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Metaphase Protein Phosphorylation in *Xenopus laevis* Eggs

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Cytoplasmic extracts of metaphase (M-phase)-arrested *Xenopus laevis* eggs support nuclear envelope breakdown and chromosome condensation in vitro. Induction of nuclear breakdown is inhibited by AMPP(NH)P, a nonhydrolyzable ATP analog, but not by ATP or γ-S-ATP, a hydrolyzable ATP analog, suggesting that protein phosphorylation may be required for M-phase nuclear events in vitro. By addition of [γ-^{32}P]ATP, we have identified in cytoplasmic extracts and in intact eggs at least six phosphoproteins that are present during M-phase but absent in G_1/S-phase. These phosphoproteins also appear in response to partially purified preparations of maturation-promoting factor. A subset of these proteins are thiophosphorylated by γ-S-ATP under conditions that promote nuclear envelope breakdown and chromosome condensation. Each of these proteins is phosphorylated on serine and threonine, and one, a 42-kilodalton protein, is also phosphorylated on tyrosine both in extracts and in intact eggs. These results indicate that activation of protein kinases accounts for at least part of the increased phosphorylation in M-phase and that both protein-serine-threonine kinases and protein-tyrosine kinases may play a role in controlling M-phase nuclear behavior.

The entry into mitosis by cells of higher eucaryotes is marked by the condensation of the interphase chromatin into metaphase (M-phase) chromosomes and the breakdown of the nuclear envelope (NE) before cell division. Upon completion of karyokinesis, the NE is again assembled around the chromosomes as they decondense, reforming an interphase nucleus in each of the daughter cells. Cell fusion experiments at different phases of the cell cycle have clearly demonstrated that NE breakdown and chromosome condensation are regulated by a cytoplasmic factor(s) that is active during late G_2- and M-phase of the cell cycle but that disappears in the daughter cells during early G_1-phase when nuclei reform (for a review, see references 42 and 43).

During the meiotic divisions of oocytes, NE breakdown and chromosome condensation are also under cytoplasmic control. In amphibians, fully grown oocytes are arrested in prophase I and resume meiosis when maturation is initiated by exposure to insulin or progesterone (for a review, and see references 28, 29, 32). The series of nuclear changes during oocyte maturation and fertilization form a simple cell cycle, comprising a G_2-to-M transition as fully grown oocytes mature and an M-to-G_1/S transition at fertilization. During maturation, the oocyte chromosomes condense, the NE breaks down, and a spindle forms. Oocytes complete the first meiotic division and continue through meiosis II to M-phase, in which they again arrest as unfertilized eggs. Fertilization or parthenogenetic activation releases the M-phase arrest, allowing completion of meiosis and the formation of interphase nuclei (pronuclei) from both the egg chromosomes and the nucleus of the fertilizing sperm. The pronuclei undergo DNA replication soon after their formation. When sperm nuclei or interphase nuclei from various somatic tissues are microinjected into the egg cytoplasm, they undergo the same events as the resident nucleus: NE breakdown, chromosome condensation, and spindle formation in M-phase-arrested eggs (14, 36, 55), but NE assembly, chromatin decondensation, and DNA replication in fertilized or parthenogenetically activated eggs (7, 11, 19).

NE breakdown and chromosome condensation in maturing oocytes are thought to be regulated by a cytoplasmic factor(s) that appears soon after hormone stimulation. The transfer of cytoplasm from maturing amphibian oocytes into immature oocytes induces the precocious breakdown of the NE and chromosome condensation without hormone treatment (33, 44). Recipients subsequently undergo the entire process of oocyte maturation; the factor that induces these nuclear changes has been called "maturation promoting factor" (MPF). MPF activity has been detected in maturing oocytes from many species of animals (20, 22, 23, 45) and has been partially purified from amphibian (52) and starfish (21) eggs. The cytoplasm of mitotically dividing cells in late G_2- or M-phase of the cell cycle also induces maturation when injected into immature oocytes (22, 37, 48–50). In all cases examined, MPF activity oscillates with the meiotic or mitotic cell cycle, reaching a high level in late G_2 and M but decreasing to an undetectable level in early G_1 and throughout interphase (see also references 5 and 10). Thus, MPF may be thought of as a widespread regulator of M-phase-specific nuclear changes in both meiotic and mitotic cells.

Although the mechanism by which MPF controls NE breakdown and chromosome condensation is not yet understood, several lines of evidence suggest that protein phosphorylation plays an important role in its action. In maturing oocytes, the level of protein phosphorylation increases shortly before NE breakdown, at a time coincident with the appearance of MPF activity (31), and oscillates with MPF activity during the meiotic divisions, decreasing again before pronuclear formation (5, 41). Hormonally stimulated oocytes that fail to undergo the initial burst in phosphorylation also fail to mature, whereas the injection of partially purified MPF elicits a precocious increase in protein phosphorylation before NE breakdown (31, 52). Although no specific protein kinase has been shown to copurify with MPF activity, the endogenous protein kinase activity of MPF preparations is enriched during purification (9, 52). Finally, when oocyte maturation is induced by either hormonal stimulation or

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MPF injection, new phosphoproteins can be identified before NE breakdown by two-dimensional polyacrylamide gel electrophoresis (30). Together, these observations support the hypothesis that a change in protein phosphorylation underlies the mechanism by which MPF triggers M-phase-specific nuclear changes. Indeed, active MPF may itself be a phosphoprotein, since its extraction from unfertilized eggs is stabilized by the presence of phosphorylated small molecules, such as β-glycerophosphate, and by NaF, ATP, and (γ-S-ATP), compounds that inhibit the action of protein phosphatases (6, 9, 17, 52). Furthermore, MPF activity stabilized by the presence of γ-S-ATP can be immunoprecipitated by antibodies against the thiophosphate moiety (9).

