Cell Cycle Dynamics of an M-phase-specific Cytoplasmic Factor in *Xenopus laevis* Oocytes and Eggs

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ABSTRACT We have examined the regulation of maturation-promoting factor (MPF) activity in the mitotic and meiotic cell cycles of *Xenopus laevis* eggs and oocytes. To this end, we developed a method for the small scale extraction of eggs and oocytes and measured MPF activity in extracts by a dilution end point assay. We find that in oocytes, MPF activity appears before germinal vesicle breakdown and then disappears rapidly at the end of the first meiotic cycle. In the second meiotic cycle, MPF reappears before second metaphase, when maturation arrests. Thus, MPF cycling coincides with the abbreviated cycles of meiosis. When oocytes are induced to mature by low levels of injected MPF, cycloheximide does not prevent the appearance of MPF at high levels in the first cycle. This amplification indicates that an MPF precursor is present in the oocyte and activated by posttranslational means, triggered by the low level of injected MPF. Furthermore, MPF disappears approximately on time in such oocytes, indicating that the agent for MPF inactivation is also activated by posttranslational means. However, in the absence of protein synthesis, MPF never reappears in the second meiotic cycle. Upon fertilization or artificial activation of normal eggs, MPF disappears from the cytoplasm within 8 min. For a period thereafter, the inactivating agent remains able to destroy large amounts of MPF injected into the egg. It loses activity just as endogenous MPF appears at prophase of the first mitotic cycle. The repeated reciprocal cycling of MPF and the inactivating agent during cleavage stages is unaffected by colchicine and nocodazole and therefore does not require the effective completion of spindle formation, mitosis, or cytokinesis. However, MPF appearance is blocked by cycloheximide applied before mitosis; and MPF disappearance is blocked by cytostatic factor. In all these respects, MPF and the inactivating agent seem to be tightly linked to, and perhaps participate in, the cell cycle oscillator previously described for cleaving eggs of *Xenopus laevis* (Hara, K., P. Tydeman, and M. Kirschner, 1980, *Proc. Natl. Acad. Sci. USA*, 77:462–466).

There is compelling circumstantial evidence that maturation promoting factor (MPF)[1] is a cytoplasmic agent responsible for the initiation of the meiotic or mitotic phase (M phase) of the eucaryotic cell cycle. This factor was first detected in unfertilized amphibian eggs, from which cytoplasm was withdrawn and microinjected into full grown amphibian oocytes, causing the oocytes to "mature" into unfertilized eggs, hence the name of the factor (for reviews, see references 13, 15, 23). In terms of the cell cycle, such eggs are naturally arrested at second meiotic metaphase, making them a convenient source of M phase cytoplasm, whereas oocytes are naturally arrested in G_2 or early meiotic prophase and lack active MPF, making them convenient recipients. A small injection of M phase cytoplasm causes the G_2 cell to initiate M phase precociously, independent of the normal stimulus by progesterone, and to complete maturation even to the extent of forming normal high levels of MPF, which can be detected by serial transfer of cytoplasm to yet another nonmatured recipient (21). More recently, MPF has been extracted in active form from oocytes,

[1] *Abbreviations used in this paper:* EB, extraction buffer, containing 80 mM sodium β-glycerol phosphate, pH 7.3, 15 mM $MgCl_2$, 20 mM sodium EGTA; EB-SATP, EB containing 0.3 mM γ-thioATP; GVBD, germinal vesicle breakdown; MPF, maturation-promoting factor; MR medium, modified Ringer's, containing 100 mM NaCl, 1.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM $NaHCO_3$.

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M phase amphibian eggs during cleavage (27), starfish oocytes and eggs (11), mammalian cultured cells (HeLa [24] and Chinese hamster ovary [19]) and cdc mutants of yeast (28), but not from interphase cells, as assayed in all cases by injection of the extract into amphibian oocytes to induce maturation. Because of its ubiquitous presence in mitotic and meiotic cells, the factor can be referred to as an "M-phase promoting factor," rather than just a maturation-promoting factor.

Partial purification of the *Xenopus* egg MPF has been accomplished; the factor behaves as a protein ~100 kdalton (kD) size (3, 26, 29). Its action as an M phase initiator is thought to depend on its ability to activate a cascade of preformed enzymes executing the specific processes of mitosis, such as nuclear membrane breakdown, chromosome condensation, and spindle formation. In support of this view, it is known that partially purified MPF can initiate the early events of M phase when injected into cycloheximide-inhibited *Xenopus* oocytes (29), or cleaving embryos (18), indicating that at least some of the agents needed for the execution of M phase are present in a latent but activatable form.

In our study, we were interested in the cell cycle dependent regulation of MPF activity. MPF activity appears at the late G_2 phase of the cell cycle and disappears at the end of mitosis. Ultimately, we would like to trace this phase-specific pattern of MPF activity to the oscillatory processes postulated to drive the cell cycle itself (8, 12). Recently, Hara et al. (6) found that aspects of the cell cycle persist in anucleate, noncleaving, colchicine-inhibited activated eggs. Namely, such eggs continue to undergo regular surface contraction waves on the schedule of the cell cycle, even though they cannot complete DNA synthesis, spindle formation, chromosome condensation, or cytokinesis. These authors suggest that these dispensable events must be classified as effects of the underlying oscillators driving the cell cycle, and not as elements of the oscillatory reactions themselves. Earlier, MPF activity had been found to cycle in activated, noncleaving eggs (27), a result that we now interpret to mean that MPF is closely linked to the oscillator, rather than to any of the various dispensable events of the cell cycle. On the other hand, there are two published results that indicate a separation of MPF activity from the cycling of the cell. If verifiable, these results would lead us to conclude that MPF cycling is itself a dispensable event of the cycle. First, Wasserman and Smith (27) report that MPF activity remains high and constant in colchicine-blocked eggs, whereas the oscillators are known to continue to cycle in such eggs (6). Second, Masui et al. (17) have mentioned that cleaving eggs arrested in mitotic metaphase with cytostatic factor (the cytoplasmic agent thought to arrest unfertilized eggs in meiotic metaphase) do not accumulate MPF, whereas one would expect MPF to be present in this M phase cell. To examine the relation of MPF to the cell cycle under a variety of conditions, we developed a small scale extraction procedure and an endpoint dilution assay for MPF. In contradiction to these previous exceptions, we report that in all cases where the cell cycle continues to function, MPF continues to cycle, whereas in cases where the cycle is blocked, MPF activity is held at the corresponding high or low level characteristic of that stage of the cycle. Thus, the pattern of MPF activity seems to be tightly linked to the cell cycle oscillator.

