

THE CYCLIC BEHAVIOR OF A CYTOPLASMIC FACTOR CONTROLLING NUCLEAR MEMBRANE BREAKDOWN

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ABSTRACT

The activity of a cytoplasmic factor (MPF), capable of inducing nuclear membrane breakdown (germinal vesicle breakdown) when injected into amphibian oocytes, has been studied during the course of early cleavage in amphibian embryos. Mature egg cytoplasm was found to contain high levels of this activity, but this was quickly lost after fertilization or artificial activation. MPF activity later reappeared in the egg cytoplasm and started to cycle with time.

The peak of embryonic MPF activity during each cycle coincided with the time the embryonic nuclei were entering the G₂-M transition, i.e., mitosis. However, in colchicine-arrested embryos, this activity remained at an elevated level and no longer oscillated. The timing of the appearance and disappearance of this activity appeared to be under the control of the cytoplasm because such behavior was still observed in enucleated eggs. Continued protein synthesis in the embryo was required for the reappearance, but not for the disappearance, of this activity.

MPF, previously thought to be restricted to oocyte maturation, may play a more general role in controlling nuclear membrane breakdown during mitosis as well as meiosis.

KEY WORDS maturation promoting factor · embryos · cell cycle · nuclear membrane breakdown

The cytoplasm of a cell has been shown to play a significant role in the control of nuclear activity. By the use of such experimental techniques as cell fusion, nuclear transplantation, and cytoplasmic transfers between cells, it has been demonstrated that DNA synthesis, chromosome condensation, and decondensation as well as nuclear membrane dissolution and reformation are all induced by substances which appear in the cytoplasm at specific times during the cell cycle. This nucleo-cytoplasmic interaction has been observed in a variety of cell systems including protozoa (3, 5, 16), cultured mammalian cells (10, 11, 17, 18, 20), and amphibian oocytes (6, 7, 27-29).

When amphibian oocytes are treated with progesterone, they undergo a process known as maturation (28). During this event, the nucleus, still

in meiotic prophase, migrates to the periphery of the oocyte and undergoes nuclear membrane breakdown (germinal vesicle breakdown [GVBD]). At about the same time, the chromosomes condense, and then align themselves on the first meiotic spindle. Subsequently, these chromosomes are aligned on the second metaphase plate of meiosis. Cytoplasm, taken from hormonally stimulated oocytes at the time of GVBD, can induce nuclear dissolution when microinjected into ovarian oocytes that have not been exposed to hormone (14, 19, 21, 22, 28). Thus, it appears that nuclear membrane breakdown is under the control of a cytoplasmic factor in these oocytes.

Similar experiments have shown that the cytoplasm controls GVBD in starfish oocytes that are induced to mature with 1-methyladenine (12). Most recently, the cytoplasmic control of GVBD has also been demonstrated in maturing mouse oocytes (1).

The cytoplasmic substance(s) controlling GVBD

in amphibian oocytes has been designated as the maturation promoting factor (MPF). Recently, this substance(s) has been extracted from mature amphibian oocytes and preliminary characterization of this material has been made (27). Early studies, employing cytoplasmic transfers, showed that MPF activity persists throughout the period of oocyte maturation and that some activity still could be detected in cleaving blastomeres (14, 19). Although it was not stated, it has been assumed that this embryonic cytoplasmic activity was residual MPF from the maturing oocyte and that this activity was eventually lost during the early cleavage stages. The objective of this study was to follow more carefully the behavior of MPF activity during early cleavage. The data show that, contrary to expectations, MPF activity could still be detected in late morula to early blastula stages. More significantly, data are presented which show that MPF activity cycles during cleavage in relation to the mitotic cell cycle.

MATERIALS AND METHODS

Defolliculated, stage 6 (4) *Xenopus laevis* oocytes were obtained as previously described (25), and were maintained in OR-2 medium (24).

