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Regulation of MPF Activity In Vitro

Martha S. Cyert and Marc W. Kirschner
Department of Biochemistry and Biophysics
University of California, San Francisco
San Francisco, California 94143

Summary

We have developed a soluble, cell-free system from premeiotic Xenopus oocytes that executes the post-translational activation of a precursor form of maturation promoting factor (MPF). We have distinguished at least two components of this ATP-dependent reaction: pre-MPF, a precursor to MPF that activates independently of added MPF and whose apparent molecular weight changes from 400 kd to 260 kd upon activation; and INH, an inhibitor of pre-MPF activation that confers MPF dependence on the reaction. We present evidence suggesting that INH is a phosphatase and that the activation of pre-MPF occurs via phosphorylation. INH activity itself seems to be regulated by another phosphatase, protein phosphatase-1. We have directly examined the pattern of protein phosphorylation during the activation reaction and have found 92 and 140 kd proteins whose phosphorylation increases when MPF activity appears. This system makes possible a direct examination of the regulation of MPF activity during the cell cycle.

Introduction

In recent years considerable attention has been given to cell cycle regulation in oocytes and eggs, particularly those of Xenopus laevis. The egg does not increase in mass or engage in transcription during its early development, and carries out a series of rapid, simplified, biphasic cell cycles consisting of only M and S phases. In the absence of a nucleus and centriole, the egg still executes periodic cortical and cytoplasmic reactions with normal cell cycle timing (Hara et al., 1980; Gerhart et al., 1984). Thus events like DNA synthesis, nuclear breakdown, and spindle formation can be distinguished from a more fundamental cell cycle clock, or oscillator, that may control them. Although the molecular nature of this oscillator is not yet understood, a fundamental regulatory component of the cell cycle, called maturation or M phase promoting factor (MPF), has been described that is either part of the oscillator or closely coupled to it.

Maturation promoting factor promotes the G2 to M phase transition in many, if not all, eukaryotic cells. It was first described as an activity in the cytoplasm of unfertilized frog eggs, cells that are naturally arrested at metaphase of the second meiotic division (Masui and Markert, 1971; Smith and Ecker, 1971). The transfer of egg cytoplasm into a premeiotic oocyte, which is naturally arrested in G2, induces the recipient to undergo meiosis (to mature) precociously, independent of the normal control by progesterone. This induction of oocyte maturation is the principal assay for MPF.

Although MPF was first discovered in eggs and oocytes, it plays the same role in mitosis as it does in meiosis. MPF activity oscillates in the mitotic divisions of cleaving embryos with the same periodicity as the cell cycle (Wasserman and Smith, 1978; Gerhart et al., 1984). It appears in late G2, peaks in mitosis, and then abruptly declines to undetectable levels by interphase.

MPF activity exists in a wide variety of eukaryotic cells: starfish oocytes and eggs (Kishimoto and Kanatani, 1976), mouse oocytes (Sorensen et al., 1985), mammalian cultured cells (Sunkara et al., 1979; Nelkin et al., 1980), and even yeast (Weintraub et al., 1982; Tachibana et al., 1987; Mark Solomon, unpublished data). In each case MPF activity, as assayed by the induction of Xenopus oocyte maturation, was detected in M phase but not interphase cells.

MPF is not only temporally correlated with M phase, it can actually promote the entry into M phase. Addition of partially purified MPF to either interphase-arrested eggs or cell-free extracts made from eggs induces nuclear envelope breakdown, chromosome condensation, and spindle formation (Miake-Lye et al., 1983; Miake-Lye and Kirschner, 1985; Lohka and Maller, 1985). Furthermore, when MPF activity declines in these systems, nuclei reform, chromosomes decondense, and DNA synthesis begins, indicating that the transition to S phase has occurred. Subsequent addition of MPF can again induce M phase events, so the presence and absence of MPF activity seems to be sufficient to produce a simplified cell cycle of alternating M and S phase states (Newport and Kirschner, 1984).

Though it is clear that MPF plays a direct role in the regulation of cell cycle events, there is still very little known about its molecular nature, its mode of action, or the regulation of its activity through the cell cycle. MPF has been partially purified from unfertilized eggs of X. laevis (Wu and Gerhart, 1980; Nguyen-gia et al., 1986) and from mitotically arrested HeLa cells (Adlakha et al., 1985), and has an apparent molecular weight of 100 kd (Adlakha et al., 1985; Gerhart et al., 1985). In extracts from both sources, MPF activity is unstable but can be preserved by the inclusion of phosphatase inhibitors and ATP. Thus MPF may be a phosphoprotein, and its phosphorylation state may affect its activity. There is more direct evidence that MPF from Xenopus eggs is a phosphoprotein. A monoclonal antibody that specifically binds to some thiophosphoproteins immunoprecipitates and inactivates MPF activity from preparations that have been incubated with ATPγS (Cyert and Kirschner, unpublished data). Therefore, MPF must have a phosphorylation site that becomes modified upon exposure to the sulphur-containing ATP analog. However, many proteins in the partially purified MPF preparation are recognized by this

antibody.

