Journal of Cell Science 109, 739-748 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
JCS7066

Cycloheximide-induced activation of mouse eggs: effects on cdc2/cyclin B and MAP kinase activities

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SUMMARY

Fertilization of metaphase II-arrested mouse eggs results in resumption of meiosis and a decrease in both cdc2/cyclin B kinase and MAP kinase activities; the decrease in cdc2/cyclin B kinase activity precedes the decrease in MAP kinase activity. Cycloheximide treatment of metaphase II-arrested mouse eggs also results in resumption of meiosis but bypasses the fertilization-induced Ca^{2+} transient. However, it is not known if cycloheximide treatment results in the same temporal changes in cdc2/cyclin B kinase and MAP kinase activities that are intimately associated with resumption of meiosis. We report that cycloheximide-treated mouse eggs manifest similar temporal changes in the decrease in both cdc2/cyclin B kinase and MAP kinase activities that occur following fertilization, although cortical granule exocytosis is not stimulated. The decrease in cdc2/cyclin B kinase activity, however, does not seem to be required for the decrease in MAP kinase activity, since the decrease in MAP kinase activity still occurs in cycloheximide-treated eggs that are also incubated in the presence of nocodazole, which inhibits cyclin B degradation and hence the decrease in cdc2/cyclin B kinase. Following removal of these drugs, cdc2/cyclin B kinase activity remains high, MAP kinase activity increases to levels similar to that in the metaphase II-arrested eggs, and a spindle(s) forms with the chromosomes aligned on a metaphase plate. Results of these experiments suggest that some other protein with a relatively short half-life, e.g. c-*mos*, a known upstream activator of MAP kinase, may be responsible for events leading to the decrease in MAP kinase activity.

Key words: Mouse egg, Cell cycle, cdc2/cyclin B kinase, Histone H1 kinase, MAP kinase, Cycloheximide-induced egg activation, Egg activation

INTRODUCTION

In contrast to somatic cells, which immediately enter interphase following mitosis, oocytes of many species, including the mouse, arrest at metaphase II following resumption of meiosis. This arrest is due to at least two factors: M-phase promoting factor (MPF) and cytostatic factor (CSF). MPF is a complex of cyclin B1/B2 and the $p34^{cdc2}$ protein kinase, and displays a cyclic activity that peaks at metaphase (Gautier et al., 1990). The MPF-associated protein kinase activity that is due to cdc2/cyclin B is also referred to as histone H1 kinase, since this enzyme displays a pronounced activity towards this substrate and is the main source of histone H1 kinase activity in oocytes and eggs during meiosis (Gautier et al., l988).

CSF is postulated to maintain elevated levels of MPF activity (Masui, 1991) and hence may be responsible for metaphase II arrest (Sagata et al., 1989). The mitogen-activated protein kinase, MAP kinase, may be a component of CSF. For example, microinjection of a stably activated thiophosphorylated MAP kinase into *Xenopus laevis* blastomeres results in cleavage arrest (Haccard et al., 1993a). MAP kinase may also modulate cdc2/cyclin B kinase. In nocodazole-arrested extracts from *Xenopus laevis* eggs, MAP kinase activity appears essential for maintaining a high level of cyclin B and histone H1 kinase activity. Furthermore, inactivation of MAP kinase in these extracts by treatment with MAP kinase phosphatase results in a decline in histone H1 kinase activity and entry into interphase (Minshull et al., 1994). Also consistent with a role for MAP kinase in modulating cdc2/cyclin B kinase is the observation that microinjection of a thiophosphorylated MAP kinase into *Xenopus laevis* oocytes results in both activation of histone H1 kinase and germinal vesicle breakdown (GVBD) (Haccard et al., 1995). Reciprocally, microinjection of an inhibitory antibody to MAP kinase kinase (MEK), the enzyme that directly activates MAP kinase (Kosako et al., 1994), inhibits the progesterone-induced increase in cdc2/cyclin B kinase and GVBD (Kosako et al., 1994).

Although MAP kinase appears to stabilize cdc2/cyclin B kinase activity, several lines of evidence suggest that the cdc2/cyclin B kinase may in turn regulate MAP kinase. For example, during maturation of mouse oocytes, the increase in histone H1 kinase activity precedes the increase in MAP kinase

activity (Verlhac et al., 1993). Furthermore, following either egg activation or fertilization in the mouse or *Xenopus laevis*, the decline in histone H1 kinase activity precedes the decrease in MAP kinase activity (Ferrell et al., 1991; Lorca et al., 1993; Verlhac et al., 1994; Moos et al., 1995). Moreover, preventing the fertilization-induced decrease in histone H1 kinase activity by nocodazole treatment of mouse eggs prevents the fertilization-associated decrease in MAP kinase activity (Moos et al., 1995); it should be noted that an intact spindle is required for cyclin destruction in the mouse (Kubiak et al., 1993; Winston et al., 1995). More direct evidence for the modulation of MAP kinase activity by the cdc2/cyclin B kinase is that injection of cyclin B into *Xenopus laevis* oocytes results in progesterone-independent maturation and the activation of MAP kinase (Gotoh et al., 1991; Haccard et al., 1993b). This cdc2/cyclin B kinase-induced activation of MAP kinase may be mediated by MEK, since cdc2/cyclin B kinase can phosphorylate and activate MEK in vitro (Van Renterghem et al., 1993).

The transient increase in intracellular calcium that occurs following fertilization or parthogenetic activation of eggs using agents such as A23187 or ethanol bypasses the maintenance of metaphase II arrest by targeting cyclin B destruction (Lorca et al., 1993) via a specific ubiquitination pathway (King et al., 1995; Sudakin et al., 1995). This targeted destruction of cyclin B results in a decrease in cdc2/cyclin B kinase activity that is followed by a decrease in MAP kinase activity (Ferrell et al., 1991; Verlhac et al., 1994; Moos et al., 1995). Treatment of mouse eggs with cycloheximide also results in egg activation, as assessed by emission of the second polar body (PB) and formation of a pronucleus (PN) (Siracusa et al., 1978; Clarke and Masui, 1983; Fulka et al., 1994). Cycloheximide-induced egg activation presumably occurs by permitting the turnover of cyclin B that results in a concomitant decrease in cdc2/cyclin B kinase activity. To our knowledge, neither the decrease in cdc2/cyclin B kinase activity nor MAP kinase activity has been measured directly in cycloheximide-treated eggs.