We examined the requirement for protein phosphorylation in NE breakdown and in chromosome condensation by using cytoplasmic extracts of *Xenopus laevis* eggs that support the nuclear changes seen in intact cells. Extracts of unfertilized eggs, which are physiologically arrested in M-phase, induce isolated sperm or somatic cell nuclei to undergo NE breakdown, chromosome condensation, and spindle formation (24, 26, 35). Because these nuclear responses are similar to those induced by the cytoplasm of intact M-phase-arrested eggs, we call these extracts "M-phase extracts." In contrast, extracts prepared from eggs that are no longer arrested in M-phase induce NE assembly, chromosome decondensation, pronuclear formation, and DNA synthesis (24, 25). We call these "G_1/S-phase extracts" since the nuclear changes they support are similar to those that occur during G_1 and S phases of the cell cycle in most proliferating cells even though early *X. laevis* embryos do not have a G_1 phase (12). M-phase extracts can be converted to G_1/S-phase extracts in vitro by the addition of Ca^{2+} ions (24, 26), the probable physiological stimulus for activation of the egg at fertilization (2, 46). Conversely, MPF activity induces NE breakdown, chromosome condensation, and spindle formation when added to sperm pronuclei formed in G_1/S-phase extracts (24, 35). Thus, the ability of extracts to support either M-phase or G_1/S-phase nuclear changes can be changed easily by the addition of Ca^{2+} ions or by MPF activity. In this paper, we used this cell-free system to investigate the involvement of protein phosphorylation in regulating nuclear behavior in vitro and in vivo.

MATERIALS AND METHODS

Preparation of sperm nuclei. *X. laevis* sperm were isolated as previously described by Stick and Hausen (47). The sperm were treated with lysolecithin (Sigma Chemical Co., St. Louis, Mo.) to remove plasma membranes and nuclear envelopes and stored at −70°C (24, 25).

Preparation of egg extracts. Both M-phase extracts and G_1/S-phase extracts were prepared as described previously (24). In most experiments, M-phase extracts were prepared by use of a modified MPF buffer (52), but in some, an extraction buffer containing 10 mM EGTA was used instead (24). M-phase cytosol was prepared by centrifugation of M-phase extracts at 250,000 × *g* for 3 h in an SW55 rotor. The supernatant obtained after centrifugation was divided into portions, frozen in a mixture of dry ice and ethanol, and stored at −70°C.

Incubation of sperm nuclei with egg extracts. Unless otherwise specified, sperm nuclei were mixed with 200 μl of egg extract to give a concentration of 10^6 nuclei per ml and the mixtures were incubated at 19 ± 1°C (24). For examination of the effect of ATP analogs, stock solutions of ATP (Sigma), γ-S-ATP, and AMPP(NH)P (both from Boehringer Mannheim Biochemicals, Indianapolis, Ind.) were prepared in 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.5, at concentrations of 10, 25, and 50 mM, with equimolar amounts of $MgCl_2$. Samples were stored at −20°C. When ATP analogs were added to G_1/S-phase extracts, 20 μl of a stock solution was added to 180 μl of extract before it was mixed with the sperm nuclei.

The effect of ATP analogs on NE breakdown and chromosome condensation was examined in the following manner. Sperm nuclei were incubated in G_1/S-phase extracts for 60 to 75 min, during which time interphase pronuclei formed. Several of the mixtures were pooled and divided again into 75-μl samples. Each sample received 15 μl of the ATP, γ-S-ATP, or AMPP(NH)P stock solution, 35 μl of MPF buffer, and 25 μl of the M-phase cytosol. The mixtures were incubated at 19 ± 1°C, and at various times samples were mixed with an equal volume of a 5-μg/ml solution of DAPI (Boehringer Mannheim). Changes in nuclear morphology were examined by phase-contrast and fluorescence microscopy with a Leitz Dialux microscope (Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N.J.).

Protein phosphorylation in egg extracts. Sperm nuclei were mixed with 200 μl of freshly prepared M-phase or G_1/S-phase extracts. A 100-μl quantity of each mixture was used to examine endogenous protein phosphorylation, and the remainder was incubated at 19°C. Only the results from extracts that supported either pronuclear formation or chromosome condensation and spindle assembly in parallel incubations are reported.

To examine endogenous protein phosphorylation, 10^8 cpm of [γ-^{32}P]ATP, prepared by the procedure of Johnson and Walseth (18), was added in a volume of 5 μl or less to 100 μl of the mixture of extract and nuclei. Samples were incubated at room temperature for 10 min, and the reaction was terminated by addition of 10 μl of stop solution (100 mM EDTA, 100 mM NaF, 100 mM sodium pyrophosphate, 10 mM sodium vanadate [pH 7.0]). Samples were placed on ice and processed for two-dimensional electrophoresis. The thiophosphorylation of proteins in each of the extracts was examined in a similar manner, except that 10^8 cpm [γ-^{35}S]ATP (specific activity, 1,167 Ci/mmol; New England Nuclear Research Products, Boston, Mass.) was added to 100 μl of extract and incubations were extended to 30 min.

The changes in protein phosphorylation that accompanied the Ca^{2+}-induced conversion of M-phase extracts to G_1/S-phase were examined as follows. Samples (10 μl) of $CaCl_2$ solutions at concentrations of 20, 30, 40, and 50 mM were added to 240 μl of M-phase extracts prepared with extraction buffer containing 10 mM EGTA. Extracts were mixed with sperm nuclei, and 100 μl of each sample was incubated with [γ-^{35}P]ATP as described above. The remainder was incubated at 19°C for 90 min, and the morphology of the incubated nuclei was examined by phase-contrast and fluorescence microscopy.