The mechanism by which MPF induces M phase events is unknown. The fact that these events can be induced in the presence of protein synthesis inhibitors (Miake-Lye et al., 1983) argues that they are regulated posttranslationally, and circumstantial evidence suggests that phosphorylation may be involved. One possibility is that MPF itself is a kinase that activates a cascade of enzymes that are responsible for the events of mitosis. There is good evidence that one event downstream of MPF, nuclear envelope breakdown, is mediated by phosphorylation. The lamin proteins, major structural proteins underlying the nuclear envelope, become hyperphosphorylated in vivo during mitosis, and in vitro after MPF addition (Gerace and Blobel, 1980; Miake-Lye and Kirschner, 1985); dephosphorylation seems to be necessary for the nucleus to reform (Burke and Gerace, 1986; Lohka et al., 1987). Other M phase–specific phosphorylations have also been described, although their physiological significance is unknown (Karsenti et al., 1987; Lohka et al., 1987; Davis et al., 1983).

Though something is known about the downstream effects of MPF, much less is known about how MPF itself is regulated during the cell cycle. Oscillations in MPF activity could be due either to periodic synthesis and degradation of the MPF protein, or to periodic activation and inactivation of continually existing polypeptides. Although there is not enough evidence to confirm or exclude either of these models, studies of meiotic induction in Xenopus oocytes indicate that MPF can be regulated posttranslationally. Injection of a small amount of MPF triggers the production of a much larger amount of MPF by the recipient oocyte, and this amplification of MPF activity occurs in the absence of any protein synthesis, suggesting that there is a preexisting inactive form of MPF in the oocyte (Wasserman and Masui, 1975; Gerhart et al., 1984). This latent MPF could be activated by many different mechanisms, including proteolysis, phosphorylation, dissociation from an inhibitor, or release from vesicles.

The ability to induce maturation in the absence of protein synthesis is unique to MPF. Progesterone and other effectors, such as the inhibitor of cAMP-dependent protein kinase, act through protein synthesis, requiring a step(s) to induce maturation (Wasserman and Masui, 1975; Maller, 1983). MPF therefore seems to act downstream of these proteins.

In this report, we describe a soluble, cell-free system derived from premeiotic Xenopus oocytes that executes the posttranslational activation of a precursor form of MPF. This development makes possible for the first time a direct examination of the control of MPF during the cell cycle. We have distinguished at least two components of the activation process whose properties suggest the presence of competing phosphorylation and dephosphorylation reactions. We have directly examined the pattern of protein phosphorylation during the course of the activation reaction, and have observed a small number of specific changes that occur when MPF activity first appears. We discuss these results in the context of a model for the regulation of the eukaryotic cell cycle.

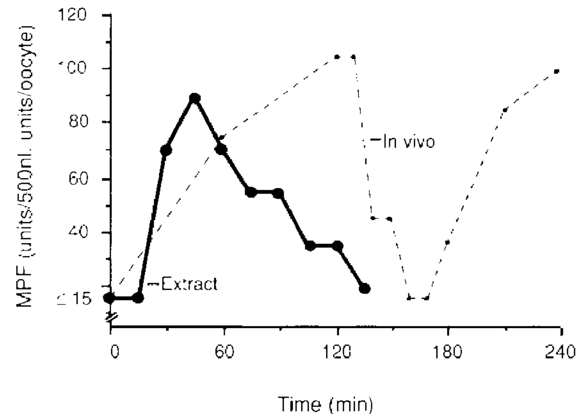

Figure 1. Amplification of MPF Activity in a Low-Speed Extract of X. laevis Oocytes and in Intact Oocytes

A low-speed extract of stage 6 oocytes (described in Experimental Procedures) was incubated at room temperature. At 0 min, an ATP-regenerating system (50 μg/ml creatine phosphokinase and 10 mM creatine phosphate) and partially purified MPF (final concentration, 20 U/μl) were added, and at the indicated times, aliquots of the extract were injected into oocytes to assay MPF activity as described in Experimental Procedures. The number of units of MPF in 500 nl of extract (the estimated volume of extract equivalent to 1 oocyte) is plotted. The dotted line, plotted as MPF units per oocyte, represents data obtained by Gerhart et al. (1984). They injected oocytes with 5 U of MPF (at time 0) and made small extracts at the indicated time points that were assayed for MPF activity.

Results

Establishment of a Crude In Vitro System That Amplifies MPF

We tested oocyte extracts to see if they contained an inactive form of MPF. In initial experiments, oocytes were gently lysed in a minimal amount of buffer and centrifuged at low speed to remove yolk platelets. The extract was then incubated at room temperature with an ATP-regenerating system and a small amount of partially purified MPF from Xenopus eggs and was assayed for production of MPF. Aliquots were withdrawn from the reaction mixture at 15 min intervals, diluted, and immediately injected into Xenopus oocytes to assay for MPF activity. After 2 hr, the percentage of oocytes that had undergone meiosis as judged by the breakdown of the germinal vesicle (GVBD) was scored. One unit of MPF activity is defined as the amount of material in 50 nl that will induce 50% GVBD in recipient oocytes (Wu and Gerhart, 1980). As shown in Figure 1, MPF activity remained undetectable (<15 units/μl) in the extract for the first 15 min and then rose quickly to a peak of 90 units/μl at 45 min. Activity then declined slowly over the next 90 min. There are two strict criteria for MPF that distinguish it from a variety of upstream effectors that can induce maturation but do not directly promote M phase. These criteria are the rapid induction of meiosis and the ability to induce meiosis in the absence of protein synthesis. We used these two criteria to verify that we were detecting bona fide MPF activity in this assay (data not shown).