With this self-consistency of results for MPF regulation in the cell cycle, we can turn to the oocyte for more detailed information on the actual steps of MPF activation (see Masui and Clarke [15] for a review). Two steps have been distinguished in maturing oocytes; one, a step in which an unidentified factor activates a small amount of MPF, and second, an amplification step in which MPF becomes auto-activating to produce high levels of itself. The first step seems clearly to require protein synthesis, since a variety of agents including progesterone, the hormone normally stimulating meiosis in the oocyte, fail to elicit MPF activation and oocyte maturation in the presence of inhibitors of protein synthesis, such as cycloheximide. It remains to be seen whether this protein-synthesis-requiring step is peculiar to the MPF activation process of the oocyte, or is general to any cell cycle which, as in the case of the oocyte, has to advance to a point where MPF can be activated. Indeed, Miake-Lye et al. (18) have found that protein synthesis is necessary for the forward progress of the cell cycle of the cleaving egg.

The second step in MPF activation, according to previous reports, may or may not require protein synthesis, and a correct resolution of the issue is important for our understanding of the MPF activation process. On the one hand, Drury and Schorderet-Slatkine (4) conclude that MPF autoamplification does not occur in cycloheximide-inhibited oocytes injected with small amounts of MPF. On the other hand, Wasserman and Masui (25) conclude the opposite in rather similar experiments. We report here than protein synthesis is *not* required for the amplification step in oocytes and that the previous discrepancies are due to the fact that MPF activity oscillates in the meiotic cycle. Therefore, MPF is amplified by posttranslational mechanisms operating on a pool of MPF precursor.

MATERIALS AND METHODS

Materials: Adult *Xenopus laevis* females and males were obtained from C. Sullivan (Nashville, TN), Nasco Co. (Modesto, CA), or raised in the laboratory. Females were injected each with 20-50 units of pregnant mare serum gonadotropin (PMSG; CalBiochem-Behring Corp., San Diego, CA) 3 d before the surgical removal of ovary for use of manually dissected oocytes in the injection assay of MPF levels. Generally oocytes were used on the day of removal. Eggs were obtained from females injected 1-3 d earlier with 50 units PMSG, and 8-12 h earlier with human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO). General procedures for the handling of eggs and oocytes have been described (14, 20, 29). MPF was partially purified from extracts of unfertilized eggs, as described by Wu and Gerhart (29), except that the arginine agarose step was omitted and replaced with a fractional precipitation with 6% polyethylene-glycol 6000 (Sigma Chemical Co.). Cytostatic factor was obtained by injecting 50 nl of 10 mM sodium EGTA, pH 7.0, into an unfertilized egg, from which ~250 nl of cytoplasm were then withdrawn in the same needle (9). This cytoplasm was then injected 50 nl each into five fertilized eggs, to block them at mitotic metaphase (17). Cycloheximide, puromycin, and nocodazole were obtained from Sigma Chemical Co.; diphtheria toxin was a gift from Dr. J. Maller (University of Colorado, Denver).

Methods: Before extraction, eggs were incubated in 5% Ficoll 400 (Sigma Chemical Co.) in 20% MR medium (modified Ringer's containing 100 mM NaCl, 1.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM $NaHCO_3$), to shrink the perivitelline space and reduce the residual volume of medium carried over into the extraction step. This precaution was not necessary for oocytes since they lack a perivitelline space. For the preparation of small volumes of extract, seven eggs or oocytes were rinsed in ice cold EB (extraction buffer containing 80 mM sodium β glycerol phosphate, pH 7.3, 15 mM $MgCl_2$, 20 mM sodium EGTA) and collected in a small cluster on a parafilm layer on a cooled aluminum block (0°C). Excess EB was removed from the cluster and then 7 μl EB-SATP (EB containing 0.3 mM γ-thioATP [Boehringer Mannheim Biochemicals, Indianapolis, IN]) were added; immediately the cells were broken in an Eppendorf pipette tip and homogenized by several passages of the suspension in and out of the tip. Various dilutions were immediately prepared from the extract, generally to a further two- and fourfold extent, by mixing the appropriate volumes of extract and EB-SATP. Each homogenate or dilution

was drawn into one-half length of a 40-μl microcap tube (Drummond Microcap Co., Oakland, CA) which had been pre-melted almost to closure at one end. The tube containing the homogenate was carefully flamed to complete closure of one end, and was centrifuged for 15 min at top speed in a Beckman Microfuge (Beckman Instruments, Palo Alto, CA), to sediment yolk to the bottom and fat to the top. The tube was then scored with a glass file and broken just beneath the fat layer, so that the clear homogenate could be drawn into the injection needle. In general, five oocytes were injected each with 40 nl of extract, for a single assay point, and scored for germinal vesicle breakdown (GVBD) after 2–2.5 h. The injection and scoring procedures have been described (14, 29) and were used with minor modifications given in the figure legends of this article.

Eggs were fertilized in vitro with testis homogenate, dejellied in 2.5% cysteine, pH 7.9, and washed extensively in 20% MR medium, as described previously (5, 20). Eggs were chosen on the basis of the dark sperm entrance point to assure fertilization. Dejellied unfertilized eggs were activated by needle puncture or by electric shock (12 V, 1 s, across a 3-cm distance, eggs in 20% MR medium).

For the staging of oocytes in meiosis, the following procedure was followed. Oocytes were injected with 5 units of partially purfied MPF (20 ng protein in 40 μl EB containing 1 mM ATP) and incubated at 23°C. GVBD occurred 65–70 min later in the majority of the oocytes and the remaining tardy oocytes were eliminated. Then, at 10- or 15-min intervals, oocytes were removed and fixed in 10% formaldehyde (by volume from a 37% stock) in 0.06 M sodium HEPES, pH 7.4, for at least 2 h, after which Hoechst dye 33258 (bisbenzimide; Sigma Chemical Co.) was added to a concentration of 5 μg/ml. Staining was allowed to proceed for several hours and then a 200-μm square patch containing the "white spot" at the animal pole of the oocyte was dissected with watchmaker's forceps in a solution containing 50% glycerol, 0.1 M Tris-HCl, pH 7.5, 2% n-propyl gallate, and 10% ethanol. The vitelline membrane, follicle cells, and excess yolk were removed from the patch, which was then mounted in a drop of the same medium on a slide with coverslip. The plasma membrane side of the patch was oriented uppermost. The Hoechst-stained chromosomes were visualized by epi-illumination on a Zeiss photomicroscope III, and were photographed through a Leitz neofluar 63× lens, with Kodak Pan 2415 film, which was developed with HC 110 dilution D. The meiotic stage of the oocyte was inferred from the thickness and arrangement of the chromosomes, and by comparison with the stages of Huchon et al. (7).

