

# Induction of Nuclear Envelope Breakdown, Chromosome Condensation, and Spindle Formation in Cell-free Extracts

MANFRED J. LOHKA and JAMES L. MALLER

Department of Pharmacology, University of Colorado School of Medicine, Denver, Colorado 80262

**ABSTRACT** Incubation of demembrated sperm chromatin in cytoplasmic extracts of unfertilized *Xenopus laevis* eggs resulted in nuclear envelope assembly, chromosome decondensation, and sperm pronuclear formation. In contrast, egg extracts made with EGTA-containing buffers induced the sperm chromatin to form chromosomes or irregularly shaped clumps of chromatin that were incorporated into bipolar or multipolar spindles. The 150,000 g supernatants of the EGTA extracts could not alone support these changes in incubated nuclei. However, these supernatants induced not only chromosome condensation and spindle formation, but also nuclear envelope breakdown when added to sperm pronuclei or isolated *Xenopus* liver or brain nuclei that were incubated in extracts made without EGTA. Similar changes were induced by partially purified preparations of maturation-promoting factor. The addition of calcium chloride to extracts containing condensed chromosomes and spindles caused dissolution of the spindles, decondensation of the chromosomes, and re-formation of interphase nuclei. These results indicate that nuclear envelope breakdown, chromosome condensation, and spindle assembly, as well as the regulation of these processes by  $Ca^{2+}$ -sensitive cytoplasmic components, can be studied in vitro using extracts of amphibian eggs.

Fully grown *Xenopus* oocytes are physiologically arrested in first meiotic prophase. Upon exposure to progesterone or insulin, oocytes synchronously complete meiotic maturation, undergoing breakdown of the nuclear envelope (germinal vesicle breakdown), chromosome condensation, spindle formation, and extrusion of the first polar body. Oocytes then proceed through meiosis II, arresting at metaphase as an unfertilized egg (see references 1 and 2 for review). After fertilization or parthenogenetic activation, the egg chromosomes decondense, a nuclear envelope reforms, and DNA replication occurs. This series of nuclear changes forms a simple cell cycle, comprising a synchronous  $G_2/M$  transition as immature oocytes undergo meiotic maturation and then an M/S transition after fertilization. Studies in several laboratories have shown that nuclei from somatic cells or spermatozoa injected into maturing oocytes or eggs cease their previous activities and begin to carry out the same processes as the oocyte nucleus (3–5). These findings suggest that the cytoplasmic components that control nuclear events through the oocyte cell cycle can act on exogenous nuclei.

The cytoplasmic control of nuclear behavior in amphibian oocytes has also been demonstrated by cytoplasmic transfer. Injection of the cytoplasm from maturing oocytes (6, 7) or of a partially purified fraction from eggs (8) into immature

oocytes induces germinal vesicle breakdown, as well as all of the other events of meiotic maturation. As a result, the activity responsible for inducing these changes has been called maturation-promoting factor (MPF).<sup>1</sup> A similar activity has been found in starfish (9) and mouse (10) oocytes undergoing meiotic maturation, as well as during mitosis in yeast (11), cleaving embryos (9, 12, 13), and cultured mammalian cells (9, 14, 15). Thus, MPF can be thought of as a widespread regulator of nuclear envelope breakdown (NEBD), chromosome condensation, and spindle formation in both meiotic and mitotic cells. Presently, however, the mechanism by which MPF induces these nuclear events remains unclear.

Recently, Lohka and Masui (16) demonstrated that cell-free cytoplasmic preparations of unfertilized *Rana pipiens* eggs could assemble a nuclear envelope around demembrated sperm chromatin and support the formation of sperm pronuclei in vitro. However, the same chromatin formed chromosomes devoid of nuclear envelopes when incubated in preparations made with the calcium ion chelator, EGTA. The apparent sensitivity of the cytoplasmic activities inducing chromosome condensation to calcium suggested that they

<sup>1</sup> Abbreviations used in this paper: MPF, maturation-promoting factor; NEBD, nuclear envelope breakdown.

might be active only at low calcium ion levels, but inactivated after an increase in free  $\text{Ca}^{2+}$  ions, which occurs at fertilization (17–19). Such regulation has also been proposed for MPF in maturing oocytes and eggs (20), and, in fact, MPF can be extracted from cells only in the presence of EGTA (13, 21).

