Mitosis-inducing Factors Are Present in a Latent Form during Interphase in the *Xenopus* Embryo

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Abstract. During the conversion to the mitotic state, higher eukaryotic cells activate a cascade of reactions which result in the disintegration of the nuclear envelope, the condensation of the DNA into chromosomes, and the reorganization of the cytoskeleton. In *Xenopus*, the induction of the mitotic state appears to be under the control of a cytoplasmic factor(s) known as mitosis-promoting factor or MPF. We have developed a rapid and highly sensitive version of an in vitro assay for MPF. The assay uses reconstituted nuclei in interphase cytoplasm from activated *Xenopus* eggs. The MPF-induced conversion from interphase to mitosis is conveniently monitored by the visual observation of the loss of the nuclear envelope from the substrate nuclei. At near saturating concentrations of MPF, nuclear breakdown requires 20–30 min. Preincubation experiments have revealed that the action of MPF requires only a few minutes and that the disassembly process itself takes up the remainder of the incubation period. Using this cell-free system, we have investigated the observation that protein synthesis is required for the progression through each successive mitotic cycle in the developing *Xenopus* embryo. A simple explanation for this finding would be that MPF is degraded after each mitosis and then resynthesized before the next mitotic cycle. However, using in vitro reactivation experiments, we have found that MPF is present in a latent, inactive form during interphase. These results suggest that the block in the cell cycle induced by inhibitors of protein synthesis is due to the lack of production of an activator of MPF.

THE higher eukaryotic cell undergoes a precisely controlled reorganization of many of its most complex structures during mitosis. The replicated genome is packaged into chromosomes for orderly transmission to each daughter cell. The nuclear membrane and its supporting lamina are dismantled (Gerace and Blobel, 1980). Organelles of the secretory machinery such as the endoplasmic reticulum and the Golgi complex are partially or completely fragmented (Warren, 1985; Lucocq et al., 1987). Finally, the cytoskeleton is refashioned to form the mitotic spindle. Despite the extent and magnitude of the structural changes that accompany mitosis, the underlying regulatory basis for the induction of the mitotic state is unknown in any molecular detail.

Collectively, cell fusion experiments (Rao and Johnson, 1970; Matsui et al., 1972) and studies of the simplified cell cycles in maturing oocytes and early embryos (for recent reviews see Ford, 1985; Kirschner et al., 1985) of a number of species have established the general principle that a dominant cytoplasmic factor governs the entry into mitosis. The activity of this agent oscillates with the same period as the cell cycle (Wasserman and Smith, 1978; Gerhart et al., 1984).

Much of this picture has emerged from the analysis of the meiotic maturation of oocytes from *Xenopus laevis* to the egg stage and the subsequent early embryonic divisions of the fertilized egg. A decided advantage of the *Xenopus* system is that during early development there is no appreciable growth. Since the progression of the cell cycle has been disengaged from growth control, the early embryonic cell divisions contain no noticeable G_1 or G_2 phases (Graham and Morgan, 1966). Thus, the cell cycle regulatory machinery has been streamlined to coordinate mainly the successive rapid rounds of DNA synthesis and the mitotic distribution of the replicated genome to each daughter cell.

In *Xenopus*, the induction of M phase is under the control of mitosis (meiosis)-promoting factor or MPF.[1] Upon exposure to progesterone, the *Xenopus* oocyte undergoes the first meiotic division and arrests in the second meiotic metaphase as an unfertilized egg. Maturation to the egg stage can also be induced artificially by the transfer of cytoplasm from the unfertilized egg to the oocyte (Masui and Markert, 1971; Smith and Ecker, 1971). MPF was originally distinguished as the cytoplasmic meiosis-inducing agent. Recent work (Miake-Lye et al., 1983; Newport and Kirschner, 1984; Gerhart et al., 1984; Miake-Lye and Kirschner, 1985; Halleck et al., 1984) has established a more general role for this *Xenopus* factor in triggering the entry into the mitotic state in cleaving *Xenopus* embryonic cells and in dividing tissue

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1. *Abbreviations used in this paper*: DHCC, 3,3′-dihexyloxacarbocyanine; EB, mitosis-promoting factor extraction buffer; MMR, modified Ringer's; MPF, mitosis-promoting factor.

culture cells. Consistently, MPF activity has been detected in mitotically arrested somatic cells from diverse species, ranging from baker's yeast to human (Sunkara et al., 1979; Weintraub et al., 1982; Kishimoto et al., 1982).

Little is known about the molecular nature of MPF or its regulation during the cell cycle. Since this factor(s) has proven refractory to purification (Wu and Gerhart, 1980; Gerhart et al., 1985; Nguyen-Gia et al., 1986), most of our knowledge is indirect and circumstantial. The active factor is most likely a phosphoprotein, since it is essential to extract MPF under conditions that are conducive to the preservation of protein–phosphate linkages (Wasserman and Masui, 1975; Drury, 1978; Wu and Gerhart, 1980). It is also known that many of the structural proteins (e.g., nuclear lamins, histones) which most likely participate directly in mitotic rearrangements are hyperphosphorylated upon the entry into mitosis (Gerace and Blobel, 1980; Paulson and Taylor, 1982). Taken together, these observations have fostered the appealing but unproven notion that MPF is a central actor in a master regulatory circuit of protein kinases. Confirmation or refutation of this model awaits the elucidation of the targets of MPF and its mode of control.

We describe here a cell-free system for mitotic conversion by MPF. The sensitivity of the system and the kinetics of the response to MPF approximate in vivo parameters. Moreover, we have discovered that MPF, although active only during mitosis, is present constantly throughout the cell cycle. This finding is surprising since protein synthesis is required for the entry into each mitosis in the cleaving embryo (Miake-Lye et al., 1983). Because the embryo contains most (if not all) of the structural components for ∿4,000 cells in a stored form, regulatory factors such as MPF were obvious candidates for the newly made proteins necessary for mitotic advancement. Instead, the potential for both the induction of the mitotic state and the execution of mitotic rearrangements is in place throughout the cell cycle in the early *Xenopus* embryo. This fact has allowed us to deduce the existence of a second mitotic regulatory factor distinct from MPF. This factor would be synthesized recurrently for each cell cycle for the cyclical stimulation of preexisting MPF.