Having confirmed that MPF activity increased in the extract during incubation, we wanted to verify that the source

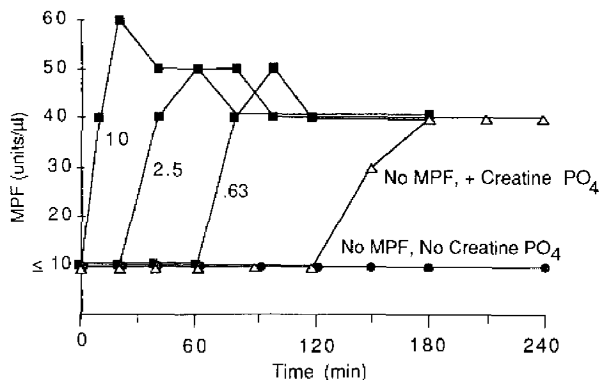

Figure 2. Amplification of MPF Activity in a High-Speed Extract of X. laevis Oocytes

A high-speed extract of oocytes, described in Experimental Procedures, was incubated at room temperature. At 0 min, the following additions were made: nothing ("No MPF, no creatine PO_4"); 50 µg/ml creatine phosphokinase and 10 mM creatine phosphate ("No MPF, + creatine PO_4"); or creatine phosphokinase, creatine phosphate, and partially purified MPF to a final concentration of 10 U/µl ("10"), 2.5 U/µl ("2.5"), or .63 U/µl (".63"). Aliquots of the reactions were taken at the indicated times and injected into oocytes to assay MPF activity as described in Experimental Procedures.

of this MPF activity was the extract itself and not a component in the small amount of added MPF. It seemed possible that the partially purified MPF contained some cryptic MPF activity that might be activated by the extract. To test this possibility, we set up serial activation reactions such that the MPF produced in one reaction was used to activate the next reaction. Partially purified MPF was added to an extract (final concentration, 10 units/µl) that was incubated to allow production of MPF (90 units/µl). An aliquot of one-tenth volume of this first reaction was used to activate a second reaction, which was then used as a source of MPF for a third reaction. The third reaction produced as much MPF activity as the first reaction of the series (90 units/µl), even though it contained less than 1% as much of the initially added partially purified MPF (0.08 units/µl) (data not shown). Thus the oocyte extract must have contributed the MPF that was generated during the incubation period.

Figure 1 compares the time course of MPF production in the extract to that in oocytes, obtained by Gerhart et al. (1984). The levels of activity in the extract and in vivo were compared by expressing the MPF activity in the extract in units per 500 nl of extract, the approximate liquid volume of an oocyte, excluding yolk platelets. By this criterion, the low-speed extract generated approximately the same amount of activity as the intact oocyte. MPF appears slightly faster in the extract than in the oocyte, perhaps because of a difference in the rate of mixing in the extract and the rate of diffusion in the oocyte. The disappearance of MPF activity is slower in the extract than in the oocyte, possibly because of the use of a buffer developed to prevent MPF inactivation (Wu and Gerhart, 1980). After extended incubation of the extract, when MPF activity had disappeared, addition of another small amount of partially purified MPF evoked no further activation response from the extract (data not shown). The extract itself, without the addition of MPF, did not produce MPF within 2 hr (data not shown).

Studies with a High-Speed Extract

There were difficulties in using a viscous, low-speed extract that contained all but the largest organelles. We found that a diluted extract that had been cleared of all organelles by spinning at 160,000 × g for 1 hr also produced MPF activity when incubated at room temperature with MPF and an ATP-regenerating system (Figure 2). This result indicated that all the components that were necessary for the reaction were freely soluble, and eliminated the possibility that active MPF was stored in a vesicle in the oocyte and was then released into the cytoplasm during M phase. The reaction proceeded in the presence of a variety of protease inhibitors, making it unlikely that the mechanism of activation was proteolysis. When no ATP-regenerating system was added to these extracts, no MPF activity was produced.

The timing of the activation reaction, but not the amount of MPF finally produced, was dependent on the quantity of MPF added at the start (Figure 2). If no MPF was added, some, but not all, extracts produced MPF spontaneously in 2.5 to 3 hr. In every extract, addition of partially purified MPF decreased the lag time for MPF production in a dose-dependent manner. With the highest levels of input MPF, the extract could produce MPF in as little as 10 min. The ability to accelerate the activation reaction seems to be specific to MPF. Extracts from interphase eggs, which presumably contain most of the components present in partially purified MPF but have no MPF activity, do not accelerate the reaction. Similarly, a number of purified kinases added at the start of the reaction—cAMP kinase, S6 kinases I and II, and phosphorylase kinase—did not stimulate the production of MPF (data not shown).