In the present study, we have prepared cytoplasmic extracts from *Xenopus laevis* eggs using conditions that preserve MPF activity and have examined their ability to regulate nuclear activities in vitro. We find that the extracts, as well as a partially purified preparation of MPF, can cause interphase nuclei to undergo NEBD, chromosome condensation, and spindle formation in vitro, and that these changes are sensitive to calcium ions.

## MATERIALS AND METHODS

**Preparation of Extracts Causing Pronuclear Formation:** Healthy *X. laevis* females were primed with 70 IU pregnant mare serum gonadotropin (Behring Diagnostics, San Diego, CA) 48–72 h before an experiment. In the evening before an experiment, animals were injected with 750 IU human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO) and placed in tap water containing 0.1 M NaCl (13). Eggs that had been laid into the tap water 12–14 h later, and those stripped from the animal, were collected, de-jellied in 2% cysteine (pH 7.8), and washed well with 0.1 M NaCl, 50 mM Tris-HCl, pH 7.0.

The de-jellied eggs were washed in an extraction buffer consisting of 100 mM KCl, 5 mM  $\text{MgCl}_2$ , 2 mM  $\beta$ -mercaptoethanol, 20 mM HEPES (pH 7.5), 0.3 mM phenylmethylsulfonyl fluoride, and 3  $\mu\text{g}/\text{ml}$  leupeptin, and transferred to 3-ml centrifuge tubes containing the same buffer. After the excess buffer was withdrawn, the eggs were crushed by centrifugation at 10,000 g for 10 min. The material between the lipid cap and the yolk in the pellet was transferred to another centrifuge tube, and cytochalasin B (Sigma Chemical Co.) was added to a final concentration of 50  $\mu\text{g}/\text{ml}$  from a stock solution at 10 mg/ml in ethanol. The extract was centrifuged again at 10,000 g for 10 min, and the supernatant was used for incubation with nuclei.

**Preparation of Extracts Causing NEBD, Chromosome Condensation, and Spindle Formation:** Extracts were prepared following the procedures described above except that the buffer either contained 10 mM EGTA or was composed of 80 mM  $\beta$ -glycerophosphate, 15 mM  $\text{MgCl}_2$ , 20 mM EGTA, 1 mM dithiothreitol, 20 mM HEPES (pH 7.5), 0.3 mM phenylmethylsulfonyl fluoride, and 3  $\mu\text{g}/\text{ml}$  leupeptin (modified MPF buffer; see reference 8). As these extracts had similar effects on nuclear behavior, they are referred to as EGTA extracts throughout this paper. The soluble fraction of these extracts was prepared by centrifugation for 3 h at 150,000 g in an SW55 rotor. The supernatant obtained after this centrifugation was either used immediately or divided into aliquots that were then frozen in a mixture of dry ice and ethanol and stored at  $-70^\circ\text{C}$ . The protein concentration of the supernatants was between 20 and 30 mg/ml, as determined by the Bradford procedure (22) using bovine serum albumin as a standard.

**Preparation of Nuclei:** Demembrated *X. laevis* sperm nuclei were prepared as described previously (23, 24) with the exception that 0.1 mM phenylmethylsulfonyl fluoride and 1  $\mu\text{g}/\text{ml}$  leupeptin were used as protease inhibitors. Nuclei from *X. laevis* brain and liver were isolated by modifications of the procedure described by Farzaneh and Pearson (25). Briefly, the brains of two animals ( $\sim 0.1$  g of tissue) were homogenized in 1.0 ml of a 2.2 M sucrose solution. All of the sucrose solutions contained 2.0 mM  $\text{MgCl}_2$ , 2.0 mM  $\beta$ -mercaptoethanol, 0.5 mM spermine, and 10 mM HEPES (pH 7.5). The homogenate was layered on a discontinuous gradient made up of 1.0 ml of a 2.5 M sucrose solution and 3.0 ml of a 2.2 M sucrose solution and centrifuged in a SW55 rotor at 100,000 g for 2 h. Nuclei were collected from the interface of the 2.2 and 2.5 M solutions, resuspended in a 0.25 M sucrose solution, and counted with a hemocytometer. The nuclear suspension was divided into aliquots containing the desired number of nuclei and centrifuged at 1,000 g for 10 min. Liver nuclei were isolated in a similar manner except that the tissue was homogenized in 2.0 ml of solution and the homogenate passed through eight layers of cheese cloth before it was layered on the sucrose gradient. The brain and liver nuclei were used within 1 h of isolation.