Materials and Methods

Extraction of MPF

Unfertilized *Xenopus* eggs were dejellied (Newport, 1987) and rinsed three times in 10 vol of M buffer (240 mM β-glycerophosphate, 60 mM EGTA, 45 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.3). The eggs were packed by gentle centrifugation (Newport and Spann, 1987) and, after the removal of excess buffer, ATPγS, leupeptin, and phenylmethylsulfonyl fluoride were added so that the final cytoplasmic concentrations upon lysis would be 0.5 mM, 10 μg/ml, and 0.5 mM, respectively (assuming a threefold dilution of the interstitial buffer upon lysis). The eggs were lysed as described (Newport and Spann, 1987). The cytoplasmic layer was removed and diluted with an equal volume of MPF extraction buffer (EB; Wu and Gerhart, 1980) supplemented with 0.5 mM ATPγS, 10 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride. EB contained 80 mM β-glycerophosphate (pH 7.3), 20 mM EGTA, 15 mM $MgCl_2$, and 1 mM dithiothreitol. The diluted cytoplasm was then centrifuged for 1 h at 55,000 rpm in the TLS 55 rotor (Beckman Instruments, Inc., Palo Alto, CA). Ammonium sulfate fractionation of this mitotic cytoplasm was performed essentially as described by Wu and Gerhart (1980). The supernatant was mixed with 0.43 vol of 3.6 M ammonium sulfate dissolved in EB. The insoluble proteins were pelleted for 10 min at 10,000 g and then redissolved in a volume of EB (supplemented with 0.1 mM ATPγS) equivalent to one-fifth of the original volume of the high speed supernatant. This fraction was dialyzed for 6–8 h against 30 vol

of EB containing 0.1 mM ATPγS. This crude MPF preparation was stable for at least 6 mo at −70°C and was resistant to several freeze-thaw cycles.

Cell-free Assay for MPF

For the in vitro assay of MPF, we prepared synthetic nuclei in interphase extracts from *Xenopus* eggs. Conversion of these extracts to the mitotic state by MPF is ascertained by the release of the nuclear membrane from the nuclei. Dejellied eggs were activated in modified Ringer's (MMR; Newport and Kirschner, 1982) containing 5% Ficoll and 2 μg/ml A23817. After activation, the eggs were transferred to fourfold-diluted MMR (1/4× MMR) containing 5% Ficoll and incubated for 30–45 min. The eggs were rinsed four times in ice-cold S buffer (0.25 M sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol). For the last rinse, cycloheximide (50 μg/ml) and cytochalasin B (5 μg/ml) were included in the buffer. The eggs were packed to remove excess buffer and then lysed by centrifugation for 5 min at 10,000 g. The cytoplasmic layer (see Newport, 1987) was removed and centrifuged again at 10,000 g for 5 min. This cytoplasm (henceforth referred to as "interphase cytoplasm") was either used immediately or rapidly frozen in liquid nitrogen and stored at −70°C. Routinely, 1 ml of interphase cytoplasm was supplemented with ATP, phosphocreatine, and creatine phosphokinase to final concentrations of 1 mM, 10 mM, and 50 μg/ml, respectively. Demembranated *Xenopus* sperm nuclei (Lohka and Masui, 1983) were then added to a final concentration of 1,000 per microliter in 0.1 ml buffer A (15 mM Pipes, pH 7.2, 0.2 M sucrose, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 7 mM $MgCl_2$). Typically, the sperm chromatin decondensed and acquired an easily discernible nuclear envelope within 1 h at 22°C. At this stage, the incubation mixture was divided into 50-μl aliquots, rapidly frozen in liquid nitrogen, and stored at −70°C. These frozen extracts were stable for at least 4 mo. For the assay of MPF action, the extracts containing reconstructed nuclei were rapidly thawed, and aliquots (5 μl) were mixed with 5 μl of EB containing the protein fraction to be assayed. After incubation at 22°C for 1 h, 3.5 μl of the mixture was removed and mixed (on a glass microscope slide) with 3.5 μl of buffer A containing 3.7% formaldehyde and 20 μg/ml Hoechst 33258. The nuclei were then examined by fluorescence or phase-contrast microscopy. Conversion to the mitotic state was routinely assessed by the loss of a phase-dense envelope around the stained DNA. Under our assay conditions, nuclear breakdown was essentially irreversible. This is due to the fact that nuclear assembly is very dilution sensitive, and occurs poorly after a twofold dilution with EB. Control experiments demonstrated that dilution with EB alone or addition of ATPγS (0.25 mM final concentration) did not elicit nuclear breakdown. Also, ATP was required to effect nuclear disassembly. Treatment of the interphase extracts with hexokinase (100 U/ml; Sigma Chemical Co., St. Louis, MO) and glucose (20 mM) abolished the ability of MPF to elicit nuclear breakdown.

For this in vitro assay, a unit of MPF activity was defined on the basis of the maximum dilution at which nuclear disassembly occurred. In the titration in Fig. 2, for example, the maximum effective dilution was 40-fold. Thus, the defined activity of this MPF preparation was 40 U per microliter or 8.9 U per microgram of protein. By necessity, this definition of a unit of MPF activity differs from that of Wu and Gerhart (1980), who used the oocyte maturation assay. According to this latter definition, an unfertilized egg contains ∿100 U of MPF in a volume of 0.6 μl (Gerhart et al., 1984). Using these values, we calculate that 40-fold diluted MPF corresponds to ∿4 U per egg equivalent of interphase cytoplasm.

The sensitivity of the extracts to MPF was in part dependent on the length of the incubation between activation of the eggs and lysis. The optimal time was 30–45 min postactivation (about halfway through the interval required for the first cleavage in fertilized eggs). Variable results were obtained with extracts that were prepared within 20 min after activation. It is known that activation mobilizes an antagonist which inactivates MPF within 8 min (Gerhart et al., 1984). It is not known how long thereafter this antagonist persists or whether protein synthesis is required for its neutralization. We have also found that extracts prepared from eggs soaked in cycloheximide (200 μg/ml) for 30 min before activation and 45 min thereafter show reduced sensitivity to MPF. Such extracts do not convert to mitosis in the presence of MPF dilutions greater than fivefold (average of three experiments). This effect could be due to failure to shut down the MPF antagonist. Alternatively, newly synthesized proteins could advance the cytoplasm to a G_2-like state and thereby reduce the threshold for MPF. Experiments to distinguish between these possibilities are in progress.