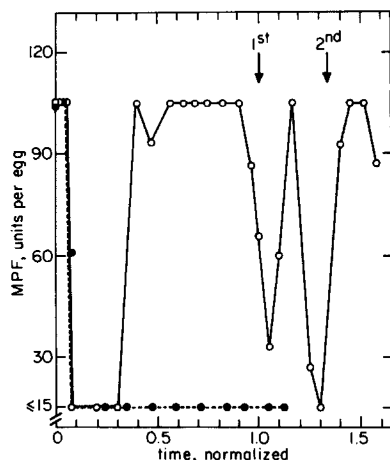


FIGURE 1 Cycling of MPF activity in artificially activated eggs of *X. laevis*, and arrest of that cycling by cycloheximide. Unfertilized eggs were dejellied in cysteine, washed in MR medium, and incubated at 19°C in MR medium with (●) or without (O) cycloheximide at 200 μg/ml, for 60 min. They were then activated by electric shock (12-V, 1-s, 3-cm separation of electrodes) and incubated in 20% MR-5% Ficoll. At each indicated time, a group of seven eggs was removed, washed, homogenized, and diluted two-, three-, or four-fold as described in Materials and Methods. The arrows marked *1st* and *2nd* indicate the times of first and second cleavage in a set of fertilized eggs from the same frog. The axis indicating time is defined with 0.0 at fertilization and 1.0 at first cleavage for a set of fertilized eggs (112 min at 19°C); for activated eggs, activation is set at 0.0 and sampling times are normalized to the 112-min interval. The units for MPF activity are defined in the Results section. The plateau of activity at 105 units per egg is a limit set by the greatest dilution used in the assay. The maximum activity never surpasses 135 U/egg, as shown in Fig. 3.

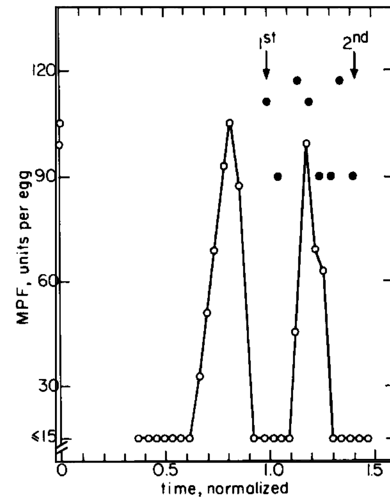


FIGURE 2 Cycling of MPF activity in fertilized eggs of *X. laevis*, and the arrest of that cycling by cytostatic factor. Eggs were fertilized in vitro, dejellied with cysteine, washed three times with 20% MR medium, and incubated at 19.5°C in 20% MR-5% Ficoll, to prevent swelling of the perivitelline space. At the indicated times, seven eggs were removed as a group, washed briefly in ice cold EB, and homogenized with 7 μl EB-SATP as described in Materials and Methods. Dilutions of two-, three-, and 4.5-fold were prepared at times when high MPF activity was expected. Cleavage occurred at 108 min. The time scale is normalized to the interval from fertilization (0.0) to first cleavage (1.0). Some eggs (●) were injected each (in the 0.5–0.7 period of the first cell cycle) with 50 μl of cytoplasm from unfertilized eggs blocked from activation by EGTA, as described in Materials and Methods, whereas others served as control (O). The arrows marked *1st* and *2nd* indicate the time of first and second cleavage in controls. The upper limit of assayable activity is 120 U/egg, set by the greatest dilution used in this experiment. The maximum activity of MPF in eggs never surpasses 135 U/egg, as shown in Fig. 3.

RESULTS

MPF Dynamics after Fertilization or Activation

Our small scale extraction method allowed us to quantify the appearance and disappearance of MPF in the first few cell cycles, with an accuracy not previously attained. We define a unit of MPF activity as that amount causing a 50% frequency of germinal vesicle breakdown in oocytes injected each with a volume of 40 nl of test material. (This definition differs slightly from the one given previously [29] in terms of the volume injected.) As shown in Figs. 1 and 2, MPF is present in unfertilized eggs at a level of at least 100 units per egg; that is, if a single egg is extracted in a volume of 4 μl, the extract will contain enough MPF to inject 100 oocytes with 40 nl each, and obtain 50% frequency of maturation. The volume of 4 μl is calculated from the end point in the dilution series prepared for the extract. The MPF activity in unfertilized eggs is actually 120–140 units per egg (see Fig. 3), but the upper limit in Figs. 1 and 2 is set at 105 units per egg by the greatest dilution used in these particular experiments.

When eggs are activated by needle puncture or electric shock, to release them from their arrest in second meiotic metaphase, MPF activity drops to undetectable levels within 8 min after egg activation. The lower limit of detection in our assay is ~15 units per egg, due to the inherent dilution of the

homogenate itself. Because the inactivation of MPF is so rapid, it was only possible to measure the time course after electrical activation, where large numbers of dejellied eggs can be activated synchronously and sampled immediately, as shown in Fig. 1.

MPF inactivation probably occurs rapidly in fertilized eggs as well, since MPF is undetectable at 20 min postfertilization, the earliest time of sampling after fertilization and dejellying. Then, MPF abruptly reappears at approximately two-thirds of the time through the first cell cycle. For an accurate comparison of cell cycle times in eggs at different temperatures, we have adopted a normalized scale, where fertilization or activation is set at 0.0 and first cleavage at 1.0. At 19.5°C, this interval would cover 108 min. On this normalized scale, second cleavage would occur at 1.4. The activation of MPF in the first cycle is seen at 0.65, as shown in Fig. 2, and MPF inactivation follows at 0.85. That is, within a 20-min period at 19.5°C, MPF returns to levels as high as in unfertilized eggs, and then disappears as rapidly as it did upon egg activation. The maximum point at 0.8 corresponds closely to the time of the "surface contraction wave" of the egg, approximately at the time of the mitotic metaphase-anaphase transition (10).