Mature *Rana pipiens* eggs were obtained by injecting females with a macerated pituitary suspension in Ringer's solution (9). The eggs were stripped from the frog, artificially fertilized, and then maintained in 10% Ringer's solution. In some instances, the eggs were not fertilized, but were artificially activated by pricking with a clean glass needle. Some of these activated *Rana* eggs were also enucleated by the method of Porter (15) as modified by King (13). The success of this enucleation procedure was tested by fertilizing other eggs, removing the egg chromosomes by the same procedure, and then scoring for androgenetic haploid development (15). This procedure was 95% successful.

Xenopus laevis eggs were obtained by injecting females with 800 IU of human chorionic gonadotropin. The eggs were stripped from the frog, artificially fertilized, and then maintained in 5% DeBoer's solution as previously described (8). The jelly surrounding the *Rana* eggs was manually cut away with iris scissors. *Xenopus* eggs were chemically dejellied with 5 mM dithiothreitol in 5% DeBoer's solution, pH 8.0, for 2 min and then washed and returned to 5% DeBoer's solution, pH 7.2.

Microinjection procedures were carried out with a Leitz micromanipulator and a multiple injection pipette system capable of delivering 40 or 80 nl per recipient oocyte (25). Eggs were assayed for MPF activity by microinjecting 80 nl of cytoplasm into each recipient oocyte and scoring for the induction of GVBD.

Embryos were injected with colchicine or cycloheximide and were simultaneously incubated in the same

concentration of the inhibitor as stated in the text. These eggs were then assayed for MPF activity.

MPF was extracted from colchicine-arrested *Xenopus* blastulae by the centrifugation-crushing method that was previously employed to extract MPF from mature *Rana pipiens* eggs (27). This procedure involved packing intact embryos with extraction medium in centrifuge tubes, spinning first at 25,000 g for 15 min followed by a second spin at 150,000 g for 2 h. The extraction medium developed for *Xenopus* eggs and embryos contained 50 mM β -glycerophosphate, 0.25 M sucrose, 10 mM $MgCl_2$, and 2 mM ethylene glycol-bis(β -aminoethyl ether)*N,N'*-tetraacetic acid (EGTA), pH 6.8.

RESULTS

The Cyclic Nature of MPF Activity in Early Cleaving Embryos

Previous studies have already demonstrated that MPF action is not species-specific (19, 27). However, of the various combinations tested, *Xenopus* recipient oocytes always underwent GVBD with the highest frequency in response to equivalent amounts of injected cytoplasm. Furthermore, GVBD in *Xenopus* oocytes normally takes place 5–6 h after progesterone stimulation; however, they exhibit a precocious response to MPF, undergoing GVBD with in 1.5–2 h after its injection (19, 27). Thus, *Xenopus* stage 6 oocytes were used as recipients in all experiments for the assay of MPF activity.

Cytoplasm taken from mature, unfertilized *Rana* or *Xenopus* eggs induced all of the *Xenopus* recipient oocytes to undergo GVBD, chromosome condensation, and polar body formation. This result was expected on the basis of earlier reports showing that MPF activity persisted through the completion of maturation (14, 19). However, after fertilization or artificial activation, MPF activity cycled as a function of time.

In *Rana* eggs, the first cell cycle lasts ~3 h at 18°C, whereas the second cell cycle lasts ~1 h (23). 80 nl of cytoplasm taken from *Rana* eggs within 30–60 min after fertilization or artificial activation contained little to no detectable MPF activity (Fig. 1A and B). However, activity was again detected in the same assay volume at 90 min, peaked at 120 min, and started to decline again at 150 min. At the time of actual cytokinesis (180 min), there was no activity detected in fertilized eggs, and only a low level of activity in the nondividing activated eggs. As the second cleavage cycle was underway, MPF activity reappeared again, reaching a peak in this case at an elapsed time of 225