Preparation of Inert MPF from Interphase Cytoplasm

Dejellied, unfertilized eggs were soaked for 1 h in MMR containing 5% Ficoll and 200 μg/ml cycloheximide. The eggs were then activated by the

addition of A23187 (2 μg/ml) for 5 min. Batches of eggs which did not activate quantitatively (as judged by the contraction of the egg pigment) were discarded. The ionophore was removed by washing with 1/4× MMR containing cycloheximide. The eggs were incubated for an additional 20 min in 1/4× MMR (containing 5% Ficoll and 200 μg/ml cycloheximide) to allow inactivation of MPF and the entry into interphase. Similar results were obtained with eggs that were incubated for 60 min after activation. After incubation, the cytoplasm was extracted and subjected to ammonium sulfate fractionation under conditions identical to those used for extraction of MPF from unactivated eggs (see above) except that ATPγS was omitted from the buffers. Neither the initial crude cytoplasmic extract from the interphase eggs nor the mock MPF preparation (i.e., the ammonium sulfate-precipitable proteins) had detectable MPF activity.

In Vitro Reactivation of Interphase MPF

100 μl of the interphase MPF preparation (4.5 mg/ml protein) was mixed with 5 μl of active MPF (4.5 mg/ml protein) and ATPγS (final concentration, 0.3 mM). In control incubations lacking ATPγS, no reactivation occurred. After a 2-h incubation at 22°C (or 8 h at 4°C), the incubation mixture was assayed for its content of active MPF in the in vitro system described above. By including tracer [³⁵S]ATPγS, we determined that ∿50% of the ATPγS was hydrolyzed during the 2-h incubation at 22°C. The hydrolysis of ATPγS was measured by TLC on polyethyleneimine-cellulose as described by Braell et al. (1984).

Preparation of Extracts from Developing Embryos

Xenopus eggs were fertilized as described by Newport and Kirschner (1982). Embryos that did not cleave normally were removed manually. To arrest the cleaving embryonic cells in interphase, we soaked the embryos in 1/4× MMR containing 5% Ficoll and 200 μg/ml cycloheximide for 2 h with gentle agitation. This treatment terminated cell division within one or two cycles (i.e., 30–60 min). Typically, cytoplasm from 1 ml of packed embryos was extracted, subjected to ammonium sulfate precipitation, and tested for latent MPF as described above for cycloheximide-arrested, activated eggs.

Miscellaneous

Protein was assayed by the method of Bradford (1976) with BSA as the standard. Nuclear membranes were stained with 3,3'-dihexyloxacarbocyanine (DHCC) as follows. DHCC was dissolved at 50 mg/ml in DMSO and then diluted 100-fold into buffer A containing 3.7% formaldehyde and 20 μg/ml Hoechst 33258. Insoluble dye was removed by centrifugation at 10,000 g for 5 min. The supernatant (saturated with DHCC) was mixed with an equal volume of nuclei-containing extracts. The stained membranes were visualized by fluorescence microscopy in the fluorescein channel. Rat liver nuclei were prepared according to the method of Blobel and Potter (1966) as modified by Newport and Spann (1987). A monoclonal antibody against Xenopus lamin L_III (Benavente et al., 1985; kindly provided by M. Kirschner [University of California Medical School, San Francisco, CA] and G. Krohne [German Cancer Research Center, Heidelberg, Federal Republic of Germany]) was labeled with rhodamine by the method of Newmeyer et al. (1986). Nuclei were stained in the presence of 50 μg/ml of the rhodamine-conjugated antibody.

Results

Efficient Cell-free Assay for MPF

To study the mechanisms controlling the entry into the mitotic state, we set out to develop a sensitive and rapid in vitro assay for MPF. Although sensitive, the traditional oocyte-microinjection assay is time-consuming and cumbersome. Two groups (Miake-Lye and Kirschner, 1985; Lohka and Maller, 1985) have reported in vitro systems that respond to MPF, but the assays required high concentrations of partially purified MPF. By exploiting some recent technical advances in the construction and disassembly of nuclei in vitro (Lohka and Masui, 1983; Lohka and Maller, 1985; Burke and Gerace, 1986; Newport, 1987; Newport and Spann, 1987), we devised an MPF assay that is more sensitive than the oocyte-microinjection assay and requires as little as 30 min to per-

form. The assay uses extracts from activated Xenopus eggs. These extracts contain a stockpile of unconstructed nuclear components (e.g., histones, lamins, nuclear membranes) that are primed for assembly around any added template DNA (Lohka and Masui, 1983; Forbes et al., 1983; Newport, 1987). A convenient source of homologous DNA is the demembranated Xenopus sperm nucleus (Lohka and Masui, 1983). Within 30–60 min after addition to the assembly extracts, frog sperm chromatin decondenses and acquires an apparently authentic nuclear membrane (Fig. 1 A). We observed that these synthetic nuclei are well-suited substrates for MPF-induced disassembly reactions. Under optimal conditions, the nuclei disassemble rapidly (∿20 min), synchronously, and quantitatively.

When we added crude MPF preparations (Wu and Gerhart, 1980; see Materials and Methods) to the interphase cytoplasm, the synthetic nuclei underwent a dramatic change in morphology. The most obvious change was the loss of the phase-dense nuclear envelope (Fig. 1). The lamin layer was also solubilized under these conditions. In the presence of 1% Triton X-100, a rigid shell surrounded the DNA in intact nuclei. This detergent-resistant lamina (Gerace and Blobel, 1980) stained with a rhodamine-labeled monoclonal antibody against the Xenopus lamin L_III (Benavente et al., 1985; Krohne and Benavente, 1986). In the presence of MPF, the lamina disappeared, and staining with the anti-lamin antibody was undetectable.

This assay system was extremely sensitive. We could dilute MPF between 20- and 50-fold, depending on the MPF preparation and the assay extract. For example, the MPF preparation (Fig. 2) used for all of the experiments in this report could be diluted 40-fold (or 80-fold if one includes the 2-fold dilution with interphase cytoplasm). The same MPF preparation induced 100% maturation in the in vivo oocyte-microinjection assay at dilutions between 10- and 15-fold. Therefore, this in vitro assay is up to 10-fold more sensitive than the traditional maturation assay.

