170
5	V	0.90	580
6	VI	1.15	655
7	VI	1.15	1,100
8	VI	1.15	600
9	Egg	1.15	1,300
	A6 cells		0.015

[a] The lysates used for Fig. 1 were analyzed in an independent experiment with an iodinated anti-mouse antibody for a quantitative detection. The resulting Western blot was analyzed with a PhosphorImager; the area of the RPB1 IIa and IIo peaks was evaluated in arbitrary units and normalized to the number of cell equivalents loaded in each lane. The oocyte volumes were estimated from their reported mean diameters (16).

[b] Experiments 1 to 6 correspond to oocytes isolated from the same female. Experiments 7, 8, and 9 each corresponded to distinct animals.

phosphorylated IIa subunit band predominated in oocytes from stage I to stage III, but bands corresponding to hyperphosphorylated forms migrating more slowly than the IIa subunit were clearly detectable (Fig. 1). The relative amount of the hyperphosphorylated forms decreased along oogenesis and became barely detectable in oocytes at stages IV, V, and VI. Oocyte maturation was characterized by a spectacular change in the RPB1 phosphorylation steady state. In the natural metaphase II-arrested oocytes (i.e., the unfertilized eggs), the band corresponding to the hyperphosphorylated IIo subunit increased markedly (Fig. 1). Stage VI oocytes in prophase can be induced to proceed to metaphase II ex vivo by simple addition of progesterone to the medium. As in metaphase II-arested eggs, the IIa and IIo subunits were found in close amounts in ex vivo-matured oocytes (Fig. 2).

Thus, phosphorylation of the RPB1 subunit is tightly regulated during oogenesis and maturation. The phosphorylation of RPB1 in fully grown oocytes is a remarkable event which can be analyzed ex vivo during progesterone-induced maturation.

Activation of a CTD kinase during oocyte maturation. Transcription is totally arrested during meiosis (31). This arrest might have affected the phosphorylation state of RPB1 during maturation, since transcription involves a cycle of RPB1 phosphorylation and dephosphorylation (10, 12). To address this hypothesis, the phosphorylation of RPB1 was investigated during oocyte maturation in the presence of a transcription inhibitor. Primer extension analysis indicated that actinomycin D (100 μg/ml) inhibited transcription from a cytomegalovirus promoter fused to the chloramphenicol acetyltransferase cDNA and microinjected in stage VI oocyte (2a). In the presence of actinomycin D (100 μg/ml), the RPB1 subunit remained hypophosphorylated in prophase-arrested stage VI oocytes, and RPB1 hyperphosphorylation occurred during progesterone-induced maturation (Fig. 2). This finding demonstrated that a simple arrest of transcription was not sufficient to ensure the change in RPB1 phosphorylation observed after progesterone treatment.

We thus examined whether a CTD kinase might have been activated during meiosis. Native extracts corresponding to the same number of oocytes were allowed to react in the presence of [γ-^{32}P]ATP with or without purified RNAPII. In an extract from metaphase II-arrested eggs, a protein comigrating with RPB1 incorporated high levels of radioactivity (Fig. 3A). In contrast, no phosphorylation of this protein was detected in extracts from stage VI oocytes (Fig. 3A), and no phosphorylation of the smaller RNAPII subunits was detected in either extract (data not shown). We next investigated the capacity of the extracts to phosphorylate the Ctd-4 peptide, which consists of four repeats of the CTD consensus motif: Ser-Pro-Thr-Ser-Pro-Ser-Tyr (9). The Ctd-4 peptide was phosphorylated in extracts from metaphase II-arrested eggs but not in extracts from prophase stage VI oocytes (Fig. 3B).

These results indicate that a CTD kinase is activated during oocyte maturation. This kinase might be responsible for RPB1 phosphorylation.