In some experiments, 20 μl of partially purified MPF, a gift of J. C. Gerhart, University of California, Berkeley, was mixed with 30 μl of MPF buffer and added to 150 μl of a G_1/S-phase extract in which pronuclei had formed during the previous 60 to 75 min. As a control, 50 μl of MPF buffer only, without MPF activity, was mixed with 150 μl of the G_1/S-phase extract. The mixtures were incubated at 19°C, and samples were examined periodically by phase-contrast and fluorescence microscopy. When all of the pronuclei had undergone NE breakdown and chromosome condensation in

extracts that had received MPF, 100 μl of each extract was removed, mixed well with [γ-^{32}P]ATP, and incubated as described above.

Protein phosphorylation in intact eggs. Eggs were collected from *X. laevis* females that had been induced to ovulate by injection of human chorionic gonadotropin 12 to 14 h earlier (24). Undamaged eggs were dejellied in small batches with 5 mM β-mercaptoethanol in a modified Barth solution (MBS; 15) and washed well with MBS. Eggs were injected with approximately 1.5×10^7 to 3.0×10^7 cpm of [γ-^{32}P]ATP in 50 to 100 nl of Tris buffer (18) and incubated at room temperature. Parthenogenetic activation during the injection procedure was prevented by incubating eggs in MBS containing 10 mM chlorobutanol (2) for 15 min before injection, during the injection, and for the entire period after injection. When activation was not suppressed, eggs were injected in MBS and transferred 10 min later to MBS containing 10 mM chlorobutanol. At 30 min after the injection of [γ-^{32}P]ATP, groups of 10 to 20 eggs were washed with 25 ml of MPF buffer and transferred to 0.5-ml Microfuge tubes containing the same buffer. Once the eggs had settled, the excess buffer was withdrawn and tubes were centrifuged for 30 s at 4°C with a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.). For each egg, 10 μl of stop solution was mixed with the supernatant above the packed yolk and pigment. The mixture was transferred to a clean Microfuge tube and centrifuged again for 5 min at 4°C, and the supernatants were processed for two-dimensional electrophoresis.

Gel electrophoresis. Samples were treated on ice with 100 μg of RNase A (Sigma) per ml for 15 min, and solid urea was added to a concentration of 9 M. An equal volume of lysis buffer (39) was added, and samples were centrifuged for 5 min in a Microfuge before they were frozen on dry ice. Two-dimensional gel electrophoresis was carried out as described by O'Farrell (39) with the following modifications. Proteins were separated in the first dimension by isoelectric focusing in a mixture of ampholytes (1.4% pH 5 to 7 and 0.6% pH 3 to 10; Serva Fine Biochemicals Inc., Garden City Park, N.Y.) and in the second dimension by a 10 to 17.5% sodium dodecyl sulfate-acrylamide gradient gel with a low level of cross-linking (53). In the first dimension, the pH gradient ranged from approximately pH 4.5 to 7.5. In the case of ^{32}P-labeled samples, gels were stained and dried and autoradiography was performed on Kodak XRP-5 film (Eastman Kodak Co., Rochester, N.Y.) at −70°C with an intensifying screen (Du Pont Co., Wilmington, Del.). For ^{35}S-labeled samples, gels were treated with 1 M sodium salicylate (3) for 30 min before drying and autoradiography.

Phosphoamino acid analysis. Proteins were radiolabeled as described above, except that 5×10^8 cpm of [γ-^{32}P]ATP was added to each 100 μl of extract, and the proteins were then separated by two-dimensional gel electrophoresis. Gels that were neither stained nor dried were wrapped in Saran Wrap, and autoradiography was performed on Kodak XAR-5 film at 4°C overnight with an intensifying screen. The proteins were cut from several gels and eluted from the gel pieces with an electrophoretic sample concentrator (Isco, Inc., Lincoln, Nebr.). Proteins were mixed with 25 μg of bovine serum albumin, precipitated overnight in 20% trichloroacetic acid, washed twice with ice-cold ether, dissolved in 1 M NH_4OH, and dried under vacuum. Samples were hydrolyzed in constant boiling HCl for 3 h at 100°C, and the hydrolysates were dried under vacuum and dissolved in distilled water containing phosphoamino acid markers. One-dimensional electrophoresis was performed on Polygram cel 300 sheets (Brinkman Instruments Co., Westbury, N.Y.) at pH 3.5 (1,000 V for 2 h), and two-dimensional electrophoresis was performed on Kodak cellulose 13255 sheets (first dimension, pH 1.9, 760 V, 1.5 h; and second dimension, pH 3.5, 760 V, 1 h).

TABLE 1. Extract Analogs on pronuclear formation and M-phase nuclear behavior

Extract	Analog added[a]		
	ATP	γ-S-ATP	AMPP (NH) P
G_1/S-phase			
NE assembly	−[b]	−[c]	+
Chromatin decondensation	−[b]	−[c]	−[c]
M-phase			
NE breakdown	+	+	−[b]
Chromosome condensation	+	+	−[b]

[a] Nuclear changes were examined and analog additions were carried out as described in Materials and Methods. + indicates that a nuclear change occurred in the presence of ATP or its analog, whereas − indicates that the nuclear change was inhibited.
[b] Inhibition at ≥2.5 mM.
[c] Inhibition at ≥1.0 mM.

RESULTS

Effect of ATP analogs on pronuclear formation and M-phase nuclear changes. *X. laevis* sperm nuclei incubated in G_1/S-phase extracts were transformed within 45 to 60 min into sperm pronuclei, a process comprising the assembly of an NE at the periphery of the sperm chromatin, chromatin decondensation, and nuclear enlargement. The addition of M-phase cytosol to pronuclei formed in these extracts resulted in the breakdown of the NE and condensation of the chromatin into M-phase chromosomes within 30 to 60 min. The effects of ATP and its analogs on these nuclear changes are summarized in Table 1. Briefly, both ATP and γ-S-ATP inhibited pronuclear formation when added during the first 15 min of incubation but had little effect at later times when the NE had already begun to form. On the other hand, neither ATP or γ-S-ATP inhibited NE breakdown and chromosome condensation. Unlike ATP and γ-S-ATP, AMPP(NH)P did not inhibit NE assembly but prevented only the decondensation of the sperm chromatin within the nascent NE. However, AMPP(NH)P either delayed or inhibited NE breakdown and chromosome condensation induced by M-phase cytosol.