As in the oocyte-maturation assay (Wu and Gerhart, 1980), there was a steep threshold for the concentration of MPF required for nuclear disassembly. MPF induced mitotic conversion at dilutions of up to 40-fold within 1 h or less (depending on the dilution). However, 60- or 80-fold dilutions of MPF did not elicit breakdown even after prolonged incubations (up to 3 h). This did not appear to be due simply to the instability of the interphase extract. For example, if interphase extracts were incubated for 1 h in the absence of MPF and then treated with MPF, disassembly occurred efficiently.

Routinely, we assayed for MPF activity by the loss of a visually discernible phase-dense nuclear envelope from the nuclei in the test extracts. Although this loss was unmistakable, we also stained the membranes with the lipophilic fluorescent dye, 3,3'-dihexyloxacarbocyanine or DHCC (Fig. 1, E and F). Nuclei which had disassembled upon the addition of MPF showed virtually no staining with DHCC, except for a small amount of membranous material that appeared to be nonspecifically adsorbed to the chromatin.

Under our standard assay conditions, the condensation of frog sperm DNA into chromosomes occurred poorly. We found that poor condensation was due to the standard two-fold dilution of the interphase extract and the presence of β-glycerophosphate (40 mM final concentration) in the assay buffer. When we modified the standard assay by diluting the
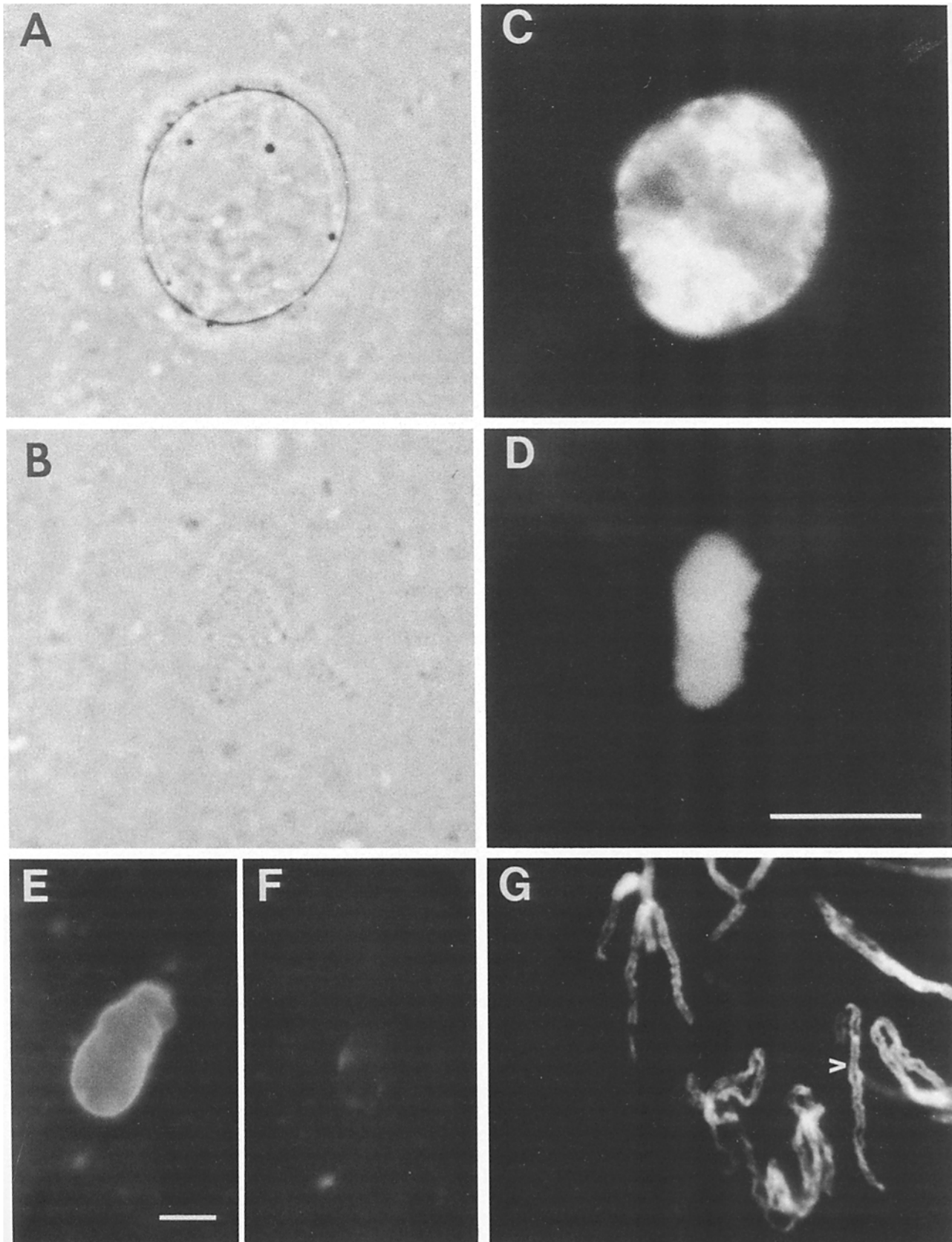
Figure 1. A rapid visual assay for conversion to the mitotic state. Interphase cytoplasmic preparations containing synthetic nuclei were incubated in the absence (*A* and *C*) or presence (*B* and *D*) of a crude preparation of MPF (see Materials and Methods for details). After a 1-h incubation at 22°C, MPF has induced the complete loss of the phase-dense membrane and lamina that envelopes the DNA. (*A* and *B*) Phase contrast. (*C* and *D*) Hoechst staining. In *E* and *F*, reconstituted nuclei were stained with DHCC, a membrane partitioning dye
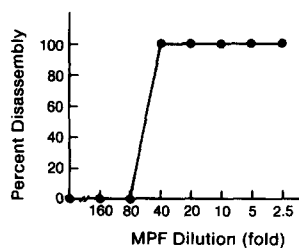
Figure 2. Titration of crude MPF preparations in the mitotic conversion assay. The high speed supernatant (10.8 mg/ml protein) from unfertilized Xenopus eggs was prepared in EB and subjected to ammonium sulfate precipitation as described in Materials and Methods. The crude MPF preparation (4.5 mg/ml protein) was enriched 7.7-fold (yield = 70%) for mitotic induction. MPF was diluted serially with EB (5 µl final volume for each dilution) and mixed with interphase cytoplasm (5 µl) containing reassembled frog sperm nuclei. Induction of the mitotic state was assessed by the loss of the nuclear membrane from the nuclei.

interphase extract by no more than 20% with the MPF-containing buffer (EB), condensation of the frog sperm DNA (Fig. 1 G) occurred efficiently.