We report here that treatment of mouse eggs with cycloheximide results in a decrease in both histone H1 kinase and MAP kinase activities and that the decrease in histone H1 kinase activity precedes the decrease in MAP kinase activity. The decrease in cdc2/cyclin B kinase activity does not seem to be requisite for the decrease in MAP kinase activity, since incubation of eggs in medium containing cycloheximide and nocodazole prevents the decrease in histone H1 kinase activity, but not the decrease in MAP kinase activity. Thus, some other protein with a relatively short half-life may be responsible for events leading to the decrease in MAP kinase activity.

MATERIALS AND METHODS

Collection of mouse eggs and one-cell embryos

Ovulated metaphase II-arrested eggs were isolated from superovulated 6-week-old CF-1 (Harlan) female mice and in vitro fertilization (IVF) was performed as previously described (Moore et al., 1994); the culture medium was Whitten's medium (Whitten, 1971). In some experiments, the *zona pellucida* (ZP) was removed by treatment with acidic Tyrode's solution prior to IVF (Hogan et al., 1986). For stimulation of eggs with calcium ionophore, eggs were incubated in the presence of 5 μM A23178 for 2 minutes, washed, and further cultured as described for inseminated eggs. Treatment of eggs and embryos with nocodazole (10 μM) was performed as previously described (Kubiak et al., 1993). For cycloheximide-induced activation, eggs were transferred into CZB medium (Chatot et al., 1989) supplemented with 20 μg/ml of cycloheximide and further cultured for the time indicated in the figures. Complete egg activation, i.e. emission of the second PB, formation of a PN, and cleavage to the 2-cell stage, was achieved by culturing the eggs for 8-15 hours in medium containing cycloheximide, transferring them to cycloheximide-free medium, and then culturing them for up to 40 hours. Cycloheximide-induced egg

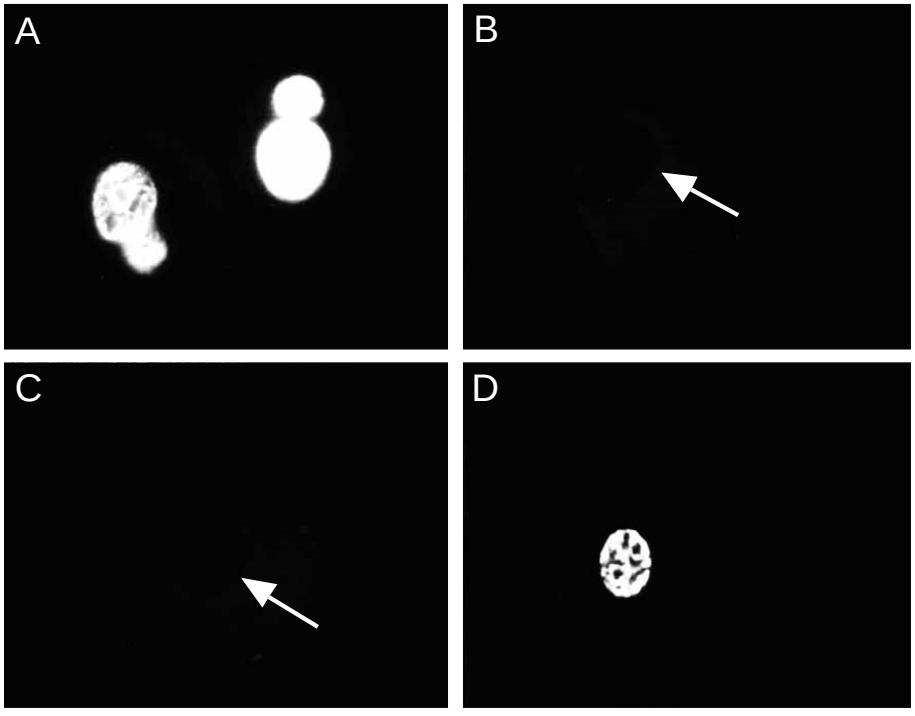

Fig. 1. Effect of protein synthesis inhibition on the onset of DNA synthesis in inseminated or cycloheximide-activated mouse eggs. (A) Fertilized egg, (B) fertilized egg cultured in the presence of cycloheximide until PN formation, (C) unfertilized egg cultured in the presence of cycloheximide until PN formation, and (D) unfertilized egg initially cultured in the presence of cycloheximide and after PN formation cultured in cycloheximide-free medium. The cells were incubated in medium containing BrdU (A,D) or BrDU and cycloheximide (B,C) for 6 hours prior to processing for immunocytochemical detection of incorporated BrdU. Shown are immunofluorescent images obtained with a laser-scanning confocal microscope.

activation was inhibited by culturing the eggs in medium containing either nocodazole (10 μM) or okadaic acid (2 μM).

Histone H1 kinase and MBP kinase assay

Histone H1 kinase activity (i.e. cdc2/cyclin B kinase activity), and MAP kinase activity were measured as previously described using histone H1 and a peptide substrate containing the MAP kinase consensus phosphorylation sequence found in myelin basic protein (MBP), respectively (Moos et al., 1995).

Electrophoresis and immunoblotting

SDS-PAGE was performed in 10% slab gels, proteins were electrophoretically transferred to Immobilon P (Millipore) and immunostained as previously described (Moos et al., 1995). Monoclonal and polyclonal antibodies against p42 and p44 MAP kinase (polyclonal, catalog no. 06182; monoclonal, catalog no. 05157), cyclin B1 (monoclonal, catalog no. 05158), and cdc2 (polyclonal, catalog no. 06194) were purchased from UBI (Lake Placid, NY). Forty eggs or embryos were loaded per lane. The ZP2 to $ZP2_f$ conversion, which is an indicator of cortical granule exocytosis (Ducibella et al., 1990), was monitored as previously described (Moos et al., 1995) using a rabbit polyclonal antiserum made against purified mouse ZP2; this antiserum recognizes both ZP2 and $ZP2_f$.