Two-dimensional gel electrophoresis of proteins phosphorylated in cell extracts. Since ATP and γ-S-ATP can be utilized by some protein kinases, whereas AMPP(NH)P cannot (54), the effects of these compounds suggested that protein phosphorylation may be required both for chromatin decondensation during pronuclear formation and for NE breakdown and chromosome condensation. For evaluation of this hypothesis, the proteins phosphorylated in extracts that supported M-phase nuclear changes were analyzed by two-dimensional electrophoresis and autoradiography and compared with those phosphorylated in extracts that supported pronuclear formation (Fig. 1). Many of the proteins phosphorylated in the two extracts were similar. However, proteins with apparent molecular weights of 110 kilodaltons (kDa) (pI, ~6.3 to 6.6), 49 kDa (pI, ~6.4), 42 kDa (pI, ~6.5 to 6.6), 41 kDa (pI, ~5.9 to 6.1), 38 kDa (pI, <5.0), and 35 kDa (pI, <5.0) were specifically phosphorylated in M-phase extracts. The pattern of protein phosphorylation was the same in all M-phase egg extracts that supported NE breakdown and

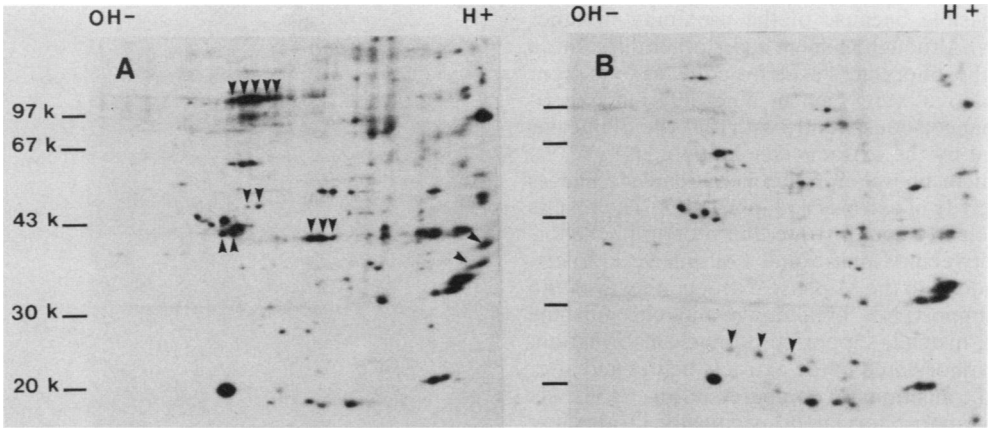

FIG. 1. Protein phosphorylation in M-phase and G_1/S-phase extracts of *X. laevis* eggs. M-phase or G_1/S-phase extracts were prepared from *X. laevis* eggs as previously described (24). Sperm nuclei were suspended in 200 μl of either extract, and [γ-^{32}P]ATP was added to a 100-μl sample of each mixture. Samples were incubated for 10 min at room temperature, at which time the reaction was stopped and samples were processed for two-dimensional electrophoresis and autoradiography. The remainder of the mixture of extract and nuclei was incubated at 19°C for 60 to 90 min to confirm that the extracts that were radiolabeled could support either chromosome condensation and spindle assembly (M-phase extracts) or pronuclear formation (G_1/S-phase extracts). (A) Proteins phosphorylated in M-phase extracts; (B) proteins phosphorylated in G_1/S-phase extracts. Arrows indicate proteins that are phosphorylated specifically in either M-phase (A) or G_1/S-phase (B) extracts.

chromosome condensation, regardless of whether they were prepared with MPF buffer or with extraction buffer containing 10 mM EGTA (24). With the possible exception of the 38- and 35-kDa proteins, the proteins appeared as two or more closely associated spots of similar molecular weight but of slightly different isoelectric points, a pattern characteristic of multiply phosphorylated forms of the same protein. In fact, as many as six adjacent spots could often be resolved in the region of the 110-kDa protein, suggesting that it may be a highly phosphorylated protein. After extracts were centrifuged at 250,000 × *g* for 3 h, the addition of [γ-^{32}P]ATP to the cytosol resulted in the phosphorylation of the same proteins, except for the 41-kDa protein, suggesting that either it or the kinase for which it is a substrate is associated with particulate material.

In contrast to the M-phase extracts, few proteins were specifically phosphorylated in G_1/S-phase extracts. Only a group of proteins with an apparent molecular weight of approximately 25 kDa were phosphorylated specifically in these extracts. None of the phosphoproteins that we detected in either M-phase or G_1/S-phase extracts were dependent on the presence of sperm nuclei. The M-phase-specific phosphoproteins that we observed were not abundant, as none were stained by Coomassie blue and even after silver staining the 110- and 41-kDa proteins could not be detected. Furthermore, discrete staining of the 49- and 42-kDa pro-