Control experiments indicated that the MPF fraction alone cannot catalyze any of the visually observable steps in nuclear disassembly. Instead, MPF must activate disassembly factors in the interphase extract. We found that isolated nuclei incubated with MPF alone did not undergo envelope dispersal or DNA condensation. For these experiments, we used rat liver nuclei, since it was difficult to remove the fragile synthetic sperm nuclei from the interphase cytoplasm in an undamaged form. Rat liver nuclei disassembled efficiently in interphase cytoplasm supplemented with MPF, but complete breakdown required ~90 min (Fig. 3 A). To rule out the possibility that interphase cytoplasm might furnish only a small molecule or cofactor, we showed that MPF could not induce disassembly of rat liver nuclei incubated in heat-denatured interphase cytoplasm (Fig. 3 B).

Two Stages in Nuclear Disassembly

The synthetic sperm nuclei disassembled quite rapidly in response to MPF. At the highest concentration of added MPF (40 U/µl, approximately the level in the unfertilized egg), all of the nuclei broke down in 20–30 min (Fig. 4 A). There was a decided lag in the onset of nuclear breakdown. In the first 10–15 min, there were no gross morphological changes in the nuclei. Then, the nuclear membranes appeared ruffled and partially vesiculated. Upon reaching this stage, the nuclei completely lost their surrounding membrane quite rapidly (within 5–10 min).

The rate of nuclear disassembly was dependent on the concentration of added MPF. However, the dependence indicated that the action of MPF was not the rate-limiting step. When 30-fold diluted MPF (1.3 U/µl, a concentration just above the required threshold) was used, nuclear breakdown required 50–60 min. At intermediate concentrations (between undiluted and 30-fold diluted), there was a nearly lin-
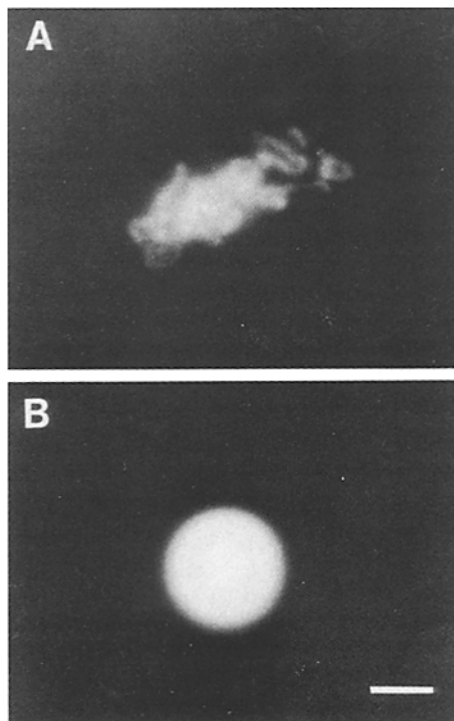
Figure 3. Crude MPF preparations are incapable of inducing any steps in nuclear disassembly in the absence of complete interphase cytoplasm. Isolated (cytoplasm-free) nuclei from rat liver were added to either untreated interphase extracts (A) or heat-inactivated interphase extracts (B). The inactivation treatment was at 60°C for 5 min. After a 90-min incubation at 22°C in the presence of MPF (1 µl of undiluted MPF per 5 µl of interphase extract), the nuclei were examined for disassembly. The final concentration of nuclei was 1,000 per µl. In A, nuclear breakdown occurred readily. The photograph depicts a cluster of condensed chromosomes from a typical disassembled nucleus. In B, there were no obvious ultrastructural changes in the nuclei. Bar, 2 µm.

ear correspondence in breakdown rates ranging from 30 to 60 min (not shown). This behavior indicated that the disassembly process itself, and not the action of MPF, was the rate-determining process. To test this notion, we carried out a series of preincubation experiments. The results of one such experiment are depicted in Fig. 4 B. Interphase extracts lacking nuclei were treated with 40 U or 1.3 U per microliter of MPF for the length of time required for nuclei in parallel extracts to disassemble completely (30 and 60 min, respectively). Then, intact sperm nuclei were added to both incubations, and the time-course of nuclear breakdown in the MPF-primed extracts was examined. In both cases, the rate of breakdown was indistinguishable, occurring within 20–30 min. This result suggests that the mechanical dismantling of

(see Materials and Methods) after a 1-h incubation in the absence (E) or presence (F) of MPF. (G) Chromosome condensation in the presence of MPF. Interphase extract (10 µl) containing reassembled sperm nuclei was mixed with 2 µl of EB containing 0.2 µl MPF (4.5 mg/ml protein). After 1 h at 22°C, the DNA was stained with Hoechst dye. The sperm DNA had undergone a round of DNA replication during nuclear assembly (Blow and Laskey, 1986; Newport, 1987). Consequently, paired sister chromatids (arrowhead) are evident upon chromosome condensation. Bars: (A–D) 5 µm; (E–G) 1 µm.
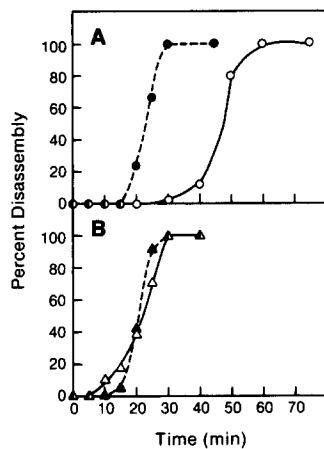
Figure 4. Two stages are required for MPF-induced disassembly. (*A*) Time-course and concentration dependence of induction of nuclear breakdown. MPF (*solid circles*, undiluted; *open circles*, 30-fold diluted) was mixed with an equal volume of interphase extract containing synthetic nuclei (1,000 per µl). At the indicated times, the percentage of disassembled nuclei was quantitated. (*B*) The length of time required for mitotic conversion by MPF. Interphase cytoplasm (100 µl) was prepared exactly described in *A*, except that no sperm nuclei were included. At zero time, the cytoplasm was mixed with undiluted (*solid triangles*) or 30-fold diluted (*open triangles*) MPF and then incubated for 30 or 60 min, respectively. Then, a small volume (5 µl) of interphase cytoplasm containing synthetic nuclei (4,000 per µl) was added. At the indicated times thereafter, nuclear disassembly was quantitated.