The second cell cycle shows similar dynamics of MPF (Figs. 2 and 3), as do later cycles, measured out to the seventh cleavage (data not shown). The MPF oscillations appear to damp out slightly as cleavage continues, perhaps because of the slight metachrony (asynchrony) of the cell cycles in different regions of the cleaving egg. However, MPF cycling is still apparent even at the seventh cleavage. These later cycles have the short period characteristic of the observed cleavages (~30-min intervals).

It is the first cell cycle that is unique from the other cycles in having a prominent G_2-like phase, that is, a long delay between the completion of DNA synthesis and the start of mitosis (18). This is reflected in the long period between MPF disappearance at fertilization and reappearance before first cleavage. As shown in Fig. 1, the pattern of MPF activity is somewhat different in activated eggs in the first cell cycle, compared to fertilized eggs. MPF appears much earlier in the first cycle, by 0.30 rather than 0.65, that is, with no delay between DNA synthesis and MPF appearance. Furthermore, in activated eggs, MPF decreases somewhat later. However, MPF cycling in the second and third cycles in these eggs is clearly demonstrable with a periodicity similar to that in fertilized eggs.

In an effort to evaluate the importance of protein synthesis for these cycling events, we treated unfertilized eggs with cycloheximide and found that MPF levels remain high, even for a 2-h period (data not shown), provided that activation does not occur. This indicates that MPF does not turn over by a process of protein synthesis and breakdown in unfertilized eggs arrested in metaphase. When cycloheximide-inhibited eggs are electrically activated, they destroy MPF activity with the same kinetics as in control eggs (Fig. 1), indicating that the MPF-inactivating system also does not require protein synthesis for its operation, but must be present, stable, and poised in an inactive form in the unfertilized, metaphase-arrested egg. It was found, however, that cycloheximide treated activated eggs could not restore MPF activity in the 0.30–0.90 period (Fig. 1), as if protein synthesis was needed to prepare new MPF precursors or to advance the cell cycle to the point of endogenous MPF activation. Miake-Lye et al.

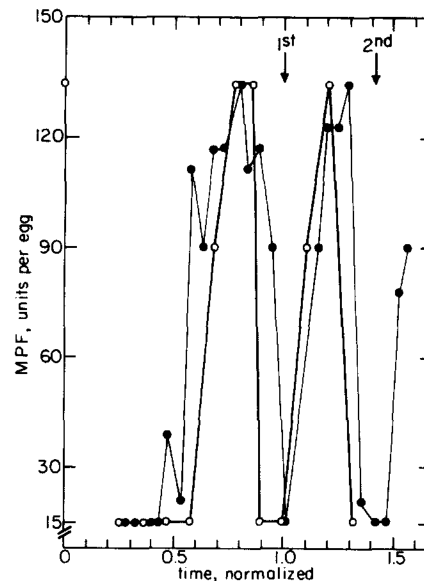


FIGURE 3 Cycling of MPF in nocodazole-treated fertilized eggs of *X. laevis*. Unfertilized eggs were incubated in MR medium with (●) or without (○) nocodazole, 10 μg/ml, for 60 min, and then fertilized in vitro, dejellied in cysteine, washed in 20% MR medium, and incubated at 19°C in 20% MR-5% Ficoll, with or without nocodazole. At each time indicted, a group of seven eggs was removed, homogenized, diluted two-, 3.5-, or fivefold, and assayed for MPF activity as described in Materials and Methods. The axes are defined in the legends of Fig. 1 and 2, and in the text. The upper limit of assayable MPF activity is ~150 U/egg, that is, in excess of the highest measured activity of 135 U/egg.

(18) have reported that the cell cycle timing of the surface contraction wave is also effectively blocked in cycloheximide treated eggs, which arrest in a G_2-like phase after the completion of DNA synthesis.

Effect of Cell Cycle Inhibitors on MPF Cycling

Fertilized eggs were incubated in solutions of the microtubule-depolymerizing drug, nocodazole, at concentrations 30-fold in excess of that needed to block cleavage. Nonetheless, as shown in Fig. 3, MPF continues to cycle with the same periodicity as in control eggs. However, in nocodazole-blocked eggs, MPF appears at 0.50, ~0.15 time units earlier than in control eggs, and disappears ~0.10 units later. In this respect of timing, the nocodazole-treated egg partially resembles the artificially activated egg. The related drug, colchicine, was injected (10 mM stock; 40 nl injected per egg) at levels 50 times above that needed to block cleavage, and MPF was found to cycle nonetheless (data not shown). As a general conclusion, it can be said that contrary to a previous report by Wasserman and Smith (27), MPF cycling was not arrested nor was MPF held indefinitely at a high level in the presence of antimicrotubule drugs. We cannot explain the discrepancy of the previous and present results, except to note that the previous studies were done with *Rana pipiens* eggs and that the assay method relied on direct cytoplasmic transfer rather than extraction. However, it remains to be seen what underlies the discrepancy. This demonstrated inability of nocodazole and colchicine to block the cycling of MPF in the egg, while interfering with spindle formation and furrow formation, is consistent with the findings of Hara et al. (6) that the periodic surface contraction wave continues to appear on time, unhindered by vinblastine or colchicine. It seems clear that anti-

microtubule drugs have a much more limited effect on the cell cycle in the egg compared to their effect in somatic cells (19), but we do not understand the bases for this difference.

In contrast to the ineffectiveness of these antimitotic drugs, the metaphase arrest factor (cytostatic factor) obtained from the cytoplasm of unfertilized eggs, is completely effective in blocking the cycling of MPF when injected into fertilized eggs in the middle (0.5 to 0.7) of the first cell cycle. Such injected eggs produce MPF on the normal schedule, and then fail to cleave. As shown in Fig. 2, they maintain for at least an hour a high level of active MPF characteristic of metaphase cells. It is known that such eggs contain condensed metaphase chromosomes on a spindle (16) and fail to initiate a surface contraction wave (Newport, J., K. Butner, and M. Kirschner, personal communication). Control experiments have shown that fertilized eggs injected with 50 nl of 2 mM EGTA (a level comparable to that present in our cytostatic factor material) do not block cleavage or keep MPF activity at elevated noncycling levels. By all these criteria, cytostatic factor appears to arrest eggs in a true metaphase state, including the presence of MPF at high mitotic levels.