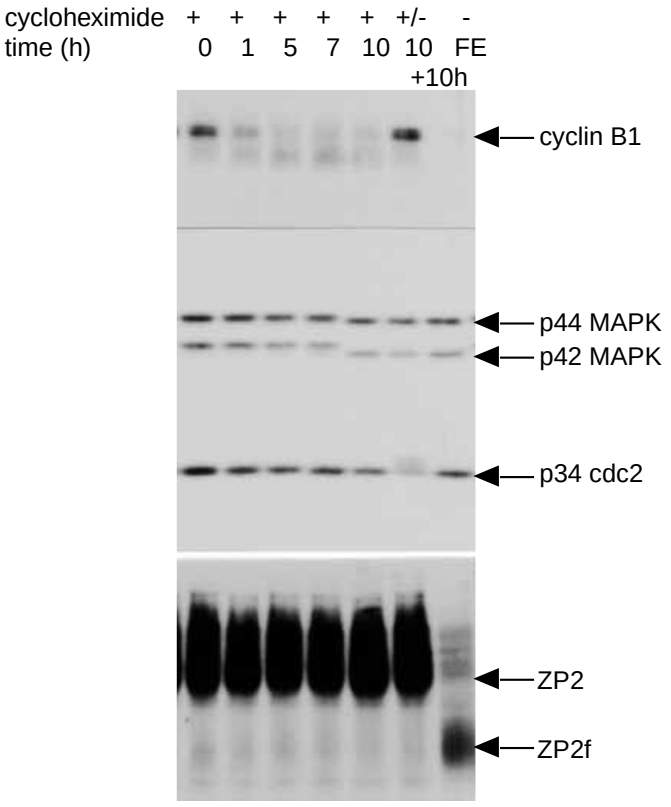

Fig. 2. Effect of cycloheximide on cyclin B concentrations, the phosphorylation state of MAP kinase and cdc2 kinase, and the ZP2 to $ZP2_f$ conversion. Ovulated mouse eggs were cultured in the presence (+) of cycloheximide for up to 10 hours, or after 10 hours cultured in the absence (+/–) of cycloheximide for an additional 10 hours (10 +10 hours). Eggs fertilized in vitro and then cultured for 10 hours (FE). Groups of 40 cells were taken at various time points shown and analyzed by immunoblotting using antibodies against MAP kinase, cdc2 kinase, cyclin B1, and mouse ZP2. All the proteins shown were detected in the same immunoblot using the same membrane. The experiment was performed three times and similar results were obtained. Shown are the results of a representative experiment.

Immunocytochemistry

DNA synthesis was monitored by incorporation of bromodeoxyuridine

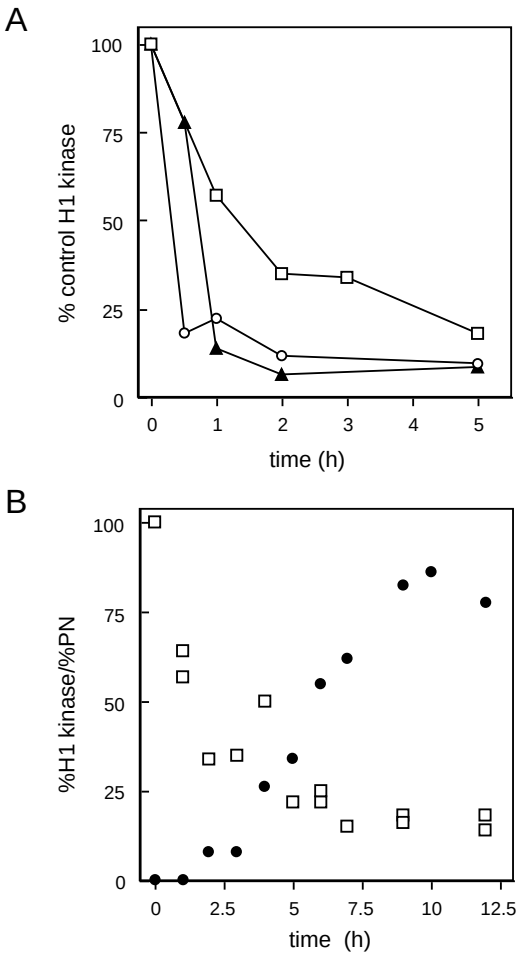

Fig. 3. Time courses of histone H1 kinase activity following insemination or activation by cycloheximide or calcium ionophore. (A) ZP-free eggs were inseminated (filled triangles) or ZP-intact eggs were activated by cycloheximide (open squares) or calcium ionophore A23187 (open circles) and then cultured for up to 5 hours. Groups of eggs were taken at the various indicated time points and analyzed for histone H1 kinase activity as described in Materials and Methods. Eighteen eggs were analyzed for each time point and the data are presented as the % histone H1 kinase activity present in the metaphase II-arrested egg. The experiment was performed three times and similar results were obtained. The values shown are the means in which the s.e.m. did not exceed 12%. (B) Metaphase II-arrested eggs were incubated with cycloheximide for up to 12 hours, and two groups of eggs were taken at each time point. One group was analyzed for histone H1 kinase activity (open squares) and the other group was washed and then cultured in cycloheximide-free medium for an additional 10 hours prior to scoring for PN formation (filled circles). At the time of PN formation, eggs are entering interphase and were considered to be activated. Nine eggs were analyzed for histone H1 kinase activity and forty eggs scored for PN formation at each time point shown. The data shown are the cumulative data from three independent experiments. Histone H1 kinase activity is expressed relative to that in the metaphase II-arrested egg.

(BrdU) that was detected with antibodies to BrdU as previously described (Moore et al., 1994), except that the fluorescence was detected using a Bio-Rad laser-scanning confocal microscope. Changes in the microtubule network and spindle assembly were detected using an α-tubulin antibody and visualized using a Zeiss epifluorescence microscope as previously described (Moore et al., 1995). To assess the state of chromatin, the eggs were stained in vivo with Hoechst 33258 (10 μg/ml) for 30 minutes, and then washed for at least 30 minutes in the culture medium prior to examination using epifluorescence microscopy.

RESULTS

Effect of inhibition of protein synthesis on DNA replication

Treatment of eggs with protein synthesis inhibitors results in emission of the second PB and PN formation but not further development. This arrest in eggs treated with protein synthesis inhibitors is likely due to a requirement of protein synthesis for DNA replication (Clarke and Masui, 1983). This is in apparent contrast to the early embryonic cell cycles in *Xenopus laevis* that do not require protein synthesis (Jackson et al., 1995). In order to examine if protein synthesis was also required for entry into S phase in inseminated mouse eggs, we examined the effects of cycloheximide on incorporation of BrdU in either inseminated or uninseminated eggs.

When compared to inseminated eggs not treated with cycloheximide (Fig. 1A) cycloheximide treatment of inseminated eggs inhibited BrdU incorporation (Fig. 1B). Likewise, although cycloheximide treatment of uninseminated eggs results in PN formation, no BrdU incorporation was observed (Fig. 1C). This inhibition was reversible, since further culture of these eggs in cycloheximide-free medium resulted in incorporation of BrdU (Fig. 1D).