**Incubation of Nuclei in Cytoplasmic Extracts:** Nuclei were mixed with 150- $\mu\text{l}$  aliquots of the egg extracts to give a concentration of  $1\text{--}2 \times 10^6$  nuclei/ml for sperm and  $1 \times 10^6$  nuclei/ml for brain and liver. The egg extracts were then incubated at  $19 \pm 1^\circ\text{C}$ . To induce NEBD, chromosome condensation, and spindle assembly, 75  $\mu\text{l}$  of extracts made without EGTA and

containing nuclei were mixed with 75  $\mu\text{l}$  of the 150,000 g supernatant from EGTA extracts and incubated at  $19 \pm 1^\circ\text{C}$ . When partially purified preparations of MPF were used (protein concentration of 11.2 mg/ml, a gift from Dr. J. C. Gerhart, University of California, Berkeley), 5  $\mu\text{l}$  of the MPF preparation was diluted with 20  $\mu\text{l}$  of MPF buffer and then mixed with 75  $\mu\text{l}$  of extracts containing sperm pronuclei. Alternatively, 5  $\mu\text{l}$  were mixed with 120  $\mu\text{l}$  of MPF buffer and 25- $\mu\text{l}$  aliquots of the mixture were added to 75  $\mu\text{l}$  of extracts containing pronuclei.

The sensitivity of condensed chromosomes and spindles to  $\text{Ca}^{2+}$  ions was examined in the following manner: 75  $\mu\text{l}$  of the 150,000 g supernatant from extracts made with 10 mM EGTA were mixed with 75  $\mu\text{l}$  of extracts containing pronuclei that had formed during the previous 60-min incubation. 1 h later, when virtually all of the pronuclei had undergone NEBD, chromosome condensation, and spindle assembly, several (4–6) incubation mixtures were pooled and separated into 100- $\mu\text{l}$  aliquots. Various volumes of a 50 mM  $\text{CaCl}_2$  solution were added to each 100- $\mu\text{l}$  aliquot, and the mixture was incubated at  $19^\circ\text{C}$  for 60–90 min before nuclear morphology was assessed.

**Cytological and Histological Procedures:** Aliquots of extracts containing nuclei were fixed in a cold mixture of ethanol and acetic acid (3:1); usually  $\sim 25\text{--}50$   $\mu\text{l}$  of extract was fixed in 1 ml of fixative. Squashes were prepared as previously described (26). In some cases, samples were fixed in Smith's solution, embedded in paraffin, and sectioned at 7  $\mu\text{m}$  for histological examination (6).

## RESULTS

Initially, we asked whether cytoplasmic preparations from *Xenopus* eggs extracted without EGTA would support, in vitro, the formation of pronuclei from demembrated sperm chromatin. When sperm chromatin was incubated in these preparations, interphase pronuclei, whose chromatin was decondensed and enclosed within a newly assembled nuclear envelope, formed within 60 min. These results were similar to those found with extracts of *Rana* eggs (16, 23, 26). In contrast, when sperm chromatin was incubated in extracts made with EGTA, pronuclei were not formed, but rather the chromatin was transformed into chromosomes, as described previously for extracts of *Rana* eggs prepared in a similar manner (16). However, unlike the chromosomes formed in *Rana* egg extracts, those formed in the *Xenopus* egg extracts were embedded in bipolar or multipolar spindles (Fig. 1) that varied greatly in size and often incorporated chromosomes from more than one nucleus. The spindles assembled in vitro did not have prominent asters, suggesting that they resemble the meiotic spindles of unfertilized eggs more closely than those of cleaving embryos.