Xp42 MAP kinase is a major CTD kinase present in extracts from unfertilized eggs. Attempts were made next to characterize the CTD kinase activity present in the native oocyte extracts. Two kinases showing CTD kinase activity in vitro are important during maturation of oocytes: the $p34^{cdc2}$ kinase (reviewed in reference 34) and the Xp42 MAP kinase (reviewed in reference 37). The $p34^{cdc2}$ kinase binds to the p13 yeast *suc1* gene product and phosphorylates histone H1 with high efficiency. To investigate the contribution of this kinase to CTD kinase activity, an extract from metaphase II-arrested eggs was fractionated on p13-agarose beads and assayed for histone H1 and Ctd-4 phosphorylation. Most of the histone H1 kinase activity (98%) was retained on the beads (Fig. 3C). In contrast, most of the Ctd-4 kinase activity (82%) remained in solution. The $p34^{cdc2}$ kinase was therefore responsible for less than 18% of the CTD kinase activity present in the extract.

To investigate the contribution of the Xp42 MAP kinase to the CTD kinase activity, a clarified extract from metaphase II-arrested eggs was fractionated by FPLC. The eluting fractions were assayed for their capacity to phosphorylate the RPB1 subunit from purified RNAPII core enzyme and the Ctd-4 peptide. The activity phosphorylating RPB1 eluted as a single peak concentrated mainly in two fractions at 180 mM NaCl (Fig. 4A, top panel). Incorporation of radioactive phosphate into RPB1 was quantified with a PhosphorImager; 69% of the polymerase kinase activity present in the extract loaded on the column was recovered as a single peak (fractions 35 to 38). The Ctd-4 kinase activities eluted in the same fractions (Fig. 4A, middle panel). The fractions showing maximal kinase activities contained the Xp42 MAP kinase in its activated form, as detected by Western blotting (Fig. 4A, bottom panel). The same fractions also showed maximal capacity to phosphorylate

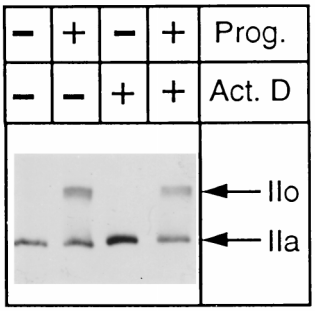

FIG. 2. Phosphorylation of RPB1 in *Xenopus* oocytes maturing ex vivo. Stage VI oocytes from a single female were sorted and treated (+) or not treated (−) with progesterone (Prog.) for 6 h. Analysis of phosphorylation of the RPB1 polymerase subunit in the presence (+) or absence (−) of 100 μg of actinomycin D (Act. D) per ml was carried out by Western blot analysis as for Fig. 1.