The buffer used routinely for extraction of inactive MPF was originally developed for the isolation of active MPF from Xenopus eggs (Wu and Gerhart, 1980), and contained 15 mM MgCl, 20 mM NaEGTA, 80 mM Naβ-glycerophosphate (pH 7.3), and 10 mM DTT. Inclusion of Mg^{++}, EGTA, and DTT was required for the production of MPF, although the concentrations could be reduced to 5 mM for Mg^{++} and EGTA and to 1 mM for DTT with little loss of activity. Other buffers could be substituted for β-glycerophosphate, but another phosphatase inhibitor, such as 5 mM NaF, had to be included. Mn^{++} could partially substitute for Mg^{++}. Treatment of the extract at 50°C for 5 min eliminated all activity.

Separation of Two Components of the MPF Activation Reaction

To begin to define and purify components involved in the MPF activation reaction, the high-speed extract was further fractionated by ammonium sulfate precipitation. A 0%–33% ammonium sulfate fraction contained the component(s) necessary to execute the activation reaction, and we shall refer to this fraction as pre-MPF. Active MPF in unfertilized eggs is precipitated from extracts by the same concentration of ammonium sulfate that precipitates

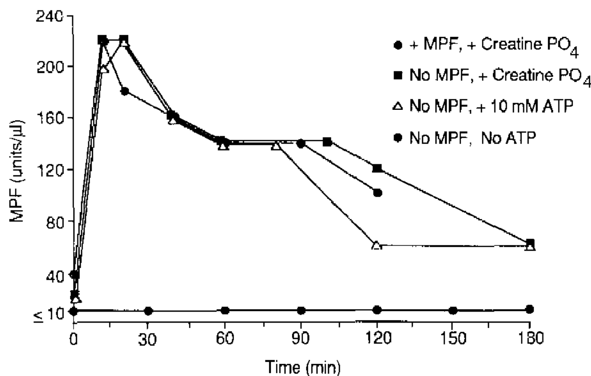

Figure 3. Activation of MPF Precursor in the 0%–33% Ammonium Sulfate Fraction of an Extract of X. laevis Oocytes

The 0%–33% fraction of a high-speed extract of oocytes was incubated at room temperature. At 0 min, the following additions were made: nothing ("No MPF, no ATP"); 1 mM ATP, 50 µg/ml creatine phosphokinase, and 10 mM creatine phosphate ("No MPF, + creatine PO_4"); ATP, creatine phosphokinase, creatine phosphate, and 10 U/µl partially purified MPF ("+ MPF, + creatine PO_4"); 10 mM ATP ("No MPF, + 10 mM ATP"). Aliquots of the reactions were taken at the indicated times and injected into oocytes to assay MPF activity.

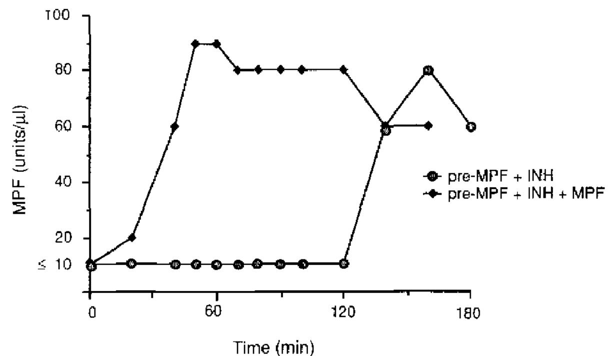

Figure 4. Reconstitution of MPF-Dependent Activation of MPF

The 0%–33% and 45%–55% ammonium sulfate fractions of an X. laevis oocyte extract were incubated together at a 1:1 volume ratio at room temperature with 1 mM ATP, 50 mg/ml creatine phosphokinase, and 20 mM creatine phosphate, with ("pre-MPF + INH + MPF") or without ("pre-MPF + INH") 5 U/µl partially purified MPF. Aliquots of the reactions were taken at the indicated times and injected into oocytes to assay MPF activity.

pre-MPF, suggesting that the active and inactive forms of MPF are similar. This fraction contained 14% of the protein and 55% of the activity of the crude extract, thereby achieving a 4-fold purification of the pre-MPF activity. After activation, such a fraction typically attained an activity of 1 unit per 55 ng. The same fraction of an unfertilized egg extract usually has a comparable activity (1 unit per 35–50 ng).

In contrast to the crude extract and the intact oocyte, the pre-MPF fraction rapidly generated MPF activity (by 10 min) even without the addition of active MPF at the start of the reaction (Figure 3). ATP was required for activation, and could be added directly (10 mM) or supplied through an ATP-regenerating system. One explanation for the unexpected MPF independence of pre-MPF activation was that the ammonium sulfate fraction no longer contained an inhibitor of the activation reaction that was present in the crude extract. To examine this possibility, the crude extract was separated into four ammonium sulfate fractions, 0%–33%, 33%–45%, 45%–55%, and 55%–66%. The 0%–33% fraction was mixed with each of the other three

Table 1. Fractionation of INH Activity

$AmSO_4$ Fractions in Reaction	MPF Activity at 20 Min (Units/µl)
0%–33%	>80
0%–33% + 33%–45%	80
0%–33% + 45%–55%	<10
0%–33% + 55%–66%	>80