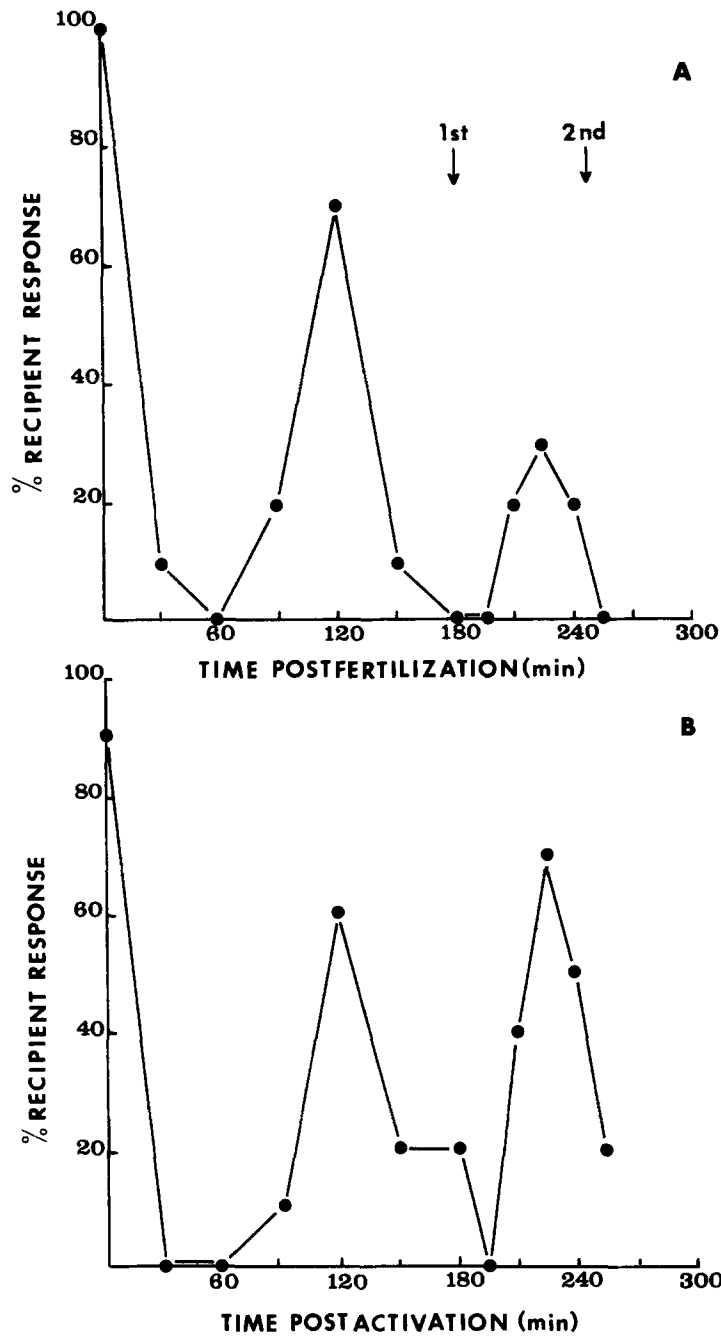


FIGURE 1 Cycling of MPF activity in fertilized or artificially activated *Rana pipiens* eggs. Abscissa: percent *Xenopus* recipient oocytes undergoing GVBD in response to 80 nl of *Rana* egg cytoplasm. Ordinate: time after fertilization or artificial activation when the *Rana* egg cytoplasm was assayed for MPF activity. (A) Fertilized *Rana* eggs. (B) Artificially activated *Rana* eggs. In all experiments, five donor eggs and 10 recipient oocytes were used at each time-point to assay for the presence of MPF activity. Arrows indicate time of cleavage.

min, and then the activity declined to a very low level (Fig. 1A). This second activity cycle was also observed in artificially activated eggs (Fig. 1B).