Chromosome condensation and spindle assembly could also be induced in newly formed sperm pronuclei. In these experiments, EGTA extracts were centrifuged at 150,000 g for 3 h, and the supernatants were added to extracts in which 70–90% of the demembrated sperm nuclei had formed interphase pronuclei during a 60–90-min incubation. As shown in Fig. 2, the supernatant from the EGTA extracts of unfertilized eggs caused NEBD, condensation of the chromatin into chromosomes, and spindle assembly. In contrast, no effect was observed when only buffer containing EGTA was added to the pronuclei. Similarly, the supernatants of extracts made from fertilized eggs 30 min after insemination, which lack detectable MPF activity (13), did not induce NEBD, chromosome condensation, or spindle assembly.

Generally, NEBD and the early stages of chromosome condensation were evident between 15 and 30 min after the addition of the supernatant. At first, the condensed chromosomes often resembled those seen at prophase or pro-metaphase, but with further incubation they became more condensed, resembling those seen in metaphase. Spindles usually formed by 60 min and often persisted up to 180 min. In the

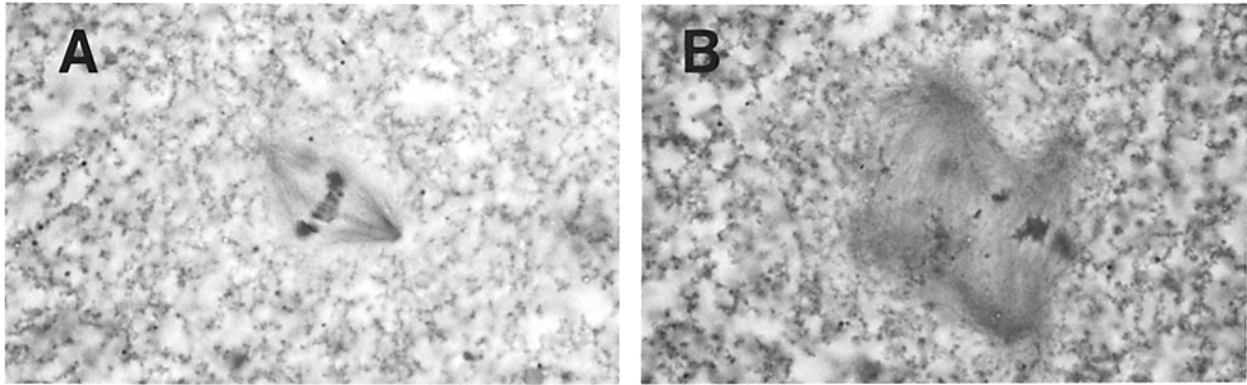


FIGURE 1 Histological section of spindles assembled during a 90-min incubation of sperm chromatin in EGTA extracts of unfertilized *Xenopus* eggs. (A) bipolar spindle; (B) multipolar spindle.  $\times 1,000$ .