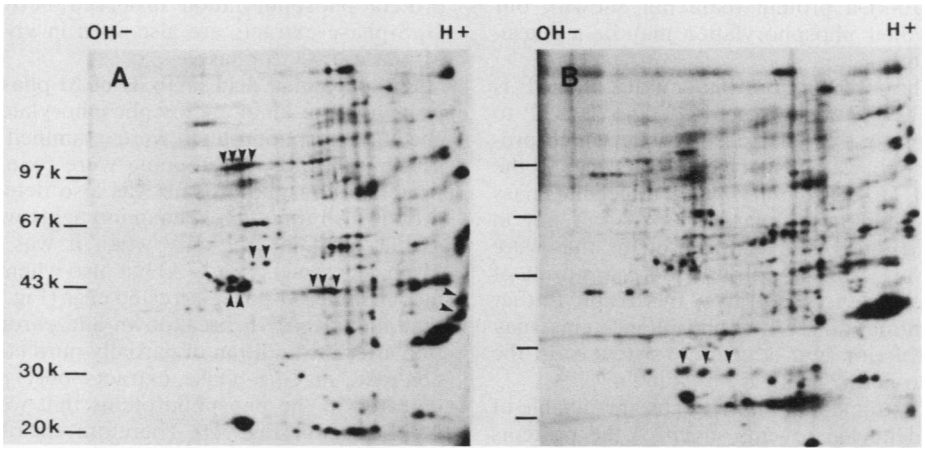

FIG. 2. Changes in protein phosphorylation induced by the addition of Ca^{2+} to M-phase extracts. M-phase extracts were prepared with an extraction buffer containing 10 mM EGTA (24). Sperm nuclei were suspended in 240-μl samples of the extract. A 10-μl portion of a $CaCl_2$ solution, at various concentrations, was added to each of the mixtures, as described in Materials and Methods. At 10 min later, [γ-^{32}P]ATP was added to 100 μl of each sample, and samples were incubated as described in the legend to Fig. 1. The remaining mixture was incubated at 19°C for 90 min to distinguish samples that supported chromosome condensation and spindle assembly from those that supported pronuclear formation. Shown are the proteins phosphorylated in an M-phase extract without (A) or with (B) added Ca^{2+}. In the experiment shown, the addition of 10 μl of a 50 mM $CaCl_2$ solution to 240 μl of an M-phase extract was sufficient to convert the extract to one supporting pronuclear formation. Arrows indicate proteins that are phosphorylated specifically in M-phase extracts before (A) or after (B) addition of Ca^{2+}.

teins was not possible because of the proximity of other abundant proteins. Although in some cases other differences in the pattern of phosphorylated proteins could also be seen, those described above were seen in nine different experiments and also changed consistently with the type of nuclear changes supported by the extracts (see below).

Changes in protein phosphorylation accompanied changes in the M-phase activity of extracts and intact eggs. The results described above clearly demonstrate that protein phosphorylation differs between M-phase and G_1/S-phase extracts. When Ca^{2+} was added to the M-phase extracts, they were no longer able to support NE breakdown and chromosome condensation but, instead, supported pronuclear formation. The proteins phosphorylated in the extracts before and after this Ca^{2+}-induced transition are compared in Fig. 2. The six proteins that were phosphorylated specifically in M-phase extracts (Fig. 2A) were not phosphorylated significantly after extracts were converted from M-phase to G_1/S-phase (Fig. 2B). Instead, the proteins of approximately 25-kDa molecular mass became phosphorylated. Thus, Ca^{2+} converted the pattern of protein phosphorylation from that seen in M-phase extracts to that of G_1/S-phase extracts, concomitant with the appearance of pronuclear forming activity.

The proteins phosphorylated after partially purified MPF was added to G_1/S-phase extracts were also examined. At the time that the sperm pronuclei underwent NE breakdown and chromosome condensation, the pattern of protein phosphorylation was similar to that of M-phase extracts (Fig. 3). The phosphorylation of the 25-kDa proteins seen in G_1/S-phase extracts was no longer detected. Instead, proteins of 110, 49, 42, 38, and 35 kDa were phosphorylated, and in some experiments the 41-kDa protein was phosphorylated as well. These changes in protein phosphorylation were not seen when MPF buffer alone was added to the G_1/S-phase extracts, nor were NE breakdown and chromosome condensation induced under these conditions. Therefore, these results show that MPF induced the pattern of protein phosphorylation seen in M-phase extracts. Since none of the M-phase-specific phosphoproteins were phosphorylated in the partially purified MPF preparations, with the possible exception of the 110-kDa protein (data not shown), our results suggest that their phosphorylation may be a consequence of MPF action.

As described in the introduction, the activity of MPF is stabilized by γ-S-ATP. Therefore, we added γ-^{35}S-ATP to M-phase and G_1/S-phase extracts to determine which proteins were thiophosphorylated specifically in each of the extracts. Proteins of 110-, 49-, and 42-kDa molecular mass were thiophosphorylated in M-phase extracts but not in G_1/S-phase extracts (Fig. 4). Since the proteins that were thiophosphorylated in M-phase extracts were a subset of those phosphorylated in these extracts, this result further supports the contention that these phosphoproteins may play a role in MPF action and is also consistent with the ability of γ-S-ATP to permit nuclear breakdown.

Although the cell extracts of *X. laevis* eggs are capable of supporting regulated nuclear events in vitro, the proteins phosphorylated in cytoplasmic extracts may not necessarily be the same as those phosphorylated in intact cells. To determine whether the pattern of protein phosphorylation observed in cell extracts is similar to that seen in intact eggs, unfertilized eggs were injected with [γ-^{32}P]ATP under conditions that either suppressed or allowed parthenogenetic activation. The proteins phosphorylated in unactivated eggs, which remain arrested in M-phase, appeared identical to those phosphorylated in M-phase extracts (Fig. 5A). In contrast, with the possible exception of the 49-kDa protein, these proteins were not phosphorylated in activated eggs, which are no longer in M-phase (Fig. 5B). Instead, the 25-kDa proteins phosphorylated in G_1/S-phase extracts were phosphorylated in activated eggs. Thus, the differences in protein phosphorylation observed between M-phase and G_1/S-phase extracts are also seen in vivo in intact eggs in M-phase or G_1/S-phase.