The first cell cycle in fertilized *Xenopus* eggs lasts ~1.5 h at 20°C, whereas the second cycle takes only 30 min (6). Thus, transition times between divisions in *Xenopus* embryos are much shorter than in *Rana* embryos. This was reflected in the behavior of MPF activity. MPF activity in fertilized *Xenopus* eggs also appeared to cycle, although on a shorter time scale. Within 15 min after fertilization, very little activity was detected. MPF activity began to increase again at 45 min, peaked at 60 min, and then decreased and peaked again during the next two cell cycles (Fig. 2). The loss of activity between cell divisions in *Xenopus* embryos was considerably less than that seen in fertilized *Rana* embryos. However, the time required for the assay procedure may surpass the short time period when MPF activity in the *Xenopus* embryo is at its lowest.

The above data show that, in both species, MPF activity disappears shortly after fertilization or artificial activation and then reappears and disappears on a time scale which correlates with the length of the cell cycle. Inasmuch as the timing of cleavage in amphibian embryos is known to be regulated by the cytoplasm, we further sought to determine whether MPF activity would cycle on the same time scale in enucleated eggs. When the cytoplasm of enucleated eggs was assayed 30 and 60 min after activation, there was little to no detectable activity (Fig. 3), as was the case for control *Rana* embryo cytoplasm (Fig. 1A). However, when the cytoplasm was assayed 120 and 150 min after activation, the activity reappeared (Fig. 3). This level of activity was approximately the same as that detected in nucleated control embryos. The activity appeared to cycle in these enucleated eggs, at least during the course of the experiment, which lasted for 5 h after activation (Fig. 3). One can conclude that the immediate presence of nuclear components is not necessary during early cleavage for the cyclic appearance and disappearance of embryonic MPF activity.

Temporal Relationship of Embryonic MPF Activity to Mitosis

In *Rana* eggs, the zygote nucleus enters mitosis about 120 min after fertilization at 18°C (23). In *Xenopus* eggs, the zygote nucleus is reported to

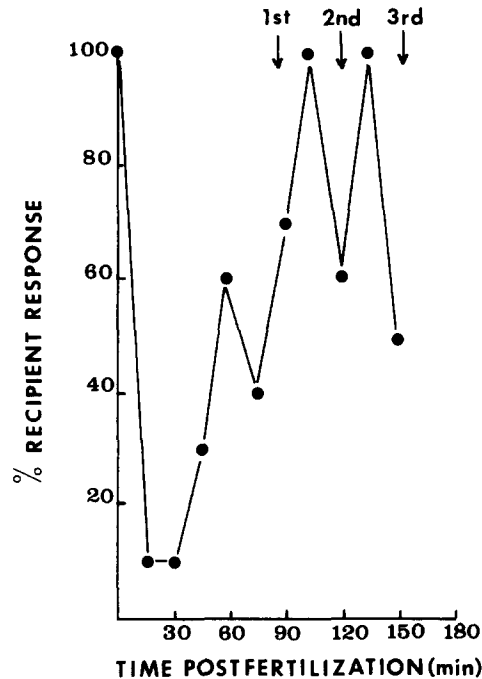


FIGURE 2 Cycling of MPF activity in fertilized *Xenopus laevis* eggs. Abscissa: percent *Xenopus* recipient oocytes undergoing GVBD in response to 80 nl of *Xenopus* egg cytoplasm. Ordinate: time after fertilization when the *Xenopus* egg cytoplasm was assayed for MPF activity. Arrows indicate time of cleavage.

enter the first mitotic division between 60 and 70 min after fertilization, whereas the second mitosis takes place at ~110 min (6). Thus, in both cases, the peak of embryonic MPF activity appears to correlate well with the time of nuclear membrane breakdown and entrance into mitosis. Cytological examination of donor *Rana* embryos during times at which MPF activity had peaked confirmed that the nuclear membrane had broken down and that the chromosomes had condensed (data not shown). To test this correlation further, two kinds of experiments were performed in which rapidly dividing cells were arrested with colchicine in mitosis.