The following reactions were incubated at room temperature with 1 mM ATP, 50 µg/ml creatine phosphokinase, and 20 mM creatine phosphate: 0%–33% ammonium sulfate fraction plus an equal volume of either EB containing 10 mg/ml BSA, 33%–45% ammonium sulfate fraction, 45%–55% ammonium sulfate fraction, or 55%–66% ammonium sulfate fraction. At 20 min, each reaction was assayed for MPF as described in Experimental Procedures.

and incubated at room temperature with an ATP-regenerating system. These reactions were assayed for MPF activity at 20 min, by which time a mixture of the 0%–33% fraction with buffer containing BSA was fully active (Table 1). All the reactions had generated some MPF activity by 20 min except the mixture of the 0%–33% fraction with the 45%–55% fraction. Therefore, the 45%–55% fraction contained an inhibitor of the activation reaction. We will refer to this activity as INH.

As shown in Figure 4, when pre-MPF and INH were combined, 2 hr of incubation was needed to generate MPF activity. The timing of this reaction varied somewhat in different preparations, and could be controlled by varying the ratio of pre-MPF to INH. Higher levels of INH resulted in longer activation times. When partially purified MPF was added to such a reaction the activation of pre-MPF was accelerated, as was previously observed in the crude extract. Thus a mixture of the pre-MPF and INH fractions reconstitutes the MPF-dependent kinetics of the crude extract. Although INH inhibits the activation of pre-MPF, this activity does not inactivate MPF once it has been activated. For example, when the 0%–33% fraction was incubated in the presence of ATP for 20 min to activate pre-MPF fully, and was then mixed with either the 45%–55% fraction or with buffer containing BSA, neither mixture showed a significant decrease in activity over the next 60 min (data not shown). INH activity in the 45%–55% fraction is somewhat heat labile. Although little activity was lost after incubation at 50°C for 15 min, 15 min of incubation at 70°C abolished all inhibitory activity.

INH May Be a Phosphatase

Since it is known that the ATP analog ATPγS stabilizes MPF activity in the early stages of purification, we examined its effect on the activation of pre-MPF. ATPγS can substitute for ATP in most phosphorylation reactions, and the resulting thiophosphorylated substrates are resistant to the action of protein phosphatases (Eckstein, 1985). We found that ATPγS could substitute for ATP in activating

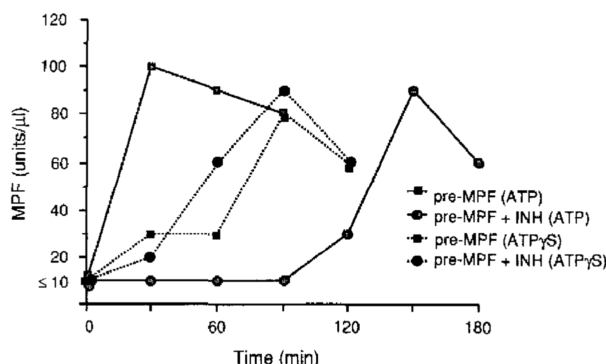

Figure 5. Inhibition of INH by ATPγS

The following reactions were incubated at room temperature: pre-MPF with 1 mM ATP, 50 µg/ml creatine phosphokinase, and 20 mM creatine phosphate ("pre-MPF (ATP)"); pre-MPF mixed 1:1 by volume with INH plus ATP, creatine phosphokinase, and creatine phosphate ("pre-MPF + INH (ATP)"); pre-MPF with 10 mM ATPγS ("pre-MPF (ATPγS)"); and pre-MPF mixed 1:1 by volume with INH plus 10 mM ATPγS ("pre-MPF + INH (ATPγS)"). Aliquots of the reactions were taken at the indicated times and injected into oocytes to assay MPF activity.

pre-MPF. The kinetics of the reaction were slower in the presence of ATPγS, but the extent of activation was comparable. Surprisingly, INH did not delay the activation reaction in the presence of ATPγS (Figure 5). Thus INH seems unable to function in the presence of ATPγS, suggesting that it might be a protein phosphatase.

We directly tested the ability of various phosphatase inhibitors to interfere with INH activity. As shown in Table 2, the addition of 50 mM NaF to a reaction that contained both the pre-MPF and the INH fractions in a 1:2 volume ratio accelerated the reaction considerably from a $t\frac{1}{2}$ of 140 min to 25 min, whereas this concentration of NaF had no effect on the rate or extent of activation of the pre-MPF fraction by itself. This supports the idea that INH is a phosphatase. Another potential phosphatase inhibitor, pyrophosphate, had no effect on the reaction.