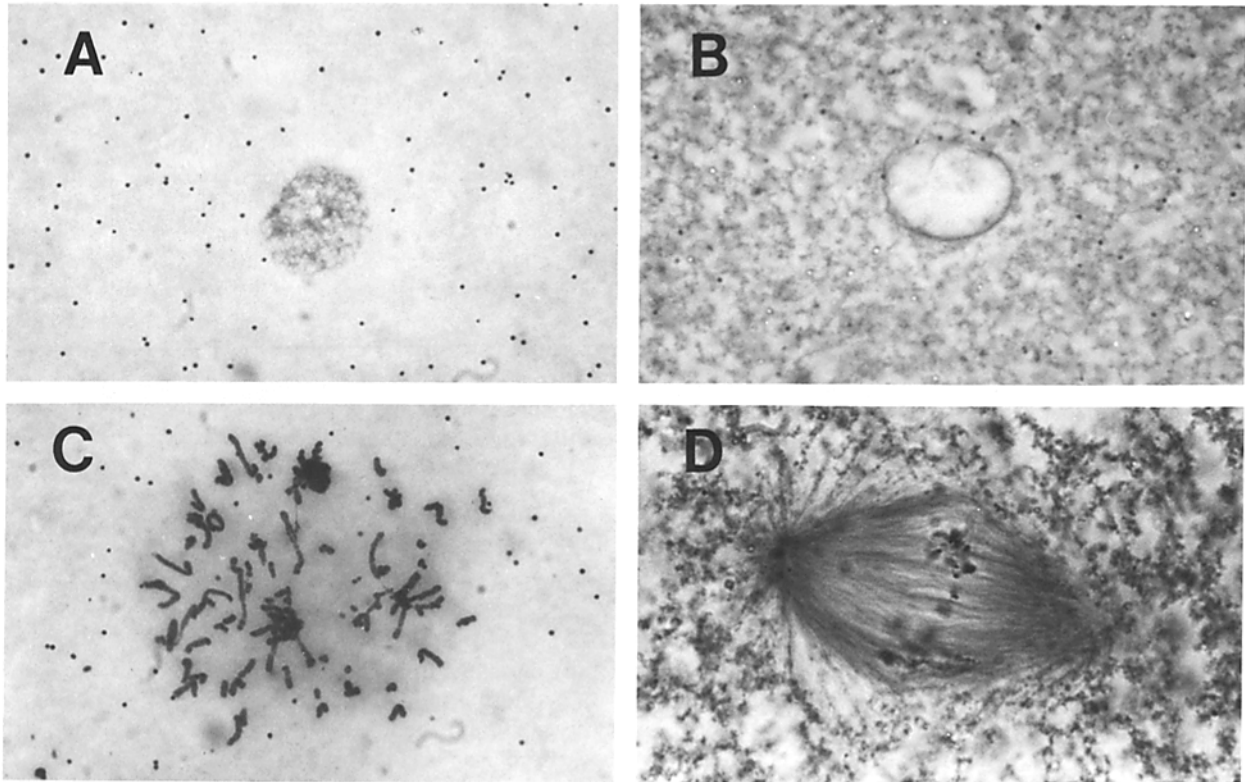


FIGURE 2 Induction of NEBD, chromosome condensation, and spindle assembly in sperm pronuclei. (A) Squash preparation of sperm pronucleus formed during a 60-min incubation in extracts made without EGTA; (B) histological section of sperm pronucleus showing intact nuclear envelope; (C) squash preparation of condensed chromosomes formed 90 min after the addition of an EGTA supernatant to extracts containing sperm pronuclei; (D) histological section of condensed chromosomes showing that NEBD and spindle assembly had occurred.  $\times 1,000$ .

presence of inhibitors of microtubule polymerization such as colchicine, nocodazole, or vinblastine sulfate (each at  $10 \mu\text{g}/\text{ml}$ ), spindle assembly was inhibited, but NEBD and chromosome condensation still occurred. In these cases, however, the chromosomes usually condensed into large intertwined clusters. Cycloheximide ( $10 \mu\text{g}/\text{ml}$ ) did not prevent any of the nuclear changes induced by the supernatants (data not shown).

An important question concerns whether the changes in nuclear behavior described above occur only in sperm pronuclei assembled in vitro. While such nuclei appear to have structurally normal nuclear envelopes (23, 26), they may be unusually sensitive to breakdown by cytoplasmic activities in

the EGTA extracts. To assess this possibility, the effect of EGTA extracts on nuclei isolated from somatic tissues was examined. In these experiments, partially purified preparations of *Xenopus* liver nuclei were mixed with cytoplasmic preparations made without EGTA. After incubations of 0 or 60 min, the  $150,000 g$  supernatant fraction of EGTA extracts was added and the nuclei incubated further. In both cases, NEBD, chromosome condensation, and spindle assembly occurred (Fig. 3). Similar results were obtained with *Xenopus* brain nuclei (data not shown).