In the first set of experiments, *Xenopus* or *Rana* eggs were fertilized, dejellied, and allowed to develop for 45 or 60 min, respectively. At this time, they were injected with colchicine, giving an estimated internal concentration of 0.5 mM. The injected eggs were then incubated with 0.5 mM colchicine for an additional 45–200 min. Colchicine-injected eggs did not cleave and were arrested in the first mitotic division. An example of

the behavior of MPF activity in colchicine-treated *Rana* eggs is shown in Fig. 4. MPF activity increased to an elevated level and was maintained at this level for the duration of the experiment (4 h). For comparison, MPF activity in fertilized eggs from the same frog that were not treated with colchicine is also shown (Fig. 4). Assay times for the control series in Fig. 4 were not the same as those in Fig. 1A. This may account for the different levels of activity that were detected around the time of first cleavage (180 min). When colchicine-treated *Xenopus* egg cytoplasm was assayed over an interval of 4 h, MPF activity again remained extremely high and did not appear to cycle or decline (Table I). Thus, in both cases, as long as the cells were arrested in mitosis, the cytoplasm exhibited high levels of MPF activity.

In the second group of experiments, fertilized *Xenopus* eggs were simply incubated in 5% DeBoer's solution containing colchicine at 0.5 mM beginning 1 h after fertilization. Under these conditions, the eggs continued to develop to late morula or early blastula stages where they arrested. This delay in arrest probably reflects the limited permeability of developing embryos to this inhibitor. These arrested blastulae were then assayed for MPF activity.

Assaying cytoplasm from individual blastula cells by the conventional microinjection procedure was technically difficult; thus, attempts were made to extract this activity from arrested em-

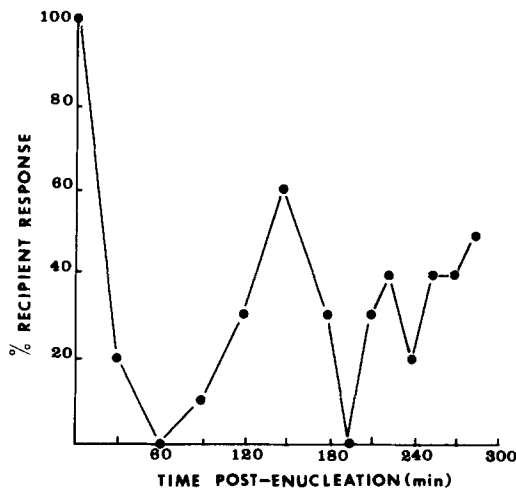


FIGURE 3 Cycling of MPF activity in enucleated *Rana* eggs. *Rana* eggs were enucleated as described in the text. Assay conditions were the same as those described for Fig. 1.

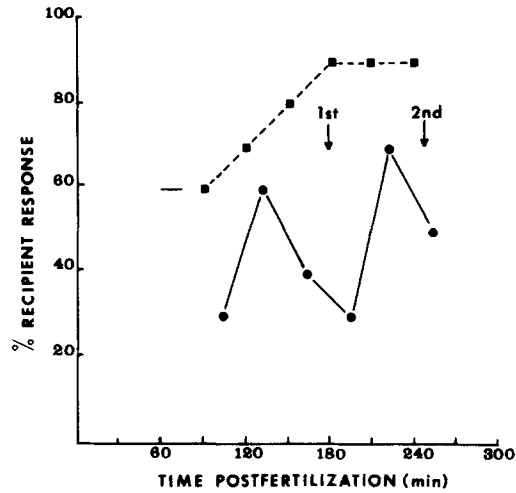


FIGURE 4 MPF activity in colchicine-arrested *Rana* eggs. *Rana* eggs were injected with colchicine, 60–75 min after fertilization, as described in the text. Assay conditions were the same as those described for Fig. 1. Colchicine-treated (■), control (●). Arrows indicate time of cleavage.

bryos. Previous work has shown that homogenization of mature oocytes leads to the quick inactivation of MPF activity (27). Therefore, the centrifugation-crushing procedure that was developed to extract MPF from *Rana* oocytes was used on these arrested blastulae. A high level of MPF activity was obtained from these colchicine-arrested blastulae in a 150,000-g cytosol preparation. Thus, MPF activity persists into the later stages of development where the mitotic rate is still high.