MPF-inactivating Factors in the Cell Cycle

As presented already in Fig. 1, MPF activity disappears within a few minutes after egg activation, due presumably to an MPF-inactivating agent that is stable and latent in the unfertilized egg and activated rapidly at fertilization or egg activation. We have assayed this agent in vivo by injecting large amounts of purified MPF into activated eggs at various times in the first cell cycle, and then extracting the injected eggs 10 min later to quantify the remaining MPF. For example, when fertilized eggs are each injected with 80–100 units of MPF, the MPF is totally inactivated (within the sensitivity of the assay, ≥85% inactivation) within 10 min in eggs in the time period of 0.3–0.6 of the first cell cycle. That is to say, the inactivating agent persists at high levels throughout the interval from fertilization to 0.6, when endogenous MPF becomes detectable before first mitotic prophase. When the injected MPF is assayed after just 5 min in the fertilized egg, it is also found that MPF is totally inactivated except at the time 0.55, that is, a few minutes before endogenous MPF appears. At this time, the injected MPF is only half-inactivated, an indication of a decline in MPF-inactivating activity. Of course, in the 0.6–0.85 period when endogenous MPF activity is high, the activity of the inactivating agent is presumably low; however, we cannot quantify the exact level by our injection assay because of the high background activity of endogenous MPF. In conclusion, the 0.0–0.6 period of the first cell cycle is characterized by a high level of an MPF-inactivating agent that is itself activated at fertilization and inactivated at the start of the first mitotic phase.

MPF-cycling in Maturing Oocytes

Full grown oocytes were injected each with 5 units of partially purified MPF (20 ng protein) to induce meiotic maturation. This amount exceeds approximately five times that needed for maturation at a 50% frequency, and causes rapid synchronous maturation in 100% of the recipients. At intervals within the subsequent 4 h, samples of oocytes were taken for homogenization and assay of the level of MPF produced during maturation. The background level of injected MPF is undetectable in our assay, but endogenously produced MPF becomes readily detectable within 30 min after injection. The level of MPF continues to increase to at least 100 units per oocyte, that is, approximately 100 times the minimal necessary injected dose, as shown in Fig. 4. After GVBD at 70–80 min, the MPF level remains high until 120 min. After this time, the level drops precipitously and becomes undetectable at 160 to 170 min at 19°C. Thereafter, it increases again and finally reaches a plateau of 100 units per oocyte by 240 min. This level is maintained for many hours and is that found in unfertilized eggs.

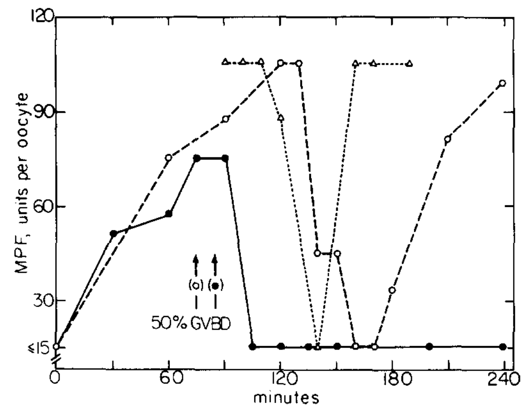


FIGURE 4 MPF activity during oocyte maturation. Oocytes were incubated for 60 min in MR, with (●) or without (○) 20 μg/ml cycloheximide. Then they were injected each with 5 units of partially purified MPF (20 ng protein) in 40 nl EB containing 1 mM ATP (time 0) and incubated at 19°C. The cycloheximide-treated oocytes, after MPF injection, underwent germinal vesicle breakdown (*GVBD*) in half the population at 85 min postinjection, whereas the nontreated oocytes reached 50% GVBD at 75-min postinjection. These times are indicated by the arrows and respective symbols. At the times indicated, five oocytes were removed as a group, washed briefly in ice cold EB, and homogenized with 5 μl EB-SATP as described in Materials and Methods. Dilutions of two-, three-, or fourfold were prepared at times where high activity was expected. Under the conditions of assay, 105 units of MPF per oocyte is the upper limit of assayable activity. The maximum MPF activity detected in oocytes, when greater dilutions were done, is 135 U/oocyte. As a separate experiment, oocytes were exposed to progesterone, 1 μg/ml in MR, and at the time of GVBD oocytes were selected in groups reaching GVBD within 5 min of one another, to achieve synchronization. Then, at regular intervals, groups of these oocytes were removed, homogenized, and diluted as described previously, and assayed for MPF activity (△). These time points are placed on the time scale, with GVBD arbitrarily set at 80 min, for comparison with the time course of MPF-induced maturation.

This pattern of rapid MPF cycling during meiotic maturation has not been observed previously. In the early studies of progesterone-matured amphibian oocytes (16, 26), it would probably have been difficult to observe cycling because the population response of the oocytes is usually heterogeneous in time and because the previous assays were too qualitative. To see if MPF goes through cycles in progesterone stimulated oocytes, we picked out a subpopulation of oocytes which had reached GVBD within a 5-min interval, ~3 h after progesterone exposure (1 μg/ml of MR). These oocytes were then extracted at closely spaced time intervals over the next 2 h. They indeed showed a disappearance of MPF at 50 min post-GVBD at 19°C, and a subsequent reappearance to ~100 units per oocyte shortly thereafter, as shown in Fig. 4. Thus, cycling of MPF occurs in both progesterone-induced and MPF-in-

duced meiotic maturation. At this time, we do not know why the progesterone-induced oocytes seem to complete the MPF cycling slightly more rapidly than do MPF-induced oocytes.