It is important to note that although chromosome condensation and spindle assembly could be induced by the supernatant of EGTA extracts when it was added to the cytoplasmic

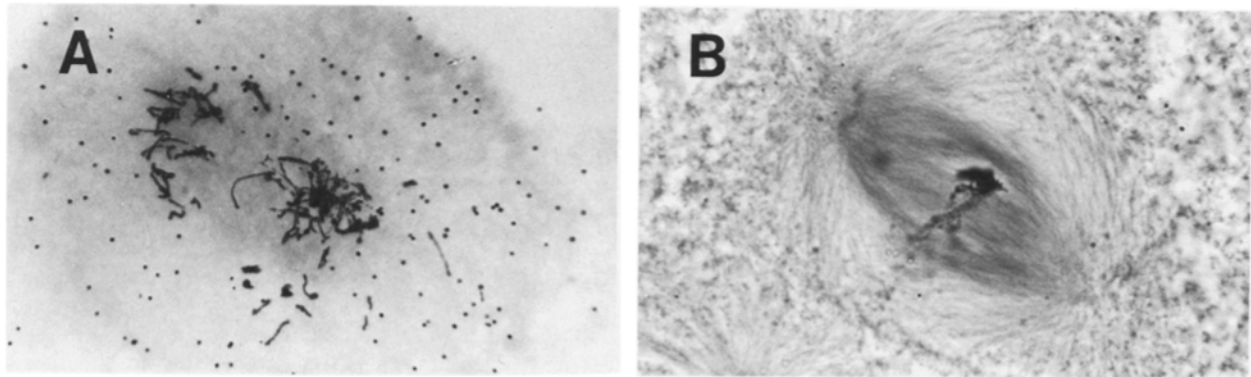


FIGURE 3 Induction of NEBD, chromosome condensation, and spindle assembly in isolated *Xenopus* liver nuclei. (A) Squash preparation of condensed chromosomes formed 90 min after the addition of an EGTA supernatant to extracts containing liver nuclei.  $\times 800$ . (B) Histological section through a spindle formed around condensed liver chromosomes.  $\times 1,000$ .

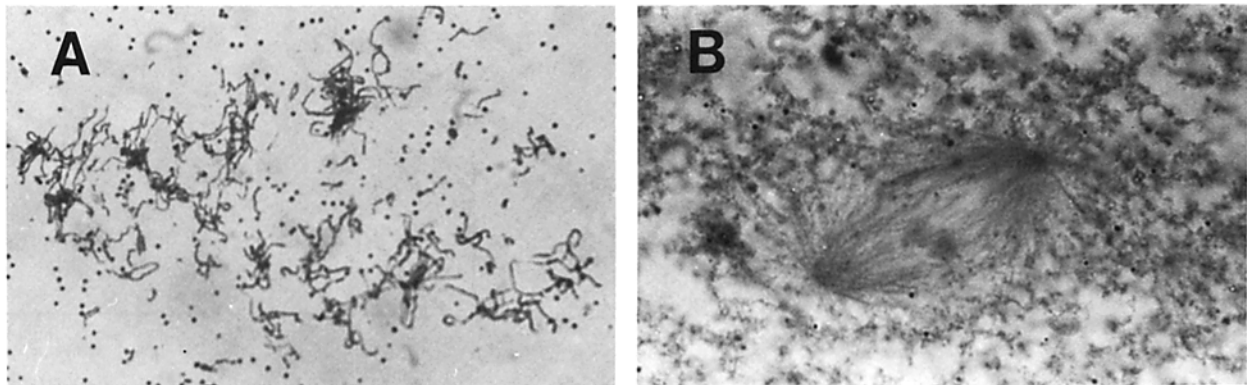


FIGURE 4 Induction of NEBD, chromosome condensation, and spindle assembly by partially purified preparations of MPF.  $5 \mu\text{l}$  of a partially purified MPF preparation was mixed with  $20 \mu\text{l}$  of MPF buffer and added to  $75 \mu\text{l}$  of an extract containing sperm pronuclei. (A) Squash preparation of condensed chromosomes formed 60 min later.  $\times 900$ . (B) Histological section through spindle formed around the condensed chromosomes 60 min later.  $\times 1,000$ .

extracts containing sperm pronuclei, brain nuclei, or liver nuclei, neither of these events occurred when the demembrated sperm chromatin or isolated nuclei were incubated in the supernatant by itself. Thus, it seems that particulate components in the cytoplasmic preparations are also required for chromosome condensation and spindle assembly in this cell-free system.