Cycling of Embryonic MPF Is Dependent on Protein Synthesis

One interpretation of the cycling behavior of MPF activity during cleavage is that MPF is synthesized and degraded during each cell cycle. To approach this possibility, *Xenopus* eggs were injected with 50 ng of cycloheximide at 30, 45, 60, and 75 min after fertilization, followed by incubation for an additional 60 min in 5% DeBoer's solution containing cycloheximide at 50 $\mu\text{g}/\text{ml}$. The internal concentration of cycloheximide was 100 $\mu\text{g}/\text{ml}$, assuming a water volume of 0.5 $\mu\text{l}/\text{egg}$, and this concentration is sufficient to inhibit protein synthesis by >95%.¹ At the end of each incubation time, 90, 105, 120, and 135 min

¹ C. O'Connor and L. D. Smith. Unpublished results.

TABLE I
Xenopus Recipient Oocyte Response to Cytoplasm from Embryos* Treated with Inhibitors

Assay time after fertilization of <i>Xenopus</i> embryo cytoplasm	Colchicine		Cycloheximide		Control	
	Oocytes‡	GVBD	Oocytes	GVBD	Oocytes	GVBD
<i>min</i>						
90	20	20	20	0	20	15
105	20	19	20	0	20	20
120	20	19	20	0	20	11
135	—	—	20	0	20	20
150	20	20	—	—	20	9
180	20	20	—	—	—	—

* All experiments were conducted with embryos from different animals.

‡ In an experiment, five donor embryos and 10 recipient oocytes were used at each time point.

after fertilization, respectively, the cytoplasm was assayed for MPF activity. None of these eggs cleaved and none exhibited any MPF activity (Table I). This negative response on the part of the recipient oocytes was not due to transferred cycloheximide from the donor embryos. As previously demonstrated, recipient oocytes treated with cycloheximide at 100 $\mu\text{g/ml}$ still exhibit GVBD when injected with MPF (26). Therefore, the cytoplasm of these cycloheximide-treated embryos must lack MPF activity.

Eggs injected with cycloheximide at 30 min after fertilization should have contained minimal MPF activity at that time; however, 60 min later, at the time of assay, activity should have been readily detectable (see Fig. 2). Thus, in the presence of cycloheximide, MPF activity does not reappear. The same conclusion applies to eggs injected 45 and 75 min after fertilization, although the basal levels of MPF activity would have already been higher at the time of the injection than those at 30 min. In this sense, the results also demonstrate that eggs containing high levels of MPF activity at the time cycloheximide was injected, especially those injected 60 min after fertilization, subsequently lose all activity during the additional 60-min incubation period before their assay. Thus, the data indicate that protein synthesis is required for the cyclic appearance of MPF activity, but not for the disappearance of the factor.

DISCUSSION

Amphibian oocytes complete their growth phase while still in the prophase stage of meiosis. Full-grown oocytes may remain in this condition for weeks or months before either continuing meiosis

or undergoing atresia. These full-grown oocytes, then, are arrested in a phase of the cell cycle that is equivalent to G_2 . The stimulus to continue meiosis is hormonal, and the first morphological event signaling the resumption of meiosis is the breakdown of the nuclear membrane (GVBD) followed by chromosome condensation and spindle formation. In this sense, the induction of oocyte maturation includes a transition at the nuclear level equivalent to the G_2 -M transition during the mitotic cell cycle.