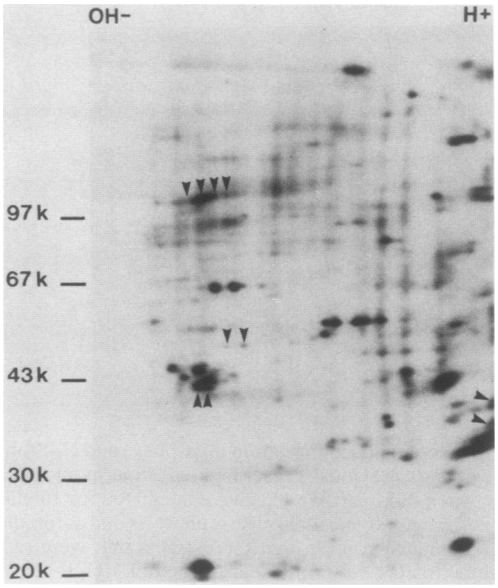

FIG. 3. Protein phosphorylation during MPF-induced NE breakdown and chromosome condensation in extracts. Sperm nuclei were suspended in a G_1/S-phase extract and incubated for 60 min. A 150-μl portion of the sample was mixed with partially purified MPF, as described in Materials and Methods. Nuclear morphology was examined periodically by phase-contrast and fluorescence microscopy after staining with DAPI. When all of the nuclei had undergone NE breakdown and chromosome condensation, approximately 80 min after addition of MPF in the experiment shown here, [γ-^{32}P]ATP was added to a 100-μl sample of the sample and incubated as described in the legend to Fig. 1. Arrows indicate proteins that were phosphorylated specifically after addition of MPF.

Phosphoamino acid analysis of M-phase-specific phosphoproteins. The amino acids phosphorylated in the M-phase-specific phosphoproteins were examined (Fig. 6). Phosphoserine and phosphothreonine were found in each of these proteins. Phosphotyrosine was also detected in the 42-kDa protein. All three phosphoamino acids were detected in the 42-kDa protein, not only when it was phosphorylated in M-phase extracts (Fig. 7A) but also when it was phosphorylated in intact M-phase-arrested eggs (Fig. 7B) and in extracts that supported NE breakdown and chromosome condensation after the addition of partially purified MPF (Fig. 7C). In contrast, in G_1/S-phase extracts only phosphoserine was detected in the phosphoproteins that were adjacent to the 42-kDa region (Fig. 7D). Therefore, the phosphotyrosine and phosphothreonine that we detected were derived from the M-phase-specific 42-kDa protein and not from contaminating proteins of similar molecular mass and isoelectric point that were phosphorylated in both M-phase and G_1/S-phase extracts.

DISCUSSION

The changes in nuclear organization that occur when a cell divides are preceded in both meiotic and mitotic cells by the

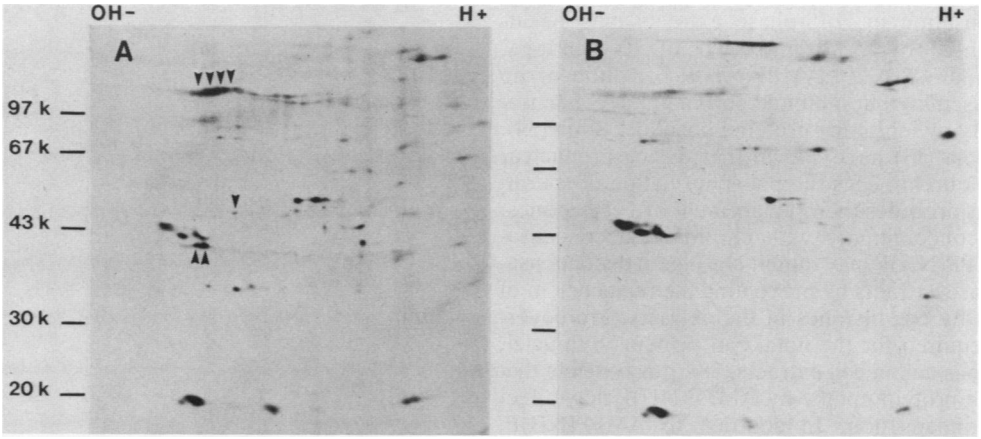

FIG. 4. Proteins labeled with [γ-^{35}S]ATP in M-phase and G_1/S-phase extracts. M-phase and G_1/S-phase extracts were prepared from *X. laevis* eggs as described previously (24). Extracts were radiolabeled with [γ-^{35}S]ATP as described in the legend to Fig. 1 for [γ-^{32}P]ATP, except the labeling period was for 30 min. (A) Thiophosphorylated proteins in M-phase extracts; (B) thiophosphorylated proteins in G_1/S-phase extracts. Arrows indicate the M-phase phosphoproteins that were thiophosphorylated in M-phase extracts (A).

appearance of MPF, a cytoplasmic factor thought to regulate nuclear behavior during M-phase. In meiotically dividing oocytes, protein phosphorylation increases severalfold when MPF appears or after partially purified MPF is injected into cells (5, 31, 41, 52). Subsequently, the level of protein phosphorylation changes with MPF activity, decreasing after M-phase, when MPF activity can no longer be detected (31, 41). The changes in protein phosphorylation that accompany MPF activity may play an important role in controlling nuclear behavior in dividing cells. Previous studies have shown that a large number of proteins are phosphorylated when cells enter M-phase and are dephosphorylated again soon after cell division, when MPF activity disappears and interphase nuclei reform in each of the daughter cells (4, 51). The hyperphosphorylation during mitosis of histones H1 and H3 and of the lamins has been shown to correlate with chromosome condensation and NE breakdown (8, 16, 34, 40). However, the M-phase-specific phosphoproteins involved in the regulation of nuclear behavior have not been identified. Since amphibian egg extracts can support in vitro the same changes in nuclear oganization that are seen in dividing cells, we used these extracts to investigate the requirement for protein phosphorylation in the cytoplasmic control of nuclear behavior.