The nuclear events observed in the cell-free extracts are similar to those that occur in oocytes after the injection of MPF. The activity in the EGTA supernatants that causes these nuclear changes may result from the action of MPF, since injection of these supernatants into immature oocytes induces meiotic maturation (data not shown). As an initial test of this possibility, we added partially purified preparations of MPF to extracts containing newly assembled sperm pronuclei. The addition of these preparations at the highest concentration induced NEBD, chromosome condensation, and spindle assembly within 60 min (Fig. 4). Lower concentrations of MPF also induced NEBD and chromosome condensation, but spindles did not always form or were greatly reduced in size, and interphase nuclei often re-formed spontaneously when the incubations were longer than 60 min.

At fertilization, the MPF activity of the egg cytoplasm disappears rapidly (12, 13). This disappearance is thought to follow the increase in free cytoplasmic  $\text{Ca}^{2+}$  ions that occurs at fertilization (17–19). To assess whether the activity in EGTA supernatants that induces NEBD, chromosome con-

TABLE I. Sensitivity of Condensed Chromosomes to  $\text{Ca}^{2+}$  Ions

Volume of $\text{CaCl}_2$ solution added $\mu\text{l}$	Nuclear morphology	
	Condensed chromosomes	Interphase nuclei
0	+	–
1	+	–
2	+	+
3	–	+
4	–	+

Extracts containing sperm pronuclei were mixed with equal volumes of the 150,000 g supernatant from extracts made using 10 mM EGTA. 60 min later, various volumes of a 50 mM  $\text{CaCl}_2$  solution were added to  $100\text{-}\mu\text{l}$  aliquots of the mixture. Nuclei were examined 90 min later. The combined results of three experiments are shown.

densation, and spindle assembly is sensitive to  $\text{Ca}^{2+}$  ions, increasing amounts of a 50 mM calcium chloride solution were added to  $100 \mu\text{l}$  of preparations in which chromosome condensation and spindle assembly had occurred during the 60-min incubation after the addition of EGTA supernatants to newly formed pronuclei. As shown in Table I, the addition of  $\text{Ca}^{2+}$  ions caused a dissolution of the spindle and decondensation of the chromosomes, resulting in the formation of interphase nuclei within 60–90 min. In each of three experiments, chromosomes remained condensed when  $1 \mu\text{l}$  or less was added. However, chromosomes decondensed and inter-

phase nuclei formed in two of three experiments when 2  $\mu$ l was added and in all cases when 3 or 4  $\mu$ l was added. The exact free  $\text{Ca}^{2+}$  concentration required to induce the transition from metaphase to interphase is not known since the amount of  $\text{Ca}^{2+}$  released during the preparation of extracts has not been determined. Although interphase nuclei re-formed after the addition of  $\text{Ca}^{2+}$ , these nuclei were usually smaller than were the pronuclei to which the EGTA supernatants had been added.

## DISCUSSION

The ability of the cytoplasm from cells in meiotic or mitotic metaphase to induce NEBD, chromosome condensation, and spindle formation has been established in a wide variety of cells by nuclear transplantation (3–5), cytoplasmic transfer (6–15), and cell fusion (27). The results described in this paper indicate that these nuclear events may also be induced in vitro when sperm pronuclei or somatic cell nuclei are exposed to cytoplasmic preparations from unfertilized *Xenopus* eggs physiologically arrested in meiotic metaphase. The ability to induce these changes in vitro may offer a novel approach for the study of the biochemical mechanisms controlling NEBD, chromosome condensation, and spindle assembly when a cell enters mitosis. In addition, the chromosomal analysis of cells with low mitotic indices may be facilitated by incubating their nuclei in the cytoplasmic preparations we have described.