The addition of a more specific phosphatase inhibitor,

Table 2. Effect of Phosphatase Inhibitors on INH Activity

Reaction	$t_{1/2}$ (Min)
Pre-MPF	<10
Pre-MPF + INH (1:2)	140
Pre-MPF + INH (1:2) + NaF	25
Pre-MPF + INH (1:2) + Pyrophosphate	140
Pre-MPF + INH (1:2) + inhibitor-1	>180
Pre-MPF + INH (1:2) + inhibitor-1 + MPF	45
Pre-MPF + INH (1:2) + MPF	45
Pre-MPF + inhibitor-1	<10
Pre-MPF + NaF	<10
Pre-MPF + INH (1:1)	20
Pre-MPF + INH (1:1) + inhibitor-1	45

Reactions consisting of the indicated components were incubated with ATP, creatine phosphate, and creatine phosphokinase at room temperature, and at various times aliquots were taken to assay for MPF. $t\frac{1}{2}$ is the approximate time at which the reaction attained half its maximal activity. Concentrations used were as follows: 50 mM NaF, 10 mM NaPyrophosphate, 3 µM inhibitor-1, 0.25 units/µl MPF.

inhibitor-1, which acts only on protein phosphatase type-1 (reviewed by Ballou and Fischer, 1986), did not accelerate the activation reaction, suggesting that INH activity is not due to protein phosphatase-1. Surprisingly, inhibitor-1 completely inhibited the activation reaction (Table 2). This inhibition was overcome by the addition of MPF at the beginning of the reaction. Inhibitor-1 had no effect on the rate or extent of activation of the pre-MPF fraction by itself, suggesting that inhibitor-1 does not interact with pre-MPF or MPF. The effect of protein phosphatase inhibitor-1 depended on the presence of the INH fraction. To examine this effect more carefully we lowered the volume ratio of INH to pre-MPF to 1:1. As shown in Table 1, this shortened the time of spontaneous MPF production from 140 min to 20 min, where pre-MPF alone activates in less than 10 min. Under these conditions inhibitor-1 does not completely abolish MPF activation but delays it from 20 min to 45 min. Given the specificity of inhibitor-1 for protein phosphatase-1, these observations suggest that there is a component in the 45%–55% fraction that is regulated by protein phosphatase-1. An activity similar to that of protein phosphatase-1 has been demonstrated in extracts of Xenopus oocytes (Andres et al., 1987). Possibly INH itself is negatively regulated by phosphatase-1, so addition of inhibitor-1 makes INH more active by blocking phosphatase-1-mediated dephosphorylation; as a consequence, the spontaneous activation of pre-MPF is delayed. Alternatively, the effect of inhibitor-1 on the reaction could be mediated by another component in the INH fraction that we have not yet defined.

Protein Phosphorylation Events during MPF Activation

The effect of phosphatase inhibitors on the activation reaction and previous observations on MPF stability suggest that phosphorylation is the likely mechanism by which MPF activity is regulated. This indirect argument predicts that phosphorylation changes should accompany MPF activation. By examining the stoichiometry of phosphate incorporation into protein we could directly determine whether any changes in phosphorylation accompanied the activation of pre-MPF, and if so, whether these changes were global or more specific. We could manipulate the activation reaction in vitro so that pre-MPF was activated at different times relative to the general kinetics of protein phosphorylation.

Initially we attempted to label proteins by adding [γ-^{32}P]-ATP to the reaction, but found that when the pre-MPF and INH fractions were combined the addition of ATP alone did not support the activation of pre-MPF; an ATP-regenerating system had to be used. This may be because adding high concentrations of ATP (10 mM) to the extract resulted in the accumulation of inhibitory levels of ATP. To ensure a constant specific activity of [γ-^{32}P]ATP during activation, therefore, we synthesized radioactive creatine phosphate and used it as the source of high energy phosphate in the reaction. We verified that the concentration and specific activity of ATP (1 mM ± 10%, 200 cpm/pmol ± 15%) did not vary during the course of the reaction.

The pattern of protein phosphorylation in a mixture of

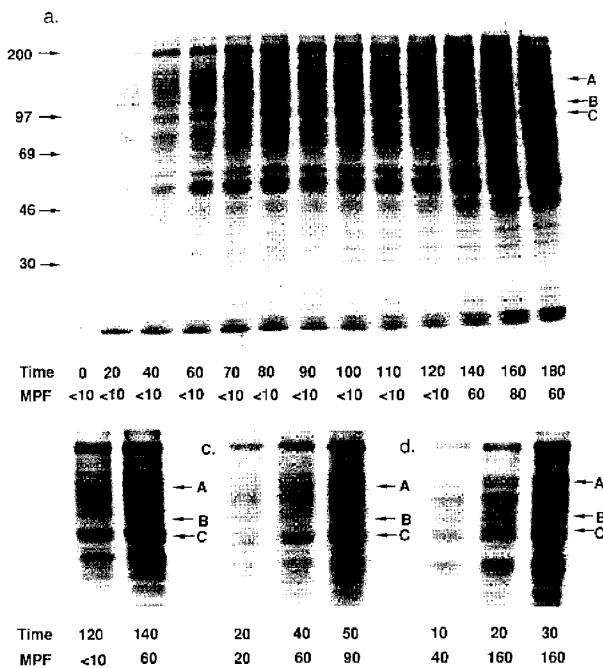

Figure 6. One-Dimensional Gel Analysis of Phosphorylation Changes Associated with Pre-MPF Activation