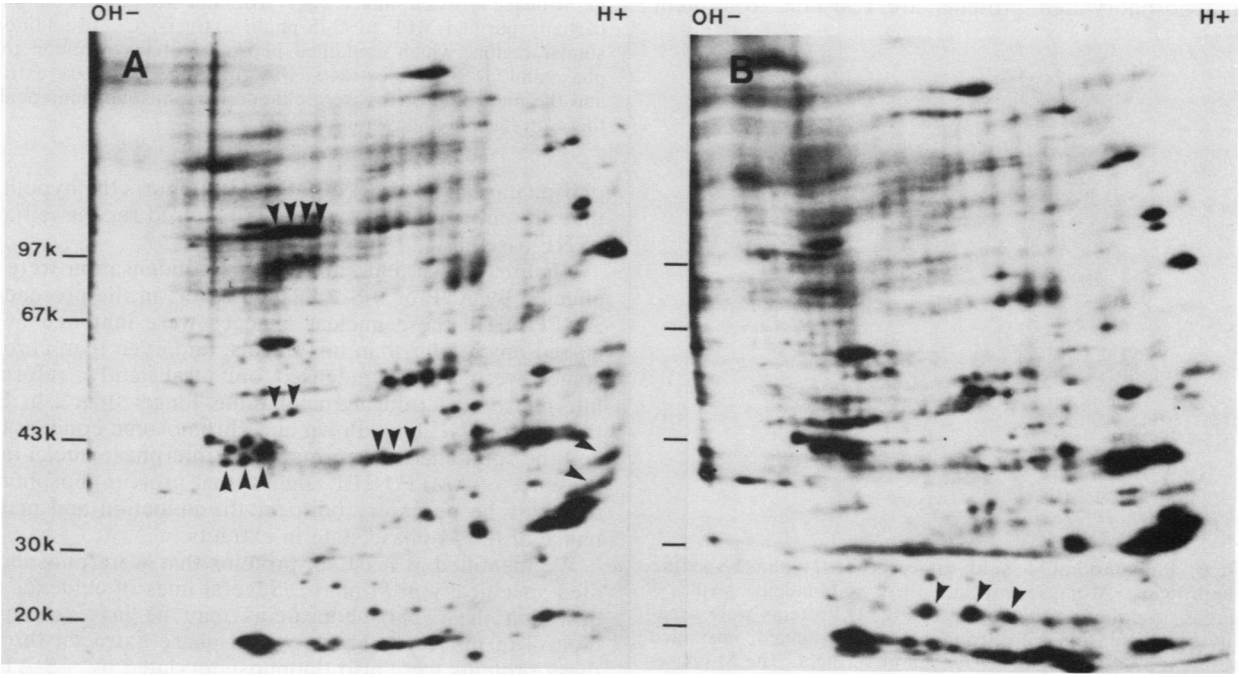

FIG. 5. Protein phosphorylation in intact eggs during M-phase or G_1/S-phase. *X. laevis* eggs were injected with [γ-^{32}P]ATP under conditions that either suppressed or allowed parthenogenetic activation (see Materials and Methods). At 30 minutes later, eggs were crushed by centrifugation and the radiolabeled cytoplasm was processed for two-dimensional electrophoresis and autoradiography. (A) Proteins phosphorylated in M-phase-arrested eggs; (B) proteins phosphorylated in parthenogenetically activated eggs, which are in G_1/S-phase. Arrows indicate proteins that were phosphorylated specifically in M-phase (A) or G_1/S-phase (B) eggs.

From our results, it appears that NE assembly and chromatin decondensation in G_1/S-phase extracts may be affected differently by ATP and two of its analogs, AMPP(NH)P and γ-S-ATP. AMPP(NH)P had little or no effect on NE assembly but inhibited chromatin decondensation, presumably by inhibiting protein phosphorylation (54). Green and Poccia (13) have shown that during pronuclear formation in sea urchin eggs the phosphorylation of sperm-specific histones precedes their replacement by egg histones, which occurs concomitantly with chromatin decondensation. Thus, AMPP(NH)P may inhibit chromatin decondensation in G_1/S-phase extracts by preventing the replacement of sperm histones by egg histones in the extracts. Moreover, since ATP is required for the uptake of proteins by nuclei, both in intact cells and in cell extracts (38), it is possible that the inhibition of protein uptake by AMPP(NH)P may affect chromatin decondensation. In contrast to AMPP(NH)P, γ-S-ATP and high concentrations of ATP inhibited both NE assembly and chromatin decondensation when they were present throughout the entire incubation period. However, since NE assembly is required for subsequent chromatin decondensation and nuclear swelling (27), the effect of γ-S-ATP and ATP may be due solely to their ability to inhibit NE assembly. Support for this hypothesis comes from the observation that γ-S-ATP and ATP inhibited NE assembly and chromatin decondensation only when they were added during the first 15 min of incubation. Presumably, once initiated, NE assembly and subsequent chromatin decondensation can continue even in the presence of γ-S-ATP and high concentrations of ATP. The effects of ATP and its analogs that we observed are similar to those seen by Burke and Gerace (1), who showed that NE assembly in extracts of mitotic CHO cells is also sensitive to γ-S-ATP and ATP but not to AMPP(NH)P. In that system, γ-S-ATP was also inhibitory only when present from the start of the incubation. Since γ-S-ATP can be utilized by protein kinases, but the thiophosphorylated proteins are resistant to protein phosphatases, the effect of γ-S-ATP supports the hypothesis that protein dephosphorylation is required for the initiation of NE assembly.