The induction in vitro of NEBD, chromosome condensation, and spindle assembly by supernatants from EGTA extracts of unfertilized *Xenopus* eggs is similar to the action of MPF in intact oocytes and in mitotically dividing cells. The possibility that the cytoplasmic component responsible for inducing these changes is, in fact, MPF is suggested by our observation that NEBD, chromosome condensation, and spindle assembly can be induced by EGTA extracts of unfertilized eggs, which have a high MPF activity, but not by EGTA extracts made from fertilized eggs 30–40 min after insemination, a time at which MPF activity cannot be detected (13). More importantly, the ability of partially purified preparations of MPF to induce all of these changes described above further supports the notion that the action of MPF is responsible for these changes. Our results are in agreement with those of others who have found that partially purified MPF preparations can cause NEBD and chromosome condensation not only when injected into oocytes (8), but also when injected into *Xenopus* embryos arrested in  $G_2$  of the cell cycle (28, 29) and when added to rat thymocyte nuclei incubated in cell-free extracts of the  $G_2$ -arrested embryos (30). However, we have extended these observations to show that spindle assembly around the condensed chromosomes is also a consequence of MPF action in vitro.

The mechanism by which MPF induces the nuclear changes associated with the entry into mitosis remains to be determined. However, MPF does not appear to act on the nucleus by itself. When sperm chromatin or somatic cell nuclei are incubated in the 150,000 g supernatants from EGTA extracts of unfertilized eggs, they did not form chromosomes or spindles. Yet the same supernatants could induce NEBD, chromosome condensation, and spindle assembly when added to extracts in which particulate components were abundant. These results suggest that particulate components are also required for these nuclear changes in vitro. While the nature of this particulate material is not yet known, it is tempting to

speculate that it includes the membrane vesicles that are found in the spindles of meiotic and mitotic cells (see reference 31 for review). Other possible roles for the particulate material remain to be identified, as does the role of nonchromosomal components such as centrioles, which may be present in the partially purified preparations of nuclei.

The spindles formed when the supernatants of EGTA extracts are mixed with pronuclei often persist for up to 3 h. However, the addition of  $\text{Ca}^{2+}$  ions to these preparations results in the disassembly of spindles and the decondensation of chromosomes, re-forming interphase nuclei. Therefore, it appears that cytoplasmic components involved in chromosome condensation and spindle assembly are inactivated by  $\text{Ca}^{2+}$  ions. A similar  $\text{Ca}^{2+}$ -sensitivity has been observed for the chromosome condensation activity in EGTA extracts of *Rana* eggs (16). Masui and co-workers (20, 32) have proposed, based on the sensitivity of several ooplasmic components to  $\text{Ca}^{2+}$ , that in metaphase-arrested eggs, the cytoplasmic activity that promotes chromosome condensation is active only when the free  $\text{Ca}^{2+}$  ion levels are low, and that the increase in free  $\text{Ca}^{2+}$  at fertilization results in the development of a cytoplasmic activity that promotes chromosome decondensation and the formation of interphase pronuclei. Our results are consistent with this proposal. However, it is not clear whether the  $\text{Ca}^{2+}$ -sensitive component in our cytoplasmic extracts is MPF or some other activity.

While partially purified MPF can induce NEBD, chromosome condensation, and spindle assembly within 60 min, at lower concentrations of MPF interphase nuclei often re-form during further incubation without  $\text{Ca}^{2+}$  addition. Similar results have been observed when partially purified MPF was either injected into  $G_2$ -arrested embryos (33) or mixed with nuclei incubated in extracts of these embryos (28). Therefore, MPF may be inactivated without the addition of  $\text{Ca}^{2+}$ . If so, the persistence of the condensed chromosomes and spindles induced by the supernatants of EGTA extracts may result from the presence of another cytoplasmic activity that has been removed during the preparation of partially purified MPF. This component could be cytostatic factor, a  $\text{Ca}^{2+}$ -sensitive ooplasmic factor thought to be responsible for the metaphase arrest in unfertilized eggs (6, 34). In fact, Newport and Kirschner (33) have recently shown that cytoplasm containing cytostatic factor can stabilize the NEBD and chromosome condensation induced by the injection of partially purified MPF preparations into  $G_2$ -arrested embryos. Therefore, in addition to facilitating the study of the mechanism by which MPF induces NEBD, chromosome condensation, and spindle assembly, the cytoplasmic preparations described in this paper may also facilitate analysis of the action of cytostatic factor.

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