Ammonium sulfate fractions of oocyte extracts were incubated with 1 mM [γ-^{32}P]ATP, 50 mg/ml creatine phosphokinase, and 20 mM creatine $^{32}PO_4$. Two aliquots were taken at the times indicated; one was immediately assayed for MPF and the other was frozen in gel sample buffer. Gel samples were boiled and equivalent amounts of radioactivity from each sample were loaded on the gel. Bands that showed consistent alterations at the time of appearance of MPF activity are labeled A (140 kd), B (115 kd), and C (92 kd). MPF activity in each sample is shown as a number of U/μl. (a) Complete gel of a reaction that contained the 0%–33% ammonium sulfate (pre-MPF) fraction and the 45%–55% ammonium sulfate (INH) fraction (1:1 mixture by volume). (b) Detail from gel shown in (a). (c) Detail from gel of a reaction that contained the pre-MPF and INH fractions (1:1 mixture) and partially purified MPF (5 U/μl) added at 0 min. (d) Detail from gel of a reaction that contained the pre-MPF fraction.

the pre-MPF and INH fractions incubated at room temperature for 180 min with the [γ-^{32}P]ATP regenerating system is shown in Figure 6a. Aliquots of the reaction were assayed for MPF activity, and these results are also indicated in the figure. A large number of polypeptides incorporated phosphate during the course of the reaction; most proteins reached steady-state levels of incorporation within 60 min. Few proteins seemed to change their incorporation of label during the interval of 120–140 min when MPF activity appears. However, at least three bands, labeled A, B, and C, showed phosphorylation differences, with A and C increasing and B decreasing the extent of labeling concomitant with MPF activation.

A similar analysis was performed by labeling a mixture of the pre-MPF and INH fractions, but adding partially purified MPF at the start of the reaction to accelerate the production of MPF activity (Figure 6c). In this case MPF was first detected at 20 min and peaked at 50 min. Again, changes can be seen in the labeling of bands A, B, and C during this interval. Thus specific phosphorylation differences correlate with pre-MPF activation under two different conditions. When pre-MPF is incubated with the radioactive ATP-regenerating system in the absence of INH, bands A, B, and C become labeled (Figure 6c), indicating that all three proteins are components of the pre-MPF fraction.

We extended our analysis of phosphorylation changes during the activation reaction to nonequilibrium two-dimensional gel electrophoresis (Figure 7) so that it would be possible to see phosphorylation changes that were masked in the one-dimensional gels by comigrating proteins. The phosphorylation patterns at several time points of the three reactions described above were compared. Figure 7a shows the pattern of phosphorylated proteins in a reaction containing pre-MPF and INH before MPF appears (60 min). Figure 7b shows a gel of material containing MPF activity, taken at 60 min from a mixture of pre-MPF and INH plus partially purified MPF. Several differences were noted. We extended this comparison to a later time point from the reaction shown in 7a after MPF activity had appeared (180 min). We also examined a gel of activated pre-MPF in the absence of INH. As found in the one-dimensional gel analysis, the overall pattern of protein phosphorylation is very similar in samples that have MPF activity and those that do not. Only one spot, corresponding to a polypeptide of 92 kd and labeled in 7a–7f, was consistently present in all samples that had MPF activity and absent in the inactive sample. This same spot was also present in unfertilized eggs, which have maximal levels of MPF activity, but not in eggs arrested after DNA synthesis with cycloheximide, which have no measurable MPF activity (data not shown). This 92 kd protein may correspond to band C seen in the one-dimensional gels. No changes that might correspond to bands A and B were seen in the two-dimensional gels.

Changes in Apparent Molecular Weight Accompanying MPF Activation

We attempted to find direct evidence for a biochemical change in pre-MPF that accompanied its conversion to active MPF. Although the active and inactive forms of MPF would have to be purified in order to establish the molecular basis of activation, comparison of their biophysical properties might provide information about the basis for the activation reaction. Pre-MPF fractions were analyzed by gel filtration on a TSK400 column both before and after activation (Figure 8). The pre-MPF was chromatographed in the absence of ATP. To identify the pre-MPF, each fraction was first activated to generate MPF and then assayed by injection into oocytes. Pre-MPF activity was found in the void volume (25% of input) and at a position corresponding to a molecular weight of 400 kd (30% of input) (Figure 8a). A different result was reproducibly obtained if the pre-MPF fraction was first activated by incubation with ATP and then fractionated on the same TSK400 column (Figure 8b). In this case, MPF activity was recovered at 260 kd (36% of the input); there was approximately 20% overlap with the position of pre-MPF-containing fractions. This molecular weight for activated MPF differs somewhat from the previous estimate of 100 kd for thio-