the nuclear envelope in the extracts requires 20–30 min. By extrapolation, undiluted MPF (40 U/µl) must act within several minutes of addition whereas 30-fold diluted MPF (1.3 U/µl) must require ∼30 min to trigger conversion to the mitotic state. An interesting implication of this finding is that cells may modulate the length of the period just before mitosis by varying the levels of MPF. In the rapidly cleaving embryo, high levels of MPF would be necessary, whereas in more slowly dividing cells, considerably lower levels of MPF may be adequate.
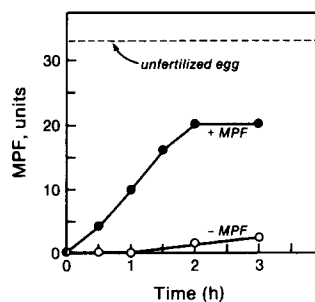
MPF Is Present in an Inert Form during Interphase

We set out to follow the fate of MPF during interphase with the hope of gleaning some clues about mitotic control. Unfertilized eggs were soaked for 1 h in cycloheximide (200 µg/ml). This treatment did not alter the concentration of MPF in the eggs, suggesting that dynamic synthesis of MPF or an activator protein is not required to sustain this special, arrested M phase (Gerhart et al., 1984). The eggs were activated parthenogenetically by treatment with calcium ionophore, and then incubated for 20 min in the continued presence of cycloheximide to prevent the translation of maternal mRNAs.

As expected (Gerhart et al., 1984), active MPF levels dropped to undetectable levels (<5% active MPF, the limit of detection) during this period. Two separate explanations for the drop in activity are that: (*a*) MPF is irreversibly destroyed, or; (*b*) MPF is reversibly modified. We reasoned that if MPF was reversibly inactivated during interphase, it might be possible to resuscitate the interphase form of MPF in vitro. Indeed, we found that active MPF was able to reactivate a reservoir of latent MPF in interphase extracts when incubated together under the appropriate conditions. This experiment is analogous to the amplification of the stored pool of oocyte MPF that occurs upon microinjection of active MPF. The ability of Cyert and Kirschner (1988) to recreate this amplification process in vitro suggested that our in-

direct approach for testing for latent MPF during interphase in the cleaving embryo might be feasible. Interphase extracts containing no active MPF were precipitated with 1.1 M ammonium sulfate in the same manner that active MPF is isolated from M-phase eggs. The precipitated proteins were redissolved and then equilibrated in EB. This protein fraction, which still had no MPF activity, was mixed with 5% by weight of active MPF in the presence of ATPγS. In six separate experiments, we observed that MPF levels regenerated to between 50 and 100% of the level in parallel extracts from unfertilized eggs which contain their entire complement of MPF in an active form (Fig. 5). Reactivation was usually complete within 1 or sometimes 2 h of incubation at 22°C. These experiments demonstrate directly that the protein or proteins which comprise MPF are present during interphase but are incapable of driving mitosis until a critical activation step occurs. The kinetics of reactivation did not suggest that the reactivation of MPF was strongly cooperative, as might be expected if MPF reactivation were strictly autocatalytic. However, since these experiments were performed with crude extracts in which competing reactions may well be occurring, firm conclusions about the mechanism of reactivation cannot be made.

For efficient reactivation of interphase MPF, it was necessary to subject the interphase extract to the first step in the MPF purification scheme, i.e., ammonium sulfate fractionation. Reactivation occurred poorly if complete cytoplasm was used. This could be due to inhibitors, either specific or nonspecific, which are removed or inactivated by the ammonium sulfate step. Alternatively, the fivefold concentration of interphase MPF which ammonium sulfate precipitation achieves may facilitate the reaction. We have also found that ATP (1 mM) without or with a regenerating system will not

Figure 5. MPF is present in a latent form during interphase. Unfertilized *Xenopus* eggs were soaked for 1 h in cycloheximide (200 µg/ml) and then driven into S phase by treatment with the calcium ionophore A-23187 in the presence of cycloheximide. The eggs were incubated for 20 min after activation, and then cytoplasmic extracts were prepared under conditions identical to those used for extraction of active MPF from unfertilized eggs. These extracts contained no detectable active MPF. The interphase extracts were precipitated with 1.1 M ammonium sulfate. The precipitated proteins were redissolved and dialyzed in EB. This mock MPF preparation (4.5 mg/ml protein) from interphase cytoplasm was mixed with 5% active MPF (also 4.5 mg/ml protein; 1.6 U/µl on the scale shown) in the presence of ATPγS (see Materials and Methods). At successive times, the level of active MPF in the incubation was determined with the in vitro assay (*solid circles*). MPF activity in a parallel incubation without exogenous active MPF was also determined (*open circles*). No reactivation occurred in the absence of ATPγS. The dotted line indicates the level of active MPF in unfertilized (mitotic) eggs from the same batch. Since the protein concentration of the latent and active MPF preparations were adjusted to be identical, MPF activity is expressed on a per volume basis (e.g., per microliter).

substitute for ATPγS. One explanation would be that there is such rapid removal of phosphate groups that active MPF cannot accumulate.