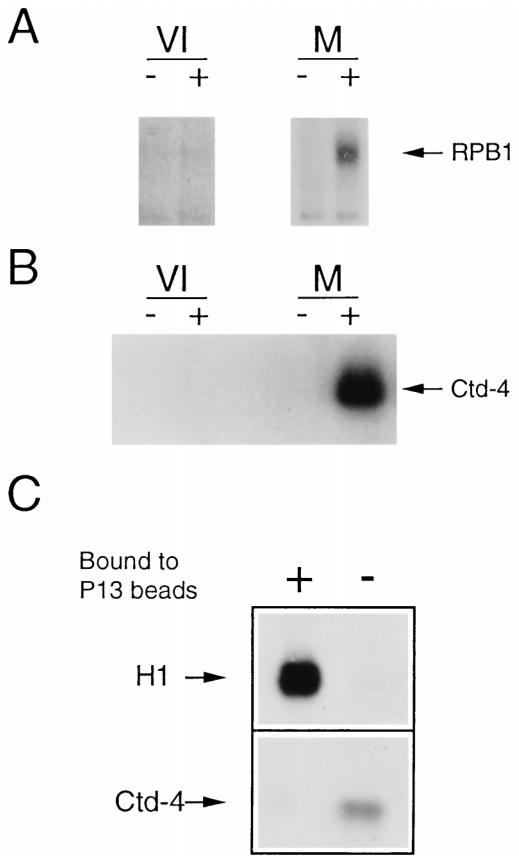

FIG. 3. Enhanced CTD kinase activity in extracts from metaphase II-arrested oocytes. (A) Extracts from stage VI oocytes (VI) or unfertilized eggs (M) were incubated with (+) or without (−) purified RNAPII in the presence of [γ-^{32}P]ATP. (B) Extracts from stage VI oocytes (VI) or unfertilized eggs (M) were incubated with (+) or without (−) Ctd-4 peptide in the presence of [γ-^{32}P]ATP. (C) An extract from unfertilized eggs was incubated with p13-agarose beads. The beads (+) and the remaining supernatant (−) were assayed for histone H1 and Ctd-4 phosphorylation in the presence of [γ-^{32}P]ATP. The reaction mixtures were electrophoresed in 6 or 15% polyacrylamide gels and processed for autoradiography. The phosphorylated RPB1 polymerase subunit migrated at a position corresponding to a molecular mass of 214 kDa. The phosphorylated Ctd-4 peptide and histone H1 migrated at positions corresponding to molecular weights of 23 and 33 kDa, respectively.

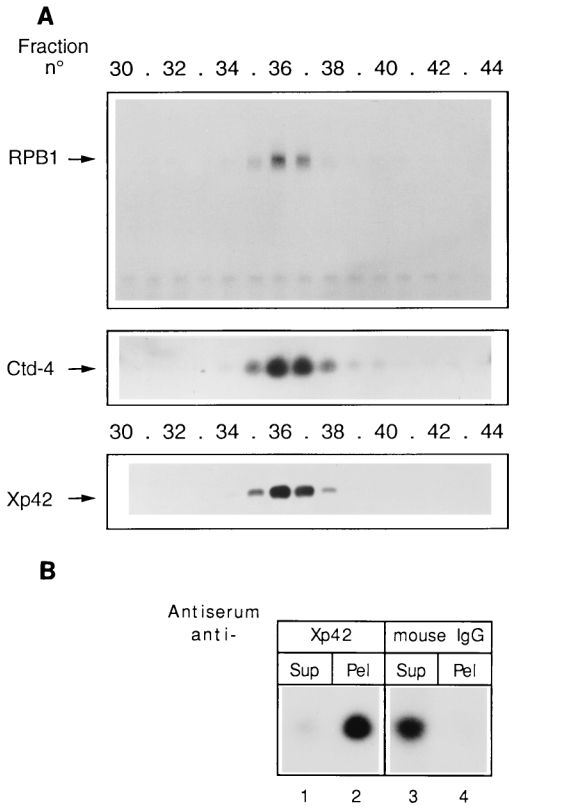

FIG. 4. Identification of a CTD kinase in matured oocyte extracts with Xp42 MAP kinase. (A) Extracts from metaphase II-arrested unfertilized oocytes were fractionated by chromatography onto a Mono Q column and eluted with a sodium chloride linear gradient. Aliquots from the Mono Q fractions were incubated with purified RNAPII (top) or with Ctd-4 peptide (middle) in the presence of [γ-^{32}P]ATP. The reaction mixtures were electrophoresed in gels, which were then autoradiographed (bottom). The presence of Xp42 MAP kinase in the fractions was probed by Western blotting with an anti-ERK1 antibody. The figure is restricted to 15 fractions, from fraction 30 (150 mM NaCl) to fraction 44 (220 mM NaCl). (B) Phosphorylation of the Ctd-4 peptide in supernatants (lanes 1 and 3 [Sup]) or pelleted beads (lanes 2 and 4 [Pel]) from fraction 36 preincubated with protein A coated with rabbit anti-Xp42 antiserum (lanes 1 and 2) or with rabbit anti-mouse immunoglobulin G (IgG) antiserum (lanes 3 and 4).

MBP, a characteristic ERK-type MAP kinase substrate (data not shown).

To demonstrate that the CTD kinase activity eluting from the column was related mainly to Xp42 MAP kinase, aliquots of fraction 36 were incubated with protein A beads precoated with an anti-Xp42 MAP kinase antiserum. Most of the Ctd-4 kinase activity was indeed immunodepleted from the supernatant and recovered with the anti-MAP kinase-coated beads (Fig. 4B). In contrast, when the protein A beads were precoated with a control anti-mouse immunoglobulin G rabbit antiserum, most of the Ctd-4 kinase activity remained in the supernatant. Hence, most of the CTD kinase activity present in extracts from metaphase II-arrested oocytes could be attributed to the Xp42 MAP kinase.