We focused on the possibility that the cycling of MPF activity in maturing oocytes reflects the first and second meiotic cycles, and therefore we attempted to correlate cytological staging of oocytes with our MPF measurements. There is only limited cytological information on the timing of meiotic stages in *X. laevis* (1, 7), and consequently we needed to develop our own normal series for MPF-injected oocytes. To this end, we fixed oocytes in formaldehyde at various times after MPF injection, and stained the meiotic chromosomes with Hoechst dye 33258 (bisbenzimide). The animal pole was dissected from the oocyte and mounted under a coverslip for observation of the fluorescent chromosomes by epi-illumination, as described in Materials and Methods. At 23°C, the injected oocytes reach GVBD by 65–70 min. Thereafter, as indicated by our cytological analysis, the first meiotic cycle is probably complete by 110–125 min, with second meiotic metaphase reached at 140 min after MPF injection at this temperature, as shown in Fig. 5. From the same series of injected oocytes, samples were taken for MPF assay, and it was found that MPF disappeared abruptly at 110–125 min after MPF injection, and rose to full activity again by 140 min. Thus, our findings suggest that the cycling of MPF does indeed reflect the progress of the two meiotic cell cycles, with MPF low at time of meiotic interphase. It is noteworthy for comparison that the disappearance and reappearance of MPF at the meiotic interphase, although rapid, is actually less rapid than occurs in the mitotic cycles after fertilization (see the second cycle of Fig. 2).

Effects of Protein Synthesis Inhibition on MPF Cycling in Maturation

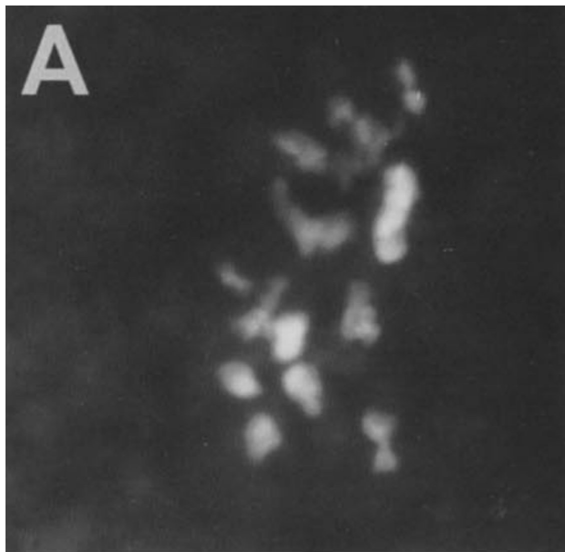
When the above experiments were repeated with cycloheximide treated oocytes, it was found that MPF-injection (5 units per oocyte) could nonetheless induce maturation at least to the stage of GVBD, as has been observed previously by others (4, 25). We find that MPF levels increase at approximately the same rate as in control oocytes (no cycloheximide), and reach an activity of 75 units per egg at 90 min at 19°C, as shown in Fig. 4. Then, in the interval between 90 and 100 min, MPF abruptly and precociously disappears and does not reappear. This same pattern of a single MPF cycle was found over a wide range of cycloheximide concentrations (5 to 50 μg/ml) and of times of exposure (0 to 120 min) of the oocytes to the inhibitor before MPF injection. Also, puromycin (50 μg/ml) and diphtheria toxin (injected at 10 pg/oocyte) had the same effects (data not shown). In a study of the time course of sensitivity to cycloheximide, we find that the reappearance of MPF is blocked completely even when the addition of the inhibitor is delayed until the end of the first meiotic period. Once MPF has begun to reappear at 180 min at 19°C, cycloheximide is no longer able to block its continued appearance. The transition to cycloheximide-insensitivity occurs abruptly, within 10 min. Thus, we conclude that the oocyte, even without protein synthesis, can activate MPF almost to the normal level during the first meiotic cycle, but is unable to achieve the activation characteristic of the second meiotic cycle.

In an attempt to induce the second cycle of MPF appearance in cycloheximide-treated maturing oocytes, we have injected MPF a second time (5 units per oocyte) at 90 min post-GVBD at 19°C. However, no MPF was detectable in extracts of such oocytes at 210 min post-GVBD; that is, at a time when noninhibited control oocytes would have shown high levels of MPF in the second meiotic cycle. Thus, MPF injections at the time of the second meiotic cycle do not cause MPF amplification in cycloheximide blocked oocytes, whereas such injections do so successfully before the first meiotic cycle. By cytological analysis, we have not been able to find first or second meiotic metaphase chromosomes in cycloheximide treated oocytes. Thus, the meiotic maturation cell cycle seems to stop shortly after GVBD in the presence of protein synthesis inhibitors. Ziegler and Masui (30) have reported that protein synthesis is needed for the condensation of meiotic chromosomes in amphibian oocytes.

DISCUSSION

We have shown that MPF activity cycles in exact correspondence with the M-phase and interphase periods of meiosis in oocytes and of mitosis in the early cleavages of the egg. When cleavage and spindle formation are interrupted with nocodazole and colchicine, MPF continues to cycle, as do the surface contraction waves that normally signify the metaphase-anaphase transition of mitosis (10). Furthermore, when the cell cycle is stopped at an interphase stage with cycloheximide, MPF levels remain low, whereas when it is stopped at metaphase with cytostatic factor, MPF levels remain high. Thus, MPF appears to be closely linked to the oscillatory reactions driving the cell cycle and not to the "dispensable" events of cleavage, spindle formation, DNA synthesis, and chromosome condensation. This correlation supports the long-standing suggestion (15, 23) that MPF behaves as a trigger of M-phase events rather than as an effect of them. From our studies, we can add three provisions to this suggestion. First,