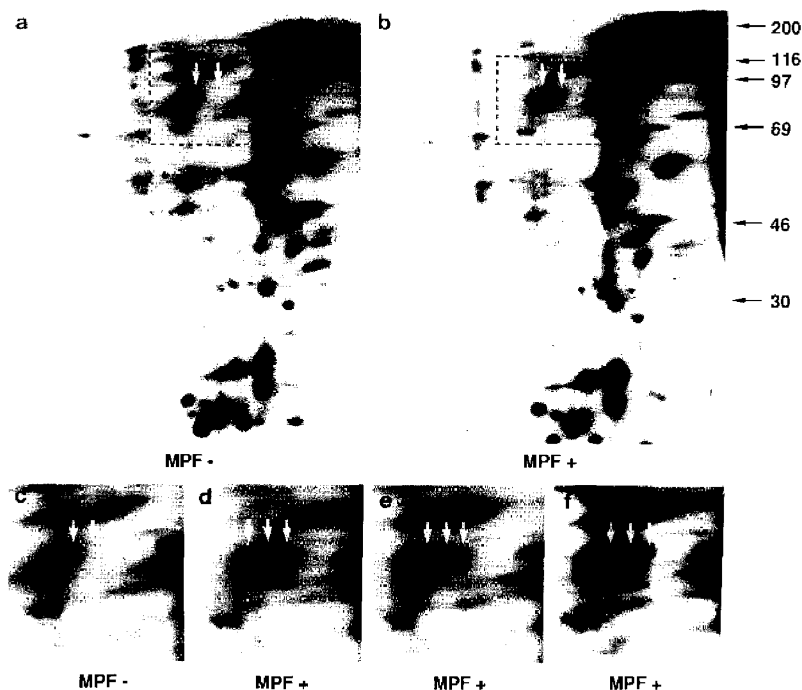

Figure 7. Two-Dimensional Gel Analysis of Phosphorylation Changes Associated with Pre-MPF Activation

Aliquots of reactions were frozen, lyophilized, resuspended in isoelectric focusing lysis buffer, and run on NEPHGE gels. The acidic side of the gel is to the right. ^{32}P labeling is as for Figure 6. (a) The entire gel of the 60 min time point of a reaction containing the pre-MPF and INH fractions (1:1 mixture by volume). This sample had <10 U/μl MPF activity. (b) The entire gel of the 60 min time point of a reaction containing the pre-MPF and INH fractions (1:1 mixture) and partially purified MPF (5 U/μl) added at time 0. This sample had 90 U/μl MPF activity. (c) The rectangle in (a) has been enlarged. (d) The rectangle in (b) has been enlarged. (e) The equivalent region of a gel of the 180 min time point from the reaction described in (a). This sample had 60 U/μl MPF activity. (f) Equivalent region of a gel of the 40 min time point of a reaction containing the pre-MPF fraction. This sample had 120 U/μl MPF activity. In all panels, the 92 kd protein whose phosphorylation correlates with MPF activity is labeled with arrows. Other phosphorylation differences (not labeled) did not consistently correlate with MPF activity in all samples.

phosphorylated MPF purified from Xenopus eggs (Gerhart et al., 1985). The discrepancy could reflect the different sizing resins used (Sephacryl 200, Biosil TSK400) or a real difference between the two preparations.

Discussion

The cycling of MPF activity appears to be an integral part of the oscillator that controls the cell division cycle. The mechanism that governs the activity of MPF during the cell cycle is not known, although previous observations made in Xenopus oocytes suggested that MPF activity might be regulated posttranslationally. We have described the development of an in vitro system derived from oocytes for the production of MPF activity that allows an examination of possible control mechanisms. The in vitro system reproduces the MPF amplification observed in the intact oocyte. Posttranslational activation of a soluble precursor form of MPF occurs in an ATP-dependent reaction; active MPF is not sequestered inside the cell and released internally. Pre-MPF is also probably not activated by proteolysis. Instead, the activation reaction contains two major components: a precursor to MPF, pre-MPF, that activates independently of added MPF; and an inhibitor, INH, that renders the reaction MPF dependent. In the presence of INH, pre-MPF activation is delayed, and addition of MPF decreases the lag time for activation in a dose-dependent manner. INH cannot function in the presence of ATPγS or sodium fluoride, two phosphatase inhibitors, suggesting that INH may be a protein phosphatase.

We suggest the following model for the role of pre-MPF and INH (see Figure 9 and Gerhart et al., 1985). MPF exists in an active (phosphorylated) and an inactive (dephosphorylated) form. In the oocyte, these forms are in equilibrium. The dephosphorylation reaction is catalyzed by INH activity and the phosphorylation reaction is catalyzed by MPF itself. The dephosphorylating activity is greater than the phosphorylating activity, so the majority of MPF exists in the inactive form. In vitro, spontaneous activation occurs in some extracts after long incubations. This activation may be the result of a loss of INH activity over time, or a slow accumulation of active MPF. The ionic conditions of the extract may favor such an accumulation because the buffer used was originally designed to maintain active MPF, and inhibits phosphatase activity. Spontaneous activation does not normally occur in vivo. When active MPF is either injected into an oocyte or added to an oocyte extract, it activates some pre-MPF by phosphorylation that can, in turn, activate more pre-MPF. This quickly overwhelms the INH activity and leads to an explosive phosphorylation of all of the pre-MPF. Maturation frequency has a very high-order dependence on MPF concentration in vivo (Wu and Gerhart, 1980), which could be explained by such a highly concerted reaction. In vitro, once MPF activity appears, it rises rapidly to its maximal level, as in the oocyte. However, in contrast to the oocyte, in vitro this phase of rapid activation is preceded by a lag phase whose length is inversely related to the amount of active MPF added at the start of the reaction. During this lag phase, MPF activity may slowly build up or INH activity may decrease such that a threshold of MPF activity needed for pre-MPF activation is reached.

Both in vitro and in the intact oocyte, MPF levels decline after reaching the maximum level. However, in vitro this process is much slower than in vivo. It is possible that the decline observed in vitro is fundamentally different from