RPB1 phosphorylation after p34^{cdc2} kinase activation requires protein synthesis. Having established that Xp42 MAP kinase is a major CTD kinase present in extracts from matured oocytes, we looked for evidence of a link between MAP kinase activation and RPB1 phosphorylation ex vivo. The microinjection of stage VI oocytes with a recombinant stable cyclin B can activate the p34^{cdc2} kinase (36) and the Xp42 MAP kinase (20, 22, 24, 40). However, in contrast to p34^{cdc2} kinase activation, Xp42 kinase activation after cyclin microinjection requires protein synthesis (25).

Stage VI oocytes were microinjected with recombinant sea urchin Δ13-cyclin B1, pooled, and lysed in buffer EB. A strong histone H1 kinase and a strong capacity to phosphorylate MBP, a MAP kinase substrate, were found in the oocytes under these conditions. The H1 kinase was retained on p13-coated agarose, as expected for p34^{cdc2} kinase, but the MBP kinase was not (Fig. 5A). In the presence of cycloheximide, however, cyclin microinjection did not activate an MBP kinase, although it did activate an H1 kinase which was retained on p13-agarose (Fig. 5A).

We thus used the cyclin microinjection method of artificial maturation to monitor changes in the phosphorylation status of RPB1. Microinjection of the recombinant cyclin B in stage VI oocytes promoted the appearance of a phosphorylated form of RPB1 and the phosphorylation of Xp42 (Fig. 5B). In the presence of cycloheximide, however, microinjection of the truncated cyclin did not promote significant RPB1 phosphorylation and prevented the phosphorylation of Xp42 (Fig. 5B).

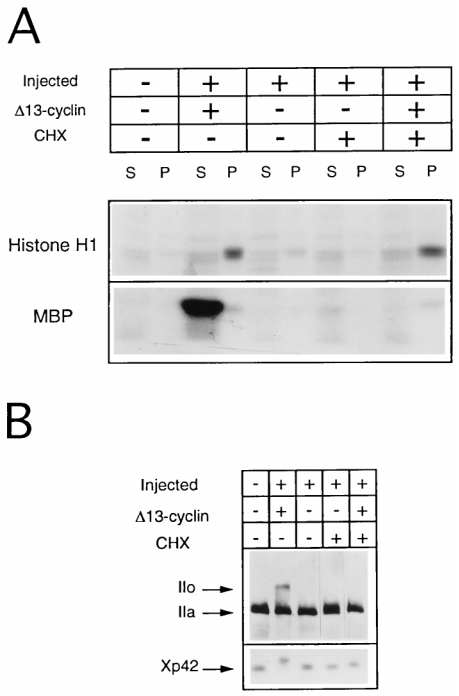

FIG. 5. Phosphorylation of the RPB1 in *Xenopus* oocytes microinjected with recombinant cyclin B1. Stage VI oocytes were microinjected (+) or not (−) with (+) or without (−) GST-Δ13 -sea urchin cyclin B1 in XB buffer, as described in Materials and Methods. The oocytes were either pretreated (+) or not (−) with cycloheximide (CHX). (A) EB buffer extracts from 10 microinjected oocytes were incubated with p13-agarose beads. Supernatants (S) or bead pellets (P) were allowed to react with histone H1 (H1) or MBP and [γ-^{32}P]ATP. Phosphorylated products were analyzed by PAGE (15% polyacrylamide) and autoradiographed. (B) Pools of five microinjected stage VI oocytes were lysed in Laemmli buffer, and aliquots corresponding to 0.1 oocyte were electrophoresed. The phosphorylation state of RPB1 was investigated with the POL3/3 or anti-ERK1 antibodies after transfer onto nitrocellulose as described in the legend to Fig. 1.