Effects of cycloheximide-induced egg activation on the amount of cyclin B1, histone H1 kinase activity, the phosphorylation state of cdc2 and MAP kinase, and conversion of ZP2

Treatment of mouse eggs with inhibitors of protein synthesis results in completion of the second meiotic division and entry into interphase with the formation of a PN (Siracusa et al., 1978; Clarke and Masui, 1983; Fulka et al., 1994). The temporal changes in the activity of histone H1 and MAP kinases that normally occur following fertilization, however, have not been analyzed under these conditions. Moreover, although the eggs apparently resume the cell cycle, the effect of this treatment on

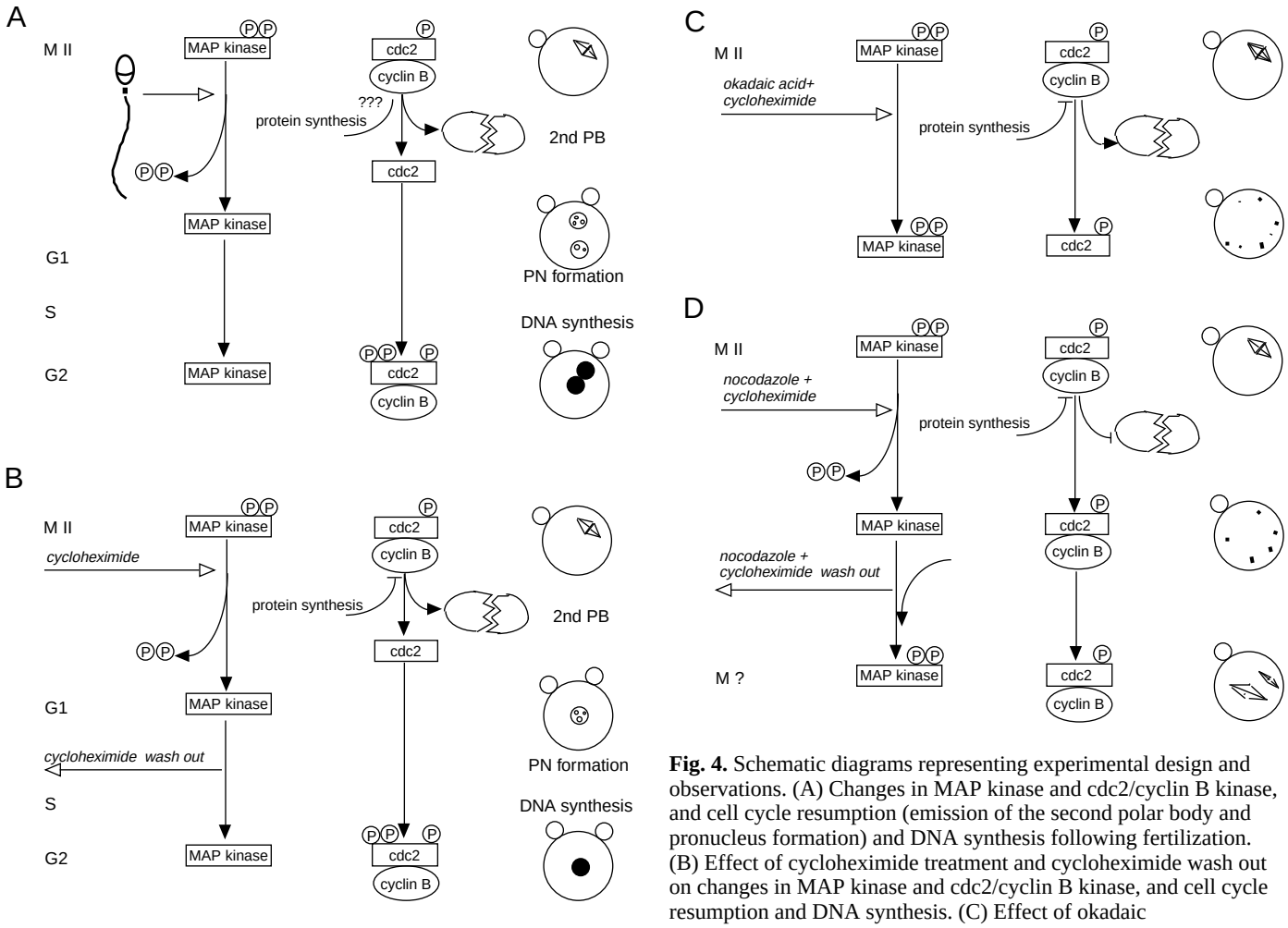

Fig. 4. Schematic diagrams representing experimental design and observations. (A) Changes in MAP kinase and cdc2/cyclin B kinase, and cell cycle resumption (emission of the second polar body and pronucleus formation) and DNA synthesis following fertilization. (B) Effect of cycloheximide treatment and cycloheximide wash out on changes in MAP kinase and cdc2/cyclin B kinase, and cell cycle resumption and DNA synthesis. (C) Effect of okadaic acid/cycloheximide treatment on changes in MAP kinase and cdc2/cyclin B kinase, and cell cycle resumption and DNA synthesis. (D) Effect of nocodazole/cycloheximide treatment and wash out on changes in MAP kinase and cdc2/cyclin B kinase, and cell cycle resumption and DNA synthesis.

cortical granule exocytosis, as assayed by the ZP2 to $ZP2_f$ conversion, is not known. Accordingly, we examined the effect of cycloheximide treatment on these parameters of egg activation.

Within 5 hours following the addition of cycloheximide to ovulated metaphase II-arrested mouse eggs the amount of cyclin B1 (Fig. 2) and histone H1 kinase activity (Fig. 3A) decreased dramatically and reached levels similar to those observed following fertilization. The time course of the decrease in histone H1 kinase activity, however, was slower than that observed with either fertilized eggs or ionophore-treated eggs (Fig. 3A). In contrast, cycloheximide treatment led to the dephosphorylation of MAP kinase, which is associated with a loss of MAP kinase activity (Shibuya et al., 1992; Verlhac et al., 1993), in a time frame similar to that following fertilization, i.e. 7-10 hours (Fig. 2) (Verlhac et al., 1994; Moos et al., 1995). Following removal of cycloheximide, the phosphorylation of cdc2, as detected by a decreased electrophoretic mobility, as well as an increase in cyclin B1 accumulation was observed after 10 hours (Fig. 2).

Cycloheximide treatment, however, did not induce the post-fertilization conversion of ZP2 to $ZP2_f$ (Fig. 2). This suggested that cycloheximide treatment did not result in an increase in the intracellular Ca^{2+}, since the fertilization-induced ZP2 conversion is triggered by a rise in the intracellular concentration of this divalent cation (Xu et al., 1994). The inability to observe the ZP2 conversion was not due to the inability of the cortical granules to undergo exocytosis in these cycloheximide-treated eggs, since subsequent treatment of cycloheximide-treated eggs with ionophore A23187 resulted in the ZP2 conversion (data not shown).