FIGURE 5 The time course of meiosis in MPF-injected *X. laevis* oocytes, as revealed by chromosome morphology. Oocytes were injected with MPF, then fixed at various times and stained with bisbenzimide (Hoechst 33258 dye). A small patch of the cell cortex was dissected from the animal pole as described in Materials and Methods, and mounted under a coverslip for visualization of the fluorescence by epi-illumination. The fluorescent chromosomes were photographed through a 63× Leitz neofluar lens. (*A*) 80 min after MPF injection; chromosomes are probably in first meiotic prometaphase, inferred from their thickened morphology and irregular arrangement. (*B*) 90 min; probable first metiotic metaphase, inferred from the approximately circular arrangement with the spindle viewed from one end. (*C*) 100 min; probable first meiotic anaphase. (*D*) 110 min; probable first meiotic telophase or interphase. (*E*) 125 min; possible second meiotic prophase. The first polar body could not be found in these preparations; it may have been removed with the vitelline membrane during dissection. (*F*) 140 min; definitive second meiotic metaphase as inferred from thin chromosomes and tight circular arrangement. The second metaphase is reported to be smaller than the first metaphase spindle (7). The meiotic figure of the unfertilized egg is identical in appearance to that of *F*. × 1,000. Bar, 10 μm.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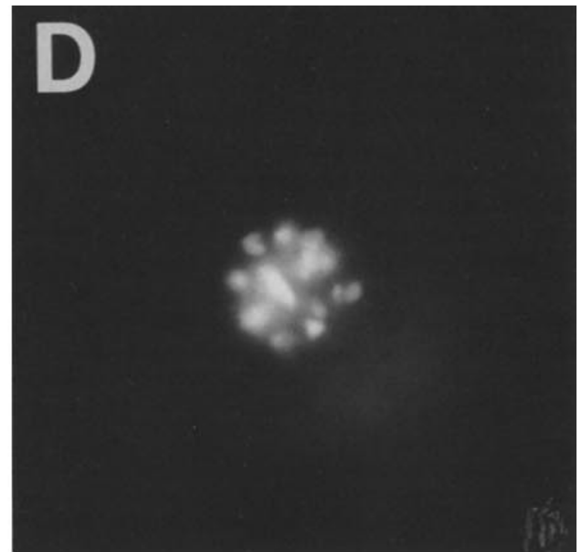
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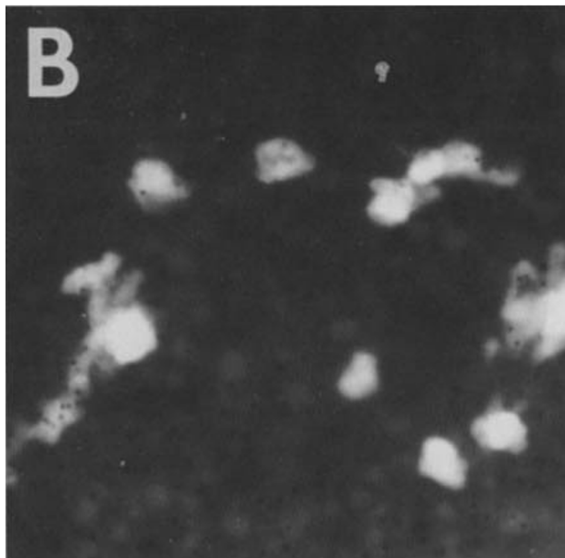
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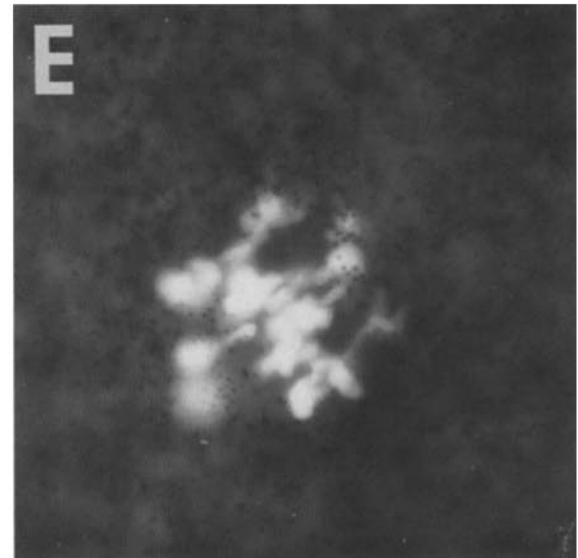
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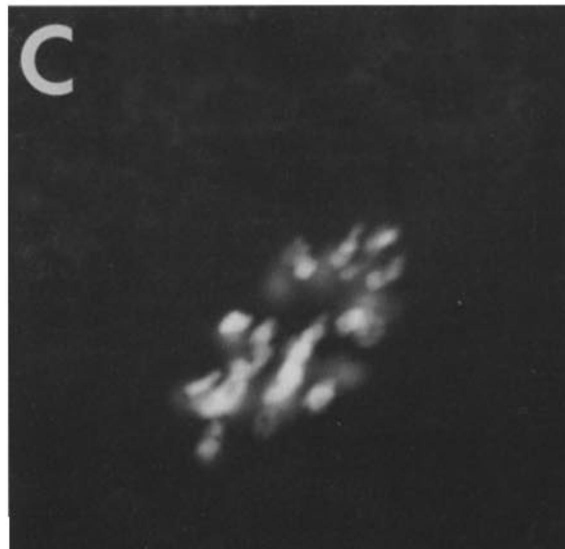
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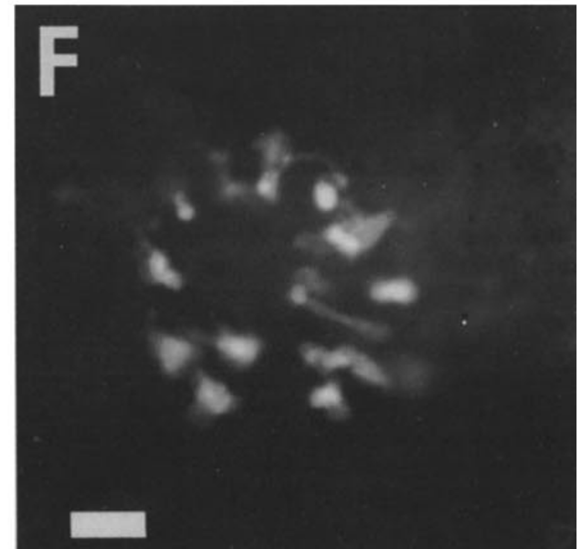
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although MPF may be a trigger of the M-phase, its own activity may be regulated by a still more fundamental and autonomous cell cycle oscillator. That is to say, MPF may function at an intermediate level of command between the oscillator and the cytologically apparent reactions of mitosis. Second, MPF is regulated at least in part by a dispensable event of the first mitotic cell cycle since artificially activated eggs show an increase of MPF level well in advance (by 0.3 time units) of the increase seen in fertilized eggs, even though the surface contraction waves occur at the normal time in both kinds of eggs (6). In reciprocal relation to MPF, the MPF inactivating system must lose activity precociously in activated eggs. These observations suggest that MPF cycling becomes partially decoupled from the oscillator in the first cell cycle. This is the only indication at present that MPF and the inactivating system are not components of the cell cycle oscillator itself, but are mediators of its effects. It would be interesting to distinguish whether precocious appearance of MPF results from the absence of the sperm centriole (and microtubule-organizing center) or from the absence of the sperm nucleus in artificially activated eggs. This could now be approached by the injection of partially purified centrioles and organizing centers. And as a third provision, it seems that MPF, even as a mediator of the oscillator's effects, must exert some feedback control on the oscillator, since an injection of a small amount of MPF into an oocyte causes the cell cycle to advance from a G_2-prophase state to second meiotic metaphase of the unfertilized egg. It seems likely that we have hardly begun to fathom the outlines of the cytoplasmic regulatory circuits controling these cell cycle events.