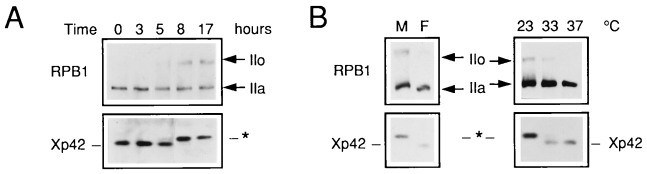

FIG. 6. RPB1 and Xp42 MAP kinase phosphorylation during progesterone-induced maturation of oocytes and after activation of mature oocytes. (A) Phosphorylation of RPB1 (top) and Xp42 MAP kinase (bottom) was analyzed in a time course after progesterone treatment of stage VI oocytes. The duration of the progesterone treatment is indicated in hours. Afte 5 h of progesterone treatment there were 0% germinal vesicle breakdowns (GVBD) and 100% germinal vesicle breakdowns after 8 h in progesterone. (B) Mature oocytes (M) were activated by fertilization (F) and lysed 60 min after sperm mixing or were activated by being heated for 60 min at the indicated temperature before lysis. Whole-cell lysates were analyzed by Western blotting as for Fig. 1, with the POL3/3 antibody or an anti-ERK1 antibody. The position corresponding to the phosphorylated Xp42 MAP kinase is indicated by a star.

This observation demonstrates that p34^{cdc2} kinase activation is insufficient per se to promote phosphorylation of RPB1 during oocyte maturation. In agreement with this observation, we did not observe changes in the steady-state phosphorylation of RPB1 in A6 cells arrested in mitosis after nocodazole treatment (data not shown). The requirement for protein synthesis is consistent with the involvement of the Xp42 MAP kinase in phosphorylating RPB1 ex vivo.

RPB1 hyperphosphorylation follows Xp42 MAP kinase activation. To further examine whether RPB1 phosphorylation correlated with Xp42 MAP kinase activation, phosphorylation of both proteins was observed during the course of oocyte maturation ex vivo. The oocytes from a single female were treated with progesterone and divided into batches which were lysed at a given time either in denaturing SDS buffer or in kinase buffer. In this experiment, the IIo subunit appeared in oocytes after 5 h in progesterone and peaked after 8 h of treatment (Fig. 6A). The active Xp42 MAPK is phosphorylated on threonine and tyrosine residues and is characterized by a slower electrophoretic migration than the inactive form. The phosphorylation of Xp42 MAP kinase appeared after 5 h in progesterone; full phosphorylation was observed after 8 h of treatment. Hyperphosphorylation of the RPB1 subunit followed the same time course (Fig. 6A).

RPB1 phosphorylation was investigated after fertilization or heat shock of mature *Xenopus* oocytes. Such treatments triggered a rapid dephosphorylation of the Xp42 MAP kinase (Fig. 5B) as described previously (20, 23, 40). RPB1 was rapidly dephosphorylated after fertilization or after a heat shock (Fig. 6B). In the latter experiments, the dephosphorylation of both RPB1 and Xp42 was more pronounced after a heat shock at 37°C than at 33°C.

These findings indicated that RPB1 hyperphosphorylation and activation of the Xp42 MAP kinase are parallel events during oocyte maturation.

DISCUSSION

In this report, we show that the RPB1 subunit is essentially dephosphorylated in growing *Xenopus* oocytes from stage IV to stage VI as previously reported (51). An abrupt change in RPB1 phosphorylation occurs during meiosis: the proportion of hyperphosphorylated subunit increases dramatically after germinal vesicle breakdown and decreases back after fertilization. Although the role of the RPB1 phosphorylation in the oocyte remains largely unknown, these observations demonstrate a developmentally regulated posttranslational modification of the basal transcription machinery.