To ascertain the length of time required for cycloheximide treatment to initiate egg activation, eggs were incubated in cycloheximide and after various time periods a portion of the sample was immediately assayed for histone H1 kinase and another portion was transferred to cycloheximide-free medium and then scored for PN formation after a 10 hour incubation. PN formation was taken as evidence that the eggs had become activated and only 5% of the eggs had developed a PN following a 6 hour culture in cycloheximide-containing medium. Results of these experiments revealed that a 4 hour cycloheximide treatment was required before the eggs could be activated and that this fraction significantly increased following a 6 hour incubation (Fig. 3B). In a parallel fashion, the level of histone H1 kinase activity was significantly reduced by 4 hours and reached essentially basal values by 6 hours (Fig. 3B). Although this decrease in histone H1 kinase activity and destruction of cyclin B were correlated with commitment to egg activation, it should be noted that this commitment occurred while MAP kinase was still phosphorylated and hence active (Fig. 2) and that MAP kinase dephosphorylation only occurred following this commitment. Results of these experiments are schematically depicted in Fig. 4A and B.

Effect of okadaic acid on cycloheximide-induced egg activation

We have previously demonstrated that okadaic acid, an inhibitor of protein phosphatases 1 and 2A, inhibits the fertilization-induced decrease in MAP kinase activity (Moos et al., 1995). In addition, following fertilization and the decrease in both histone H1 and MAP kinase activities, treatment of these fertilized eggs with okadaic acid results in the reactivation of MAP kinase in the absence of any increase in histone H1 kinase activity (Moos et al., 1995). We examined if okadaic acid treatment elicited similar responses in eggs activated by cycloheximide treatment. Results of these experiments, which are schematically depicted in Fig. 4C, indicated a loss of cyclin B and a decrease in histone H1 kinase activity (Fig. 5A),and disassembly of the spindle (data not shown). This treatment, however, did not result in an inactivation of MAP kinase (Fig. 5B), chromatin decondensation (compare Fig. 8A and B), and PN formation. Thus, in these cycloheximide-activated eggs, as with fertilized eggs (Moos et al., 1995), an elevated MAP kinase activity appears to be incompatible with the presence of a PN. In addition, the maintenance of the condensed chromosomes under these conditions in which

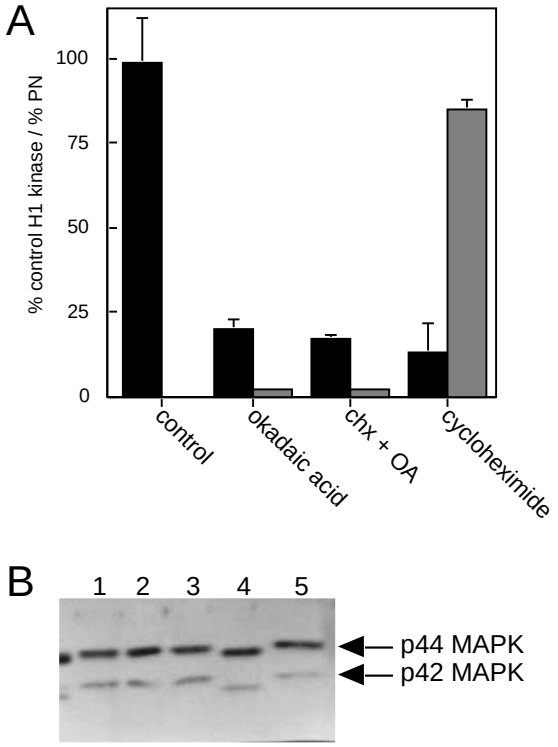

Fig. 5. Effect of okadaic acid and cycloheximide on changes in histone H1 kinase activity, percentage of PN formation, and phosphorylation status of p42 MAP kinase in mouse eggs. (A) Ovulated mouse eggs were incubated alone (control), in cycloheximide (cycloheximide), in okadaic acid (okadaic acid), or in okadaic acid and cycloheximide (chx + OA) containing medium for 18 hours. Histone H1 kinase activity (filled bars) was measured following these different treatments and the activity is expressed as a percentage of histone H1 kinase activity in ovulated eggs. In each experiment two samples of three eggs each was assayed and the experiment was performed three times. The data are expressed as the mean ± s.e.m. The percentage of cells displaying a PN (open bars) is shown as mean ± s.e.m. (n=3 experiments). At least 50 eggs were scored for each point in each experiment. (B) Changes in phosphorylation status of p42 MAP kinase assessed by immunoblotting with anti-MAP kinase antibody. Lane 1, ovulated eggs incubated in CZB medium for 18 hours; lane 2, eggs incubated in okadaic acid for 18 hours; lane 3, eggs incubated in cycloheximide and okadaic acid for 18 hours; lane 4, eggs incubated in cycloheximide for 18 hours; and lane 5, freshly ovulated eggs that were not cultured. The experiment was performed three times and similar results obtained in each case. Shown are the results of a single experiment.

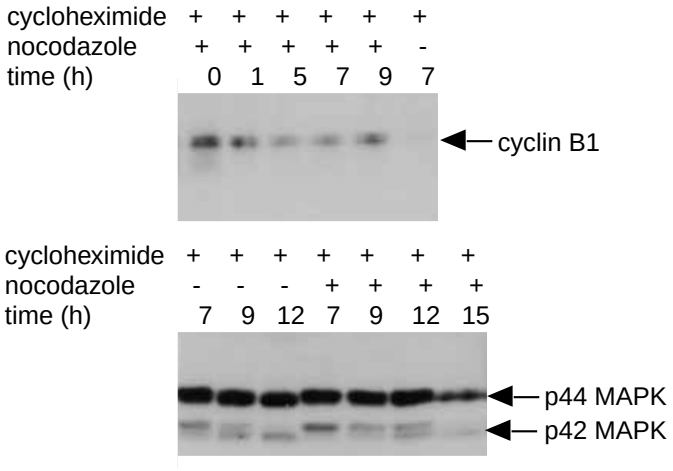

Fig. 6. Effect of spindle disruption by nocodazole on cycloheximide-induced changes in cyclin B1 concentration and phosphorylation status of MAP kinase. Ovulated mouse eggs were cultured in the presence of cycloheximide in either the presence (+) or absence (–) of nocodazole for up to 15 hours as indicated. Groups of 40 cells were taken and analyzed by immunoblotting for cyclin B1 or the phosphorylation status of MAP kinase. Nocodazole was added to a final concentration of 10 μM 30 minutes before the addition of cycloheximide. The experiment was conducted three times and similar results obtained in each case. Results of a single experiment are shown. It should be noted that no changes were observed in the phosphorylation status of cdc2 at these time points (data not shown). The apparent increase in the amount of cyclin at 9 hours is due to experimental variability and was not observed in the other two experiments.