We now turn to the question of the mechanism of MPF activation. A final step of this process may be MPF autoactivation, following the activation of a small threshold amount of MPF via the cell cycle oscillator. Information about autoactivation comes mostly from our studies of cycloheximide-treated oocytes. First, it seems clear that G_2 arrested oocytes contain abundant MPF in a latent form that can be activated by posttranslational mechanisms when a small amount of active MPF is injected into the oocytes. This posttranslational mechanism probably drives the autoactivation phase of MPF activation. A similar conclusion was reached by Doree (2) for starfish oocytes. We suggest that Drury and Schorderet-Slatkine (4) were led to conclude that protein synthesis is needed for autoactivation of MPF in amphibian oocytes because they were unaware of MPF cycling during meiosis and chose to test oocytes 2 h after MPF injection, a time when cycloheximide treated oocytes have already destroyed their amplified MPF of the first meiotic cycle. While we agree with Wasserman and Masui (25) that protein synthesis is unnecessary for MPF autoamplification, we cannot reproduce their finding that cycloheximide treated oocytes contain transferable levels of MPF 7 h after they were injected with MPF to initiate maturation. In our experience, MPF is detectable only within the first 90 min of maturation in cycloheximide-blocked oocytes. At later times, the oocytes undergo abnormal surface changes of the sort described by Schuetz and Sampson (22), and by 7 h they reach a state of discoloration and cytolysis. There are differences in the experimental procedures used by us and by Wasserman and Masui (25), and these may explain the different results. For example, they obtained oocytes from frogs receiving less hormonal stimulation, and matured them at a slightly lower temperature (18°C), both procedures causing slower maturation than in our experiments. In fact, they noted that in some cases maturation had not reached GVBD by 7 h in cycloheximide-treated oocytes, as if the inhibitor had retarded the rate of maturation still further. Also, they note that their results were variable; only two of four frogs gave oocytes that contained MPF at the time of 7 h after maturation in the presence of cycloheximide. It seems possible that the negative oocytes may have matured more quickly and destroyed all their MPF by the time of sampling, or may have failed to mature by the time of sampling. In any event, we could not obtain active MPF from our treated oocytes at such long times after maturation.

As a second finding about MPF activity changes, we can say that oocytes contain abundant reserves of MPF-*inactivating* agents in latent form, which, like MPF, are activated by posttranslational processes when MPF is injected in small amounts. These inactivating agents operate shortly after GVBD, that is, approximately on schedule at the end of the first meiotic cycle even in cycloheximide treated oocytes. A similar conclusion applies to unfertilized eggs: they too must contain latent MPF-inactivating agents, which are activated by posttranslational means at fertilization.

A third finding from these studies is that cycloheximide-blocked oocytes fail to activate MPF at the time of the second meiotic cycle. In a parallel situation, unfertilized eggs treated with cycloheximide fail to activate MPF for the first mitotic cycle after egg activation. There are various possibilities for this failure; foremost among these, the cell cycle may be stopped by cycloheximide and may never advance to the point where MPF would be activated. In fact, we anticipate this to be the case since cycloheximide is known to prevent the cyclic appearance of the surface contraction waves normally occurring at metaphase of activated enucleated eggs (18). If the cell cycle does not advance, the oocyte or egg might then lack the primary stimulus to activate a small amount of latent MPF, and so MPF would never reach its autoactivating phase. Also, if the cell cycle is arrested, perhaps the MPF-inactivating system is itself never destroyed, and would continue to oppose MPF-activation. We favor this possibility since we find in preliminary experiments that high levels of MPF are rapidly destroyed when injected into cycloheximide-treated oocytes 90 min after GVBD, that is, at a time when these treated oocytes continue to lack endogenous MPF activity, whereas control oocytes would have acquired MPF activity in the second meiotic cycle. As a final possibility, cycloheximide-treated maturing oocytes may not be able to activate MPF in the second meiotic cycle, for lack of MPF precursor. This would be the case if MPF is regularly proteolyzed in the course of each cyclic inactivation, and then activated from newly synthesized precursor material. Unfortunately, the continued presence of the strong MPF inactivating system in cycloheximide-treated oocytes after GVBD precludes our testing of this possibility.

In conclusion, we have attempted to view the cell cycle of the oocyte and cleaving egg as basically the same, driven by an endogenous oscillator that depends on protein synthesis for its regular cycling, but does not depend on the "dispensable" elements of the cycle, such as DNA synthesis, chromosome condensation, spindle formation, and cleavage. We look on the nonmatured oocyte as a specialized case where the oscillator is blocked in the late G_2 phase by an arrest system, which can be neutralized by a progesterone-dependent release mechanism, as has been proposed by Maller and Krebs (13). The arrest system would be specific to those few cell types,

such as the oocyte, that arrest in the G_2 phase, whereas the progesterone-dependent release system might be specific to the oocytes of amphibia. In contrast, the oscillator is likely to be general to all meiotic and mitotic cell cycles. After release, the oscillator would progress to the point of stimulating MPF activation. Then MPF autoactivation would take over, and the cytological events of maturation would follow as a result of the MPF stimulus. However, if protein synthesis is blocked, the oscillator cannot advance, and therefore MPF activation cannot occur. Despite this, if active MPF is injected, the dependence on the oscillator can be bypassed, and MPF is activated directly. According to this model, protein synthesis would not be needed for the progesterone-dependent release system, as has been proposed (12, 13), but for the advance of the cell cycle to the point of MPF activation. Then, when the maturing oocyte reaches second meiotic metaphase, as an unfertilized egg, another specialized arrest system appears; namely, the cytostatic factor that blocks the cell cycle at metaphase, a stage where MPF activity is high and the MPF-inactivating system is suppressed. Fertilization or activation removes this arrest, and MPF is inactivated. Thereafter the oscillator is free to cycle 12 times on a regular 30 min schedule of M and S phases until the midblastula transition occurs (20). At this time the cell cycle becomes slower and more complex, and MPF activity presumably comes under new cellular controls.

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