The MPF activity regenerated in interphase extracts was indistinguishable from authentic MPF. Reactivated MPF induced both nuclear envelope disassembly and chromosome condensation. It also showed heat sensitivity similar to MPF from unactivated eggs. All activity was lost upon a 10-min incubation at 45°C. Reactivated MPF also met the classical definition for genuine MPF (Wasserman and Masui, 1975; Drury and Schorderet-Slatkine, 1975; Gerhart et al., 1984). The regenerated factor induced maturation of both untreated and cycloheximide-arrested oocytes with an efficiency that matched its potency in the in vitro system. This distinguishes reactivated MPF from agents such as the R subunit of cAMP-dependent protein kinase (Maller and Krebs, 1977) and *ras* protein (Birchmeier et al., 1985). These polypeptides can induce oocyte maturation, but only in conjunction with the synthesis of new protein.

In a standard 2-h incubation at 22°C, reactivation was stimulated by exogenous active MPF in a concentration-dependent manner (Fig. 6). We found that addition of as little as 2.5% active MPF initiated complete regeneration. At lower concentrations of active MPF, regeneration was proportionally less. However, occasionally there was significant reactivation without any added active MPF. The level was usually low ($\sim$2.5 U/μl), but occasionally was as high as 10 U (about one-third of the mitotic level). There are several reasonable explanations for these observations. The interphase extracts may contain some residual active MPF. This active MPF may have escaped destruction during the 20-min incubation after egg activation. Alternatively, a small percentage of the eggs may not have activated. Finally, it is possible that a small fraction of MPF becomes thiophosphorylated by the promiscuous action of one or more kinases during in vitro reactivation. It is well known that kinases vary widely in their degree of target specificity (Taylor, 1987). Since as little as 2.5% by weight of active MPF results in complete reactivation, our experiments would be very sensitive to the adventitious presence (or production) of even traces of active MPF in the interphase preparations.

Latent MPF Levels Are Unchanged at Least until the Gastrulation Stage

We undertook a series of experiments to ascertain whether the observation that MPF is present in the first interphase af-

Figure 6. Concentration dependence of the stimulatory effect of active MPF on the regeneration of latent MPF. The same protocol was followed as in Fig. 5, except that the percentage of added, active MPF was varied (*solid circles*). Mock (interphase) MPF preparations were incubated for 2 h at 22°C in the presence of ATPγS and the indicated amounts of active MPF. The level of active MPF at the end of the incubation was determined (*solid circles*). For comparison, the activity level of the exogenously added (active) MPF is shown (*open circles*).
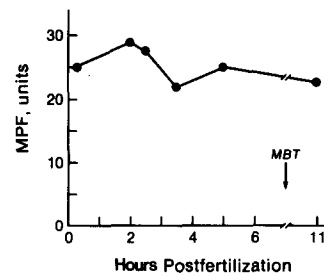
Figure 7. Inactive MPF is present in interphase-arrested embryos at least through the gastrulation stage. Fertilized *Xenopus* embryos were blocked in interphase at the following developmental stages (1 cell, 4 cell, 8 cell, 32 cell, 256 cell, and 11-h gastrula) by a 2-h treatment with 200 μg/ml cycloheximide (see Materials and Methods). Inactive MPF in extracts from each group of embryos was reactivated in vitro, and then tested in the mitotic conversion assay. The activity in each extract was normalized according to a protein concentration of 4.5 mg/ml. The activity of MPF from unfertilized eggs assayed under the same conditions was 50 U. *MBT*, midblastula transition.

ter fertilization was applicable to embryonic cells at later stages of development. About 7 h after fertilization, the *Xenopus* embryo undergoes a major developmental change known as the midblastula transition (Newport and Kirschner, 1982). At this stage, many of embryonic cell division times become longer. This lengthening is due to the introduction of G_1 and G_2 phases. Might this basic alteration in the cell cycle regulatory machinery be reflected in the levels of MPF in the embryo or in its ability to be reactivated? To address this issue, we prepared cleaving embryos at various stages of development (until gastrulation) and treated them with cycloheximide (200 μg/ml) for 2 h to arrest all the embryonic cells before mitosis. By squashing the embryos in Hoechst dye, we verified that cycloheximide treatment reduced the mitotic index to much less than 0.1%. Cytoplasmic extracts were prepared, subjected to ammonium sulfate fractionation, mixed with 2.5% active MPF, and then incubated at 22° in the presence of ATPγS. We found that the store of MPF was unchanged up until the MBT and at least several hours thereafter (Fig. 7). These experiments do not rule out changes in the activator of MPF at the MBT.

After gastrulation, the next dramatic developmental event is the process of neurulation. Between gastrulation and neurulation (i.e., from 10 to 20 h after fertilization), the number of cells per embryo increases from about 20,000 to 50,000 (Gerhart and Keller, 1986). This rate of cell division is sharply lower than the rate in the previous 10 h. By the late neurula stage of embryogenesis, the cell division rates of the embryonic cells are typical of exponentially growing somatic cells. The average length of the cell cycle has increased to 8–10 h (Graham and Morgan, 1966), and the number of cells in mitosis at any given time is <0.5%. We prepared interphase extracts from neurula-stage embryos ($\sim$20 h old), and subjected them to the MPF reactivation protocol. In three separate experiments, we found that the levels of reactivated MPF were $\sim$2.5 U/μl or $\sim$10-fold lower than in earlier embryos. Although we cannot rule out an artifact in the extraction of MPF from these later embryos, there might be an interesting correlation with growth and cell division rates. In more slowly dividing cells, lower concentrations of MPF might be sufficient to sustain the slower division cycles. A related question is whether interphase cytoplasm from these later stage embryos is as sensitive to MPF as the cytoplasm from earlier stages. We prepared interphase extracts from

neurula-stage embryos and examined the breakdown of their endogenous embryonic nuclei in response to MPF. Nuclear disassembly occurred at concentrations of MPF very similar to those required in the early embryonic or egg extracts (i.e., ~1 U of MPF per μl). This suggests that the factors that respond to MPF are still present at levels comparable to those in earlier embryos.

Discussion

We have described here a modified in vitro assay for the mitosis-inducing agent MPF. In this modified form, the assay is more sensitive and rapid than previous systems (Wu and Gerhart, 1980; Miake-Lye and Kirschner, 1985; Lohka and Maller, 1985). In addition, since the extracts are stable to freezing and thawing, the same preparation can be stored in small aliquots for continued use over several months. The ease and manipulability of the cell-free system have allowed us to establish rigorously some indirectly surmised characteristics of MPF. Furthermore, we have uncovered some new insights into the regulation of MPF in the mitotic cycle.