The nature of the events which are stimulated by hormones and which lead to GVBD during oocyte maturation is not yet clear. However, considerable evidence has accumulated to support the view that the nuclear events of maturation are brought about by a cytoplasmic factor(s) designated as the maturation promoting factor or MPF (28). Thus, cytoplasm or cytosol from maturing oocytes can induce GVBD and the subsequent events of meiosis when injected into oocytes not previously exposed to steroid hormones. In addition, these events occur much earlier in oocytes injected with MPF compared with those exposed to steroids. In fact, cytological studies have revealed that visible changes occur in the oocyte nuclear membrane as early as 30 min after the injection of MPF.² Undetected changes in the germinal vesicle membrane may take place even earlier than 30 min. Inasmuch as some time is required for the injected material to diffuse to its site of action, the remaining short response interval suggests that MPF may be acting directly on the nucleus.

The current experiments clearly show that cy-

² W. Wasserman. Unpublished results.

toplastm from cleaving embryos contains a factor which is equivalent to the MPF found in maturing oocytes. Both require protein synthesis for their appearance, both appear in the absence of a nucleus, and both induce GVBD precociously after injection into *Xenopus* oocytes. The only major difference between oocyte MPF and early embryo MPF is that, in the latter case, the activity cycles as a function of the mitotic cell cycle. MPF reaches a peak in activity at about the time of nuclear membrane breakdown, chromosome condensation, and formation of the spindle, i.e., at the G₂-M transition of the mitotic cell cycle. In this regard, we should point out that after the first meiotic division during oocyte maturation, a nuclear membrane does not reform. Rather, the chromosomes remain condensed and realign rapidly on the second meiotic spindle. In this sense, MPF activity in colchicine-treated embryos is analogous to the situation in post-GVBD oocytes that are arrested at the second metaphase of meiosis.

We cannot eliminate the possibility that the MPF activity present during cleavage represents material retained from oocyte maturation which is used throughout early development. Ample precedent exists for the synthesis and accumulation during oogenesis of materials which are inherited by the embryo and utilized during early embryogenesis (2). To be consistent with the cycloheximide experiments, such a possibility would require that oocyte MPF be activated during each cleavage cycle by a protein-synthesis-dependent mechanism. Alternatively, oocyte MPF could be degraded rapidly after fertilization, followed by *de novo* synthesis of the factor itself, coupled with turnover, at each cell cycle. Regardless of which possibility is more likely, the current results indicate that oocytes completing meiosis and embryonic cells undergoing mitosis contain a factor(s) that can induce nuclear membrane breakdown, chromosome condensation, and spindle formation.

If, as suggested earlier, dividing embryonic cells contain a factor, analogous to oocyte MPF, which can regulate the entry of the oocyte nucleus into a meiotic division, then the factor present in oocyte cytoplasm might be expected to regulate the entry of embryonic nuclei into a mitotic division. This has not yet been tested directly. However, it is known that brain nuclei, normally arrested in G₀ of the cell cycle, are induced to undergo nuclear membrane break-

down and chromosome condensation within 60 min after their injection into amphibian oocytes that have undergone GVBD (7, 29). Recently, Balakier and Czolowska (1) have shown that anucleate fragments of maturing mouse oocytes that were fused to interphase blastomeres induced precocious nuclear membrane breakdown and chromosome condensation in the blastomere nuclei.

The ability of the cytoplasm to induce nuclear membrane breakdown and chromosome condensation certainly is not restricted to oocytes and embryos. Studies involving fusion of several cultured cell types in different phases of the cell cycle have shown that nuclear membrane breakdown and chromosome condensation factors appear in the cytoplasm at the end of G₂, persist throughout M, and disappear as the cells progress into G₁ of the next cell cycle (11, 20).

Taken together, these observations suggest that MPF, previously thought to be associated only with oocyte maturation, may be more ubiquitous. Perhaps, MPF may play a more general role in controlling nuclear membrane breakdown during mitosis as well as meiosis. The results also suggest that the amphibian oocyte will provide a useful bioassay system to test certain cellular substances for their ability to initiate a nuclear division.

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