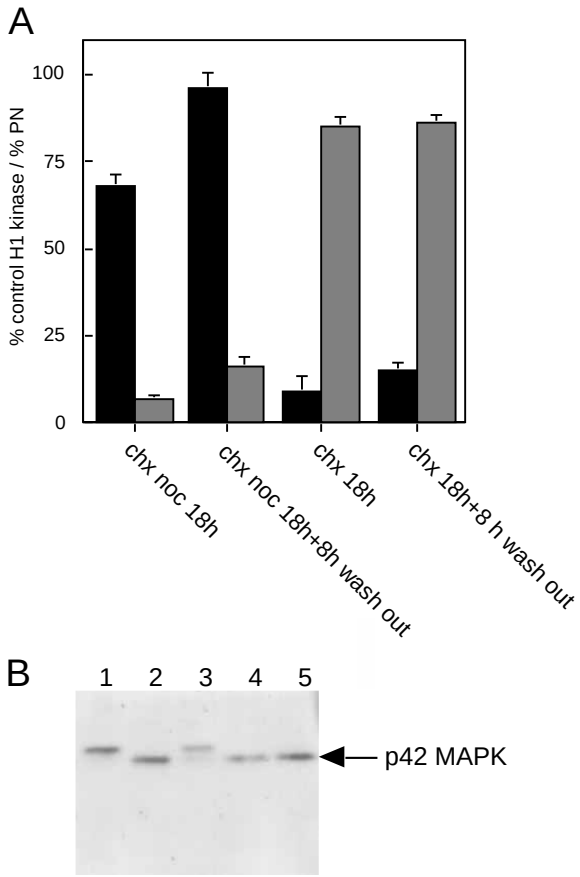

Fig. 7. Changes in histone H1 kinase activity, percentage of PN formation, and phosphorylation status of p42 MAP kinase in mouse eggs following removal of cycloheximide and nocodazole. Ovulated mouse eggs were incubated in cycloheximide (chx 18 hours) or in nocodazole and cycloheximide (chx noc 18 hours) containing medium as described in the legends to Figs 2 and 4 for 18 hours. The eggs were then washed and cultured in inhibitor-free medium for an additional 8 hours (+ 8 hours med). (A) Histone H1 kinase activity (closed bars) is expressed as a percentage of histone H1 kinase activity in ovulated, untreated eggs. In each experiment two samples of three eggs each was assayed and the experiment was performed three times. The data are expressed as the mean ± s.e.m. The percentage of cells displaying a PN (open bars) is shown as mean ± s.e.m. (n=3 experiments). At least 50 cells were scored for each point in each experiment. (B) Changes in phosphorylation status of p42 MAP kinase as assessed by immunoblotting. Lane 1, control ovulated eggs; lane 2, eggs treated with cycloheximide and nocodazole for 18 hours; lane 3, eggs treated with cycloheximide and nocodazole for 18 hours followed by an 8 hour incubation in inhibitor-free medium; lane 4, eggs treated with cycloheximide for 18 hours; and lane 5, eggs treated with cycloheximide for 18 hours followed by an 8 hour incubation in inhibitor-free medium. The experiment was performed three times and similar results obtained in each case. Shown are the results of a single experiment.

cdc2/cyclin B kinase activity is low and MAP kinase activity is high, suggests that other proteins, e.g. MAP kinase, could be involved in either the initiation of chromosome condensation or the maintenance of the condensed state (Guo et al., 1995).

Because okadaic acid treatment is not readily reversible (okadaic acid is not effectively washed out of cells placed in okadaic-free medium; Picard et al., 1989), it was not possible to discern if eggs treated with both cycloheximide and okadaic acid had become committed to egg activation. To address this issue, eggs were incubated in cycloheximide for 6 hours to permit histone H1 kinase activity to decrease to basal levels prior to the addition of okadaic acid, which maintains MAP kinase in its phosphorylated and active form. Even after culture for up to 15 hours, PN never formed, and the chromatin remained in a condensed state (data not shown). Therefore, even though cycloheximide treatment for 6 hours can result in commitment to egg activation as assessed by PN formation, maintenance of MAP kinase activity at elevated levels prevented this commitment step.

Effect of nocodazole on cycloheximide-induced egg activation

As described in the Introduction, several lines of evidence suggest that cdc2/cyclin B kinase may be involved in maintaining elevated levels of MAP kinase activity. The ability of cycloheximide to mimic the temporal changes in the decrease in each of these kinase activities that normally occurs following fertilization by fostering the turnover of cyclin B is also consistent with this proposal. Nevertheless, it is formally possible that changes in turnover of a protein(s) that regulates MAP kinase, and is not cyclin B, is responsible for initiating the decrease in MAP kinase. Since cyclin B degradation requires an intact spindle (Kubiak et al., 1993), we treated eggs with both cycloheximide and nocodazole, which disrupts the spindle, and assayed the eggs for cyclin B1, histone H1 kinase activity, and MAP kinase phosphorylation status in order to determine if MAP kinase is dephosphorylated under conditions that maintain elevated levels of histone H1 kinase activity.