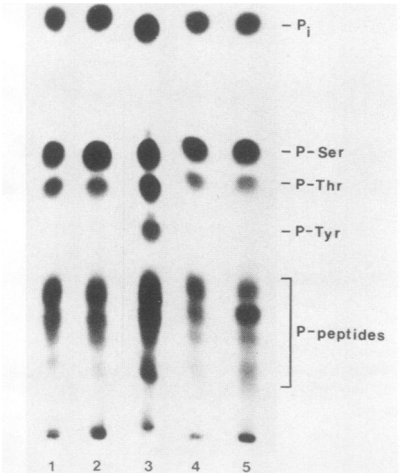

FIG. 6. Phosphoamino acid analysis of M-phase-specific phosphoproteins. M-phase extracts were radiolabeled with [γ-^{32}P]ATP as described in the legend to Fig. 1. Proteins were separated by two-dimensional electrophoresis, and unfixed, unstained gels were exposed to Kodak XAR-5 film overnight. The M-phase-specific phosphoproteins were eluted from the gel and subjected to one-dimensional phosphoamino acid analysis, as described in Materials and Methods. Shown are the phosphoamino acids of proteins with the following apparent molecular masses (in kilodaltons): lane 1, 110; lane 2, 49; lane 3, 42; lane 4, 41; lane 5, 38 and 35.

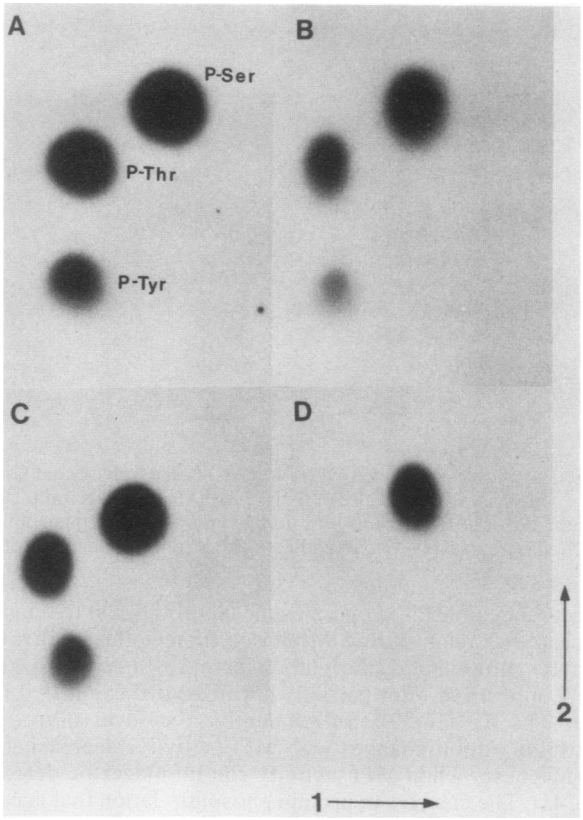

FIG. 7. Phosphoamino acid analysis of the 42-kDa M-phase-specific phosphoprotein. The 42-kDa protein phosphorylated during various M-phase conditions was eluted from two-dimensional gels and subjected to two-dimensional phosphoamino acid analysis, as described in Materials and Methods. Shown are the phosphoamino acids of the 42-kDa protein phosphorylated in M-phase extracts (A), in M-phase-arrested intact eggs (B), and after the addition of partially purified MPF to G_1/S-phase extracts (C). In addition, a similar region, which contained phosphoproteins common to M-phase and G_1/S-phase extracts, was cut from G_1/S-phase extracts, and the phosphoproteins were eluted. The phosphoamino acids in these proteins are shown in panel D.

NE breakdown and chromosome condensation were not affected by ATP or γ-S-ATP. However, in the presence of AMPP(NH)P these nuclear changes were inhibited or occurred much later than in controls, and even then chromosomes were often condensed only transiently, reforming interphase nuclei during incubations longer than 2 h. Both the delay in NE breakdown and chromosome condensation and the spontaneous reformation of interphase nuclei in the presence of AMPP(NH)P suggest that protein phosphorylation may be necessary both for the induction and maintenance of the M-phase state in extracts.

We identified at least six proteins that were phosphorylated specifically in M-phase. Several lines of evidence suggest that these phosphoproteins may be involved in the control of nuclear behavior. In M-phase extracts, three of these proteins were also thiophosphorylated by γ-S-ATP, a compound known to stabilize MPF activity and support NE breakdown. After the transition from M-phase to G_1/S-phase induced by Ca^{2+} ions, the phosphorylation of these proteins no longer occurred or was greatly reduced. Conversely,

induction of M-phase by the addition of partially purified MPF to G_1/S-phase extracts resulted in the phosphorylation of these proteins. Moreover, proteins of similar molecular weights and isoelectric points were phosphorylated in intact M-phase-arrested eggs but not in parthenogenetically activated eggs, which are no longer in M-phase. Finally, these proteins were not phosphorylated in extracts of immature oocytes but were phosphorylated in extracts of occytes that were in M-phase after progesterone-induced oocyte maturation (M. J. Lohka and J. L. Maller, unpublished observations). Therefore, in all cases examined, the phosphorylation of the six proteins that we have described was strictly correlated with M-phase, both in extracts and in intact cells. However, since we are not yet able to identify the unphosphorylated forms of these proteins on silver-stained gels, we cannot determine whether this M-phase-specific phosphorylation represents changes in the absolute level of phosphorylation of these proteins or an increased phosphate turnover. We think that the latter is unlikely, since the M-phase-specific phosphoproteins were only a small fraction of the total phosphoprotein and were totally absent in G_1/S-phase.

In M-phase extracts, each of the specific phosphoproteins contains phosphoserine and phosphothreonine. Phosphotyrosine is also detected in the 42-kDa protein under all M-phase conditions that we examined. Thus, it is evident that during M-phase both protein serine threonine kinase(s) and protein tyrosine kinase(s) are likely to play a role in controlling M-phase nuclear behavior. The identification of these protein kinases and their substrates will be important in determining the mechanism of MPF action.

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