Since the thiophosphorylated MPF used in the assay remains stable in the absence of ATP, we have been able to demonstrate directly that MPF requires ATP to effect nuclear disassembly. The stage or stages that require ATP are not known. Many proteins are hyperphosphorylated during mitosis (Davis et al., 1983). Some examples include the nuclear lamins (Ottaviano and Gerace, 1985; Miake-Lye and Kirschner, 1985) and histones H1 and H3 (Paulson and Taylor, 1982; Ajiro and Nishimoto, 1985). Although direct causal proof that phosphorylation is responsible for any particular structural or regulatory change during mitosis is still lacking, a preponderance of circumstantial evidence suggests that phosphorylation is an important component of mitotic control. Increased phosphorylation could in principle be due to the activation of selective protein kinases and/or the attenuation of specific protein phosphatases. In the case of kinase activation, ATP might be required for the activation process and for utilization by the stimulated kinase.

With the added flexibility of an in vitro system, we have been able to demonstrate directly that MPF activates nuclear disassembly factors but itself plays no immediate role in catalyzing mitotic rearrangements. Even crude MPF preparations cannot cause nuclear envelope release or chromatin condensation without the intermediary action of enzymes in interphase cytoplasm. Consistently, MPF-induced nuclear disassembly has at least two kinetic phases that we can discern. The first is the activation or priming of interphase cytoplasm for nuclear dismantling. This stage occurs in only a few minutes at the level of MPF equivalent to that in the egg during mitosis. The second phase involves the direct remodeling of nuclear constituents by MPF-activated enzymes. This stage may well require the continued presence of active MPF, but we cannot demonstrate this due to our inability to remove MPF from ongoing incubations. This second stage in the breakdown process presumably requires the activated forms of chromosome-condensing enzymes, lamin-specific kinase(s), and a proposed membrane-vesicularization factor (Miake-Lye and Kirschner, 1985; Suprynowicz et al., 1986; Lohka and Maller, 1985; Newport and Spann, 1987). The activities which can be activated by MPF appear

to be present in cells in which mitosis represents a small part of the total cell cycle (late neurula stage cells). This might enable these "mitotic activities" to play a role in modulating nuclear structure in interphase cells.

Recent work has identified a small family of proteins in early clam and sea urchin embryos known as the cyclins. These polypeptides are synthesized continuously throughout each interphase and then abruptly destroyed at the meta-phase–anaphase transition (Evans et al., 1983; Swenson et al., 1986). One of the cyclins, cyclin A from clams, has been shown to induce breakdown of the germinal vesicle (i.e., the nucleus) in *Xenopus* oocytes (Swenson et al., 1986). It is not yet known whether isolated cyclin A can also induce entry into the second meiotic metaphase or can elicit mitosis in cleaving frog embryos. Also, since Swenson et al. (1986) injected cyclin A mRNA, not the purified protein, into oocytes, it was not possible to test whether cyclin A, like MPF, induces germinal vesicle breakdown without prerequisite protein synthesis. Nonetheless, the striking and important finding that cyclin A can induce M phase events suggested that this protein might be related to MPF. Although analogues or homologues to the cyclins have not yet been reported in *Xenopus*, our finding that MPF is present constitutively throughout the cell cycle eliminates the possibility that MPF itself is a cyclin.

These studies emphasize the pivotal role that protein translation may play in driving the oscillation of the simplified embryonic cell cycle. Since the complete machinery for catalyzing mitotic events is poised for activation throughout the entire cell cycle, the ability of the cell to execute cell division would be dependent upon the synthesis of an activator of MPF. Once this activator has accumulated to the requisite level and had sufficient time to act, it would trigger the preexisting MPF. The surmised dynamics of the proposed activator of MPF are very similar to those of the cyclins. It is not known whether any of the identified cyclins correspond to the activator of MPF, but it seems probable that this activator would behave as a cyclinlike molecule.

If this putative mobilizer of MPF is required for both the production and maintenance of active MPF, then the destruction of the activator protein would elicit the exit from mitosis and the beginning of the ensuing interphase. The destruction of the activator could be due to the recruitment of a previously inactive degradative enzyme. An alternative mechanism would also be workable. In this scheme, the egg would continuously synthesize and degrade the activator protein during the interphase period, but synthesis would outpace proteolysis. In this event, a simple mechanism for inactivation of the mitotic state would be to dampen translation of the mRNA for the activator protein.

It has been known for some time that MPF exists in an inert form in the immature oocyte (Wasserman and Masui, 1975; Gerhart et al., 1984, 1985). The injection of a small amount of MPF into an oocyte leads to the rapid elevation of MPF levels to approximately the amount in the mature egg. This increase occurs even in the presence of protein synthesis inhibitors. Moreover, Cyert and Kirschner (1988) have demonstrated that this process will occur in cell-free extracts from oocytes. It has been unclear whether this amplification of stored MPF was a peculiarity of the specialized meiotic divisions of the oocyte or was instead a general feature of

MPF in both meiotic and mitotic regulation. It now appears that the latter is the case.

The presence of MPF during interphase in the *Xenopus* embryo is seemingly inconsistent with the observation of Miake-Lye et al. (1983), who found that MPF injected into cycloheximide-arrested embryos did not undergo amplification. Miake-Lye et al. also observed that the injected MPF was unstable, and suggested that a potent MPF inactivation system was in place. This inactivating agent would presumably be the same one operating during the activation of the egg (Gerhart et al., 1984). We suggest that protein synthesis may be required for the removal of this inactivator. In the in vitro reactivation protocol, the ammonium sulfate precipitation step may remove or inactivate this inhibitor (Cyert and Kirschner, 1988). This model would also be consistent with our finding that mitotic conversion extracts prepared from cycloheximide-treated, activated eggs are 5- to 10-fold less sensitive to MPF.

In summary, we have developed an in vitro system in which MPF converts interphase cytoplasm to the mitotic state at physiological concentrations and at close to in vivo rates. Using this assay, we have deduced that the cellular mitotic regulator contains at least two discernible components. One is MPF, a constitutive factor, which plays an executive role in triggering M phase. The other component is an activator of MPF, which the embryonic cells make anew for each cleavage to turn on MPF in lockstep with the mitotic cycle.

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