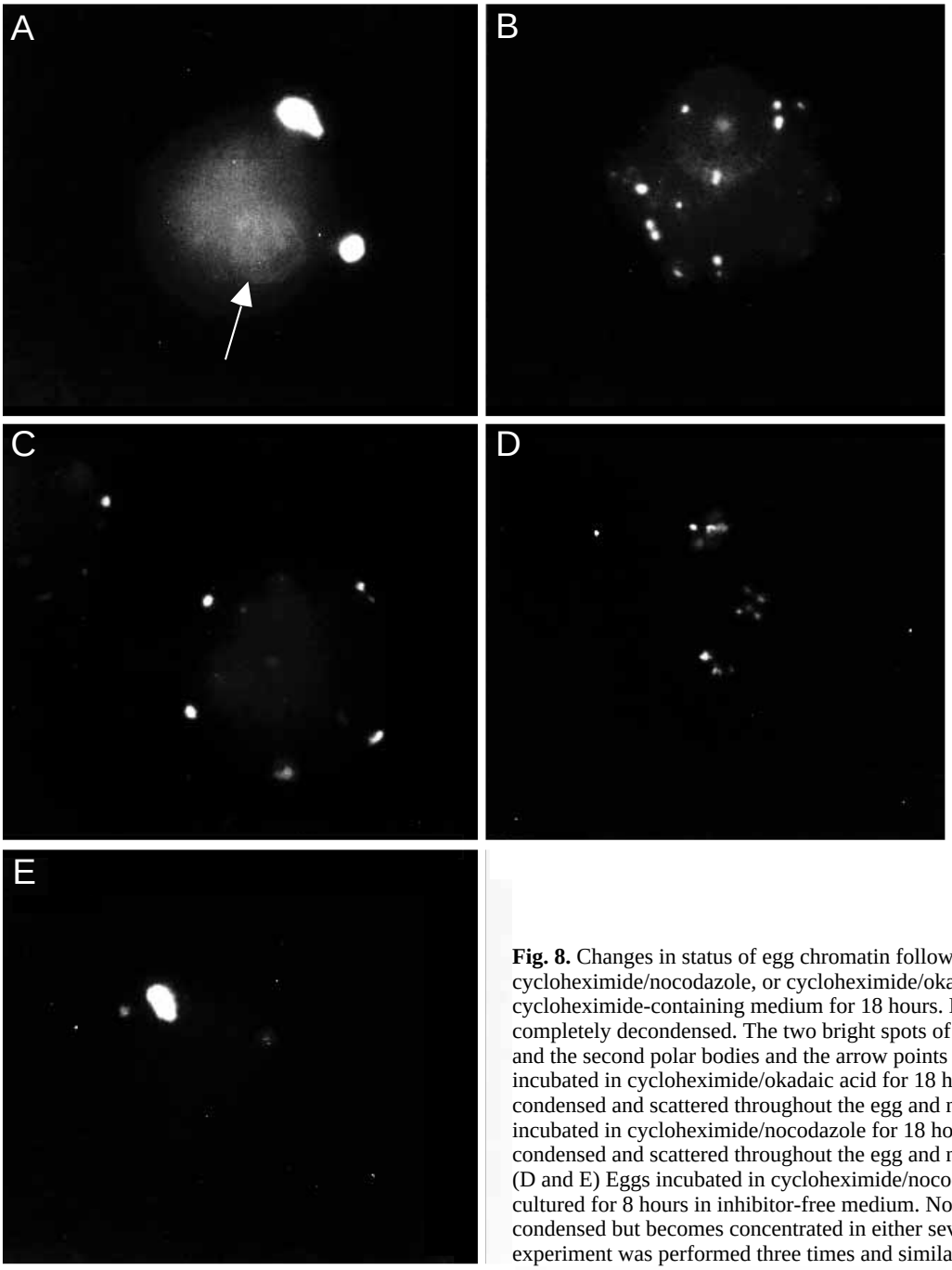

Fig. 8. Changes in status of egg chromatin following incubation with cycloheximide, cycloheximide/nocodazole, or cycloheximide/okadaic acid. (A) Eggs incubated in cycloheximide-containing medium for 18 hours. Note that the chromatin is completely decondensed. The two bright spots of condensed DNA represent the first and the second polar bodies and the arrow points to the pronucleus. (B) Eggs incubated in cycloheximide/okadaic acid for 18 hours. Note that the chromatin is condensed and scattered throughout the egg and no pronucleus is formed. (C) Eggs incubated in cycloheximide/nocodazole for 18 hours. Note that the chromatin is condensed and scattered throughout the egg and no pronucleus is formed. (D and E) Eggs incubated in cycloheximide/nocodazole for 18 hours and then cultured for 8 hours in inhibitor-free medium. Note that the chromatin remains condensed but becomes concentrated in either several (D) or single (E) locations. The experiment was performed three times and similar results were obtained in each case.

As expected, treatment of eggs with both cycloheximide and nocodazole substantially inhibited cyclin B1 degradation (Fig. 6) and the drop in histone H1 kinase activity (Fig. 7A). Interestingly, the dephosphorylation of MAP kinase was only modestly delayed (Fig. 6). Results of these experiments suggested that the decrease in MAP kinase activity was not directly coupled to the destruction of cyclin B and the concomitant loss of histone H1 kinase activity.

In contrast to the eggs incubated with only cycloheximide, co-incubation of the eggs with cycloheximide and nocodazole blocked PN formation (Fig. 7A) as well as chromatin decondensation (compare Fig. 8A and C). This inhibitory effect was reversible, since further culture of the eggs in cycloheximide- and nocodazole-free medium resulted in the rephosphorylation of MAP kinase (Fig. 7B), and concentration of the scattered condensed chromosomes to a few areas or to even a single area (Fig. 8D and E). Moreover, compared to eggs cultured in cycloheximide (Fig. 9A) or cycloheximide and nocodazole (Fig. 9B) for 18 hours, subsequent culture in cycloheximide- and nocodazole-free medium resulted in the formation of either multiple metaphase spindles (Fig. 9C) or a single metaphase spindle (Fig. 9D) associated with chromosomes.

DISCUSSION

Our results indicate that, as with sperm-induced egg activation

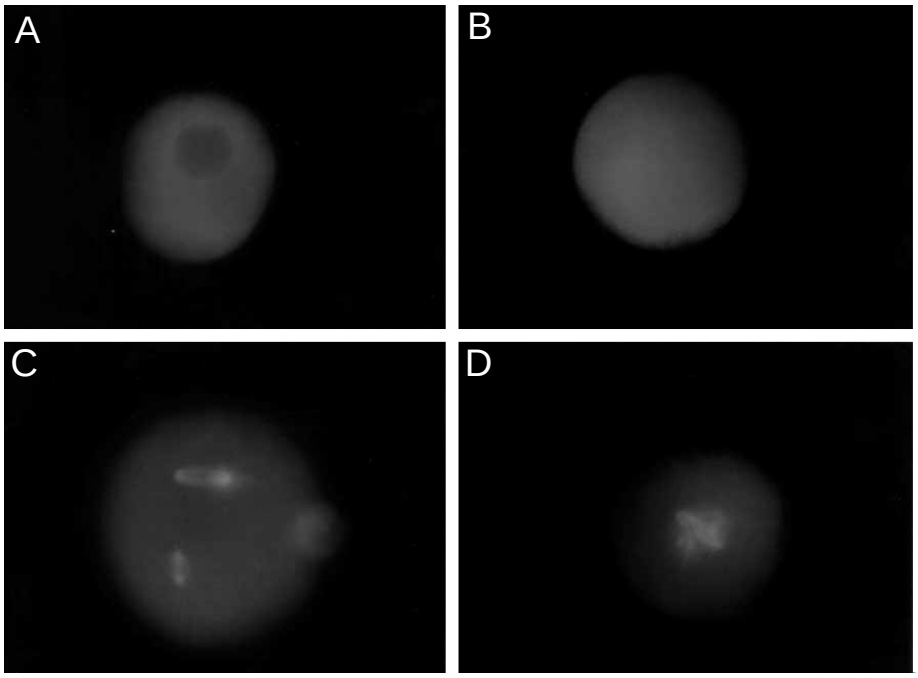

Fig. 9. Changes in microtubule organization in metaphase II-arrested eggs following cycloheximide or cycloheximide/nocodazole treatment. (A) Eggs incubated in cycloheximide-containing medium for 18 hours. (B) Eggs incubated in cycloheximide/nocodazole for 18 hours. (C and D) Eggs incubated in cycloheximide/nocodazole for 18 hours and then washed and cultured in inhibitor-free medium. Note the presence of multiple spindle-like structures (C) or a single spindle-like structure (D). The experiment was performed three times and similar results were obtained in each case.