Phosphorylation of the RPB1 subunit by the Xp42 MAP kinase. In somatic cells, the steady-state proportion between hypophosphorylated and phosphorylated forms of RPB1 relies on a balance between CTD kinases and CTD phosphatases, depending on transcription. The increased RPB1 phosphorylation during maturation is likely due to an increased CTD kinase activity, since it occurs in the presence of the transcriptional inhibitor actinomycin D. Many protein kinases phosphorylate the CTD in vitro, but few may function as CTD kinases in vivo. The general transcription factor TFIIH phosphorylates the CTD in vitro (19, 44). Inactivation of the yeast TFIIH kinase, Kin28, results in RPB1 dephosphorylation in vivo (53). In cultured cells exposed to TFIIH kinase inhibitors, the RPB1 subunit is dephosphorylated (15). The TFIIH kinase cdk7 (MO15) is present in *Xenopus* oocytes and embryos (21, 39). However, no variations in cdk7 kinase activity have been described during oocyte maturation and early development (4). Furthermore, the RPB1 subunit was phosphorylated upon progesterone-induced maturation in the presence of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (100 μM) (data not shown), which has been shown to inhibit the cdk7-driven RPB1 phosphorylation (15, 56). Environmental conditions (stress or growth factors) alter the IIa/IIo ratio (14, 55). In both cases, the involvement of activated ERK1 and ERK2 MAP kinases has been proposed. The Xp42 MAP kinase is an ERK homolog (22); the present data strongly suggest that it

phosphorylates the RPB1 subunit during oocyte maturation. First, most of the CTD kinase activity in the extracts was dependent on the presence of Xp42 MAP kinase. Second, during progesterone-induced maturation and after fertilization, the phosphorylation of RPB1 correlated with the presence of an activated Xp42 MAP kinase. Third, in cyclin B-injected oocytes, phosphorylation of RPB1 and activation of Xp42 MAP kinase both required protein synthesis. Thus, these results emphasize the parallel between the signal transduction pathways involved in the somatic cell responses and the hormonal triggering of maturation.

RNAPII is stored in large amounts during oogenesis. From stage I to stage IV, the oocyte volume increases about 40-fold, and from stage IV to stage VI, it increases about 2-fold. Meanwhile, the chromosomal templates remain in a constant amount. The amount of RNAPII engaged in transcription on the lampbrush chromosomes is equivalent to that found in 2×10^3 somatic cells (13). We estimated that the oocytes contain as much RPB1 subunit as 4×10^4 to 8×10^4 A6 cells. These values were in agreement with initial reports: stage VI oocytes showed as much RNAPII activity as 10^5 kidney cells (43). Importantly, this finding strongly suggests that the specific activities of RNAPII are approximately the same in a somatic cell and in an oocyte. The RNAPII stored at the latest stages of oogenesis is in a large excess over the RNAPII engaged in transcription; it might be required to support the zygotic transcription from the midblastula transition up to the neurula stage.

Developmental regulation of RPB1 phosphorylation. In *Xenopus* A6 cells, like in mammalian fibroblasts (15), the hyperphosphorylated and hypophosphorylated forms of the RPB1 subunit are present in equal amounts. In contrast, the RPB1 subunit was essentially dephosphorylated in growing *Xenopus* oocytes from stage IV to stage VI. Phosphorylation of RPB1 is incomplete in the mature oocyte; at least half of the RPB1 molecules remain hypophosphorylated. Two alternative hypotheses may be considered. (i) The mature *Xenopus* oocyte is heterogeneous; part of the RPB1 molecules might not be accessible to MAP kinase. (ii) CTD phosphatases might counteract the action of MAP kinase. In support of the latter hypothesis, the amount of IIo forms decreases rapidly after fertilization when Xp42 MAP kinase is turned off.

The significance of major posttranslational changes affecting a fraction of the RNAPII is puzzling. Because of the large excess of polymerase stored in the oocyte, a minor proportion of the RPB1 subunits might be involved in transcription. A global control of RNA synthesis by aberrantly phosphorylated RPB1 has previously been suggested to occur during herpesvirus infection (42). It has recently been proposed that promoters recruit RNAPII holoenzymes to initiate transcription (29, 38). The RNAPII holoenzymes are complexes formed between the RNAPII core enzyme and the general transcription factors. In particular, the general transcription factors TFIID, TFIIE, and TFIIH have been proposed as the constituents of the mammalian holoenzymes. Furthermore, it has been shown that phosphorylation of the CTD prevents the interaction of RPB1 with the basal transcription factors TFIID and TFIIE (35, 52). The RPB1 subunit in the holoenzyme is not phosphorylated (38). Phosphorylation of the CTD, supposedly by the Xp42 kinase, might destabilize the holoenzyme and hence would contribute to abolish transcription in the *Xenopus* meiotic oocyte. This issue is currently under investigation.

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