(Verlhac et al., 1994; Moos et al., 1995), cycloheximide-induced activation of mouse eggs results in a decrease in both cdc2/cyclin B kinase and MAP kinase activities and that the decrease in cdc2/cyclin B kinase precedes the decrease in MAP kinase. Two lines of evidence indicate that these changes are unlikely due to a cycloheximide-induced increase in the intracellular Ca^{2+} concentration. First, we do not observe the ZP2 conversion, which is triggered by a transient increase in intracellular calcium following fertilization (Xu et al., 1994), and second, measurements of intracellular Ca^{2+} in fura-2-loaded mouse eggs do not detect an increase in this divalent cation in cycloheximide-treated eggs (Bos-Mikich et al., 1995; Moses and Kline, 1995). Thus, in the absence of protein synthesis the turnover of critical regulatory components of the cell cycle machinery, e.g. cyclin B, is likely responsible for this Ca^{2+}-independent egg activation. Consistent with this interpretation is the observation that the time course for the decrease in cdc2/cyclin B kinase activity is significantly slower in the cycloheximide-treated eggs when compared to fertilized eggs or eggs activated with the calcium ionophore A23187. This likely reflects the rapid Ca^{2+}-activated ubiquitin-mediated proteolysis of cyclin B in fertilized or ionophore-treated eggs in contrast to the slower turnover of cyclin B in the cycloheximide-treated eggs.

When eggs are treated with cycloheximide and nocodazole, cyclin B turnover is markedly inhibited, since proteolysis of cyclin B in mouse eggs requires an intact spindle (Kubiak et al., 1993; Moos et al., 1995), and consequently histone H1 kinase activity remains elevated. Nevertheless, we observe that MAP kinase is still dephosphorylated. In these cycloheximide- and nocodazole-treated eggs, the spindle is destroyed and the condensed chromosomes are scattered throughout the cytoplasm. Although this result seems to contradict a recent report that mouse eggs treated with cycloheximide and colcemid, which also disrupts the spindle, form a PN (Moses and Kline, 1995), it should be noted that in that study the eggs were cultured for 32 hours. We have observed that cycloheximide- and nocodazole-treated eggs cultured for this length of time also form a PN, but that this is correlated with a decrease in histone H1 kinase to basal levels (J. Moos et al., unpublished results). Thus, a prolonged culture does presumably result in degradation of cyclin B even in the absence of a spindle, i.e. cyclin B degradation still occurs when a spindle is not present, albeit at a much slower rate than when the spindle is present. Last, it should be noted that this situation in the mouse apparently differs from that found in *Xenopus laevis*, in which addition of a MAP kinase phosphatase to nocodazole-treated *Xenopus laevis* egg extracts induced both the decrease in MAP kinase and histone H1 kinase (Minshull et al., 1994), and maintenance of MAP kinase activity in metaphase II-arrested *Xenopus laevis* eggs does not require protein synthesis (Haccard et al., 1993b).

Although a decrease in cdc2/cyclin B kinase activity occurs prior to that of MAP kinase in both fertilized and artificially activated eggs, the decrease in cdc2/cyclin B kinase activity is apparently not causally related to the decrease in MAP kinase activity. In both fertilized and artificially activated eggs, the decrease in cdc2/cyclin B kinase activity is clearly due to the decrease in the amount of cyclin B. Nevertheless, the decrease in MAP kinase activity in the cycloheximide- and nocodazole-treated eggs occurs in the presence of elevated levels of cdc2/cyclin B kinase. It should be emphasized that the decrease in MAP kinase activity is not due to turnover of MAP kinase, since the results of the immunoblotting experiments indicate little, if any, decrease in the amount of MAP kinase present in these treated eggs. These results suggest that some other protein(s) that is required for activation of MAP kinase is degraded and that loss of this protein during the culture period leads to the decrease in MAP kinase activity.

Two logical candidates are the upstream activators of MAP kinase, c-*mos* and its target, MEK (Nebreda and Hunt, 1993; Posada et al., 1993; Shibuya and Ruderman, 1993). MEK is unlikely to be the cycloheximide sensitive component, since MEK is so far the only known direct activator of p42 and p44

MAP kinases and okadaic acid can still induce rephosphorylation and activation of inactive MAP kinase in fertilized eggs cultured in the presence of cycloheximide (Moos et al., 1995). In contrast, c-*mos* is an attractive candidate for several reasons. In *Xenopus laevis*, c-*mos*, which is a potent upstream activator of the MAP kinase pathway (Nebreda and Hunt, 1993; Shibuya and Ruderman, 1993), is degraded following fertilization in *Xenopus laevis* eggs (Watanabe et al., 1991). c-*mos* is also likely to be a component of CSF, since oocytes in *c-mos*-deficient mice do not arrest at metaphase II following oocyte maturation, but rather become parthogenetically activated (Hashimoto et al., 1994; Colledge et al., 1994). Thus, fertilization may induce the destruction of both cyclin B and c-*mos*, each of which leads to the loss of cdc2/cyclin B kinase and MAP kinase activity, respectively. Differences in the timing of their degradation would account, therefore, for the differences in the timing of the decrease in their activities. It should be noted that in the cycloheximide- and nocodazole-treated eggs, the time course for the decrease in MAP kinase activity is slower than that in cycloheximide-treated or fertilized eggs (Moos et al., 1995). This could reflect the modulation of MAP kinase activity by cdc2/cyclin B kinase, and as described in the Introduction, results of several lines of experimentation suggest that cdc2/cyclin B kinase plays such a role. Nevertheless, if cdc2/cyclin B kinase modulates MAP kinase activity, it does not counter the loss of the cycloheximide-sensitive component that is responsible for maintenance of active MAP kinase in MII arrested mouse eggs.

Results of these experiments suggest that c-*mos* could be the cycloheximide-sensitive component. To date we have been unsuccessful in determining if c-*mos* degradation precedes the loss of MAP kinase, since the c-*mos* antibodies have not proven satisfactory for immunoblotting using mouse egg extracts. Nevertheless, with the advent of suitable antibodies, this prediction of the model should be testable.

This research was supported by a grant from the NIH (HD 22732) to G.S.K. and R.M.S.; J.M. was supported by a grant from the Agency for International Development (A.I.D. 12.061E).

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(Received 27 November 1995 - Accepted 26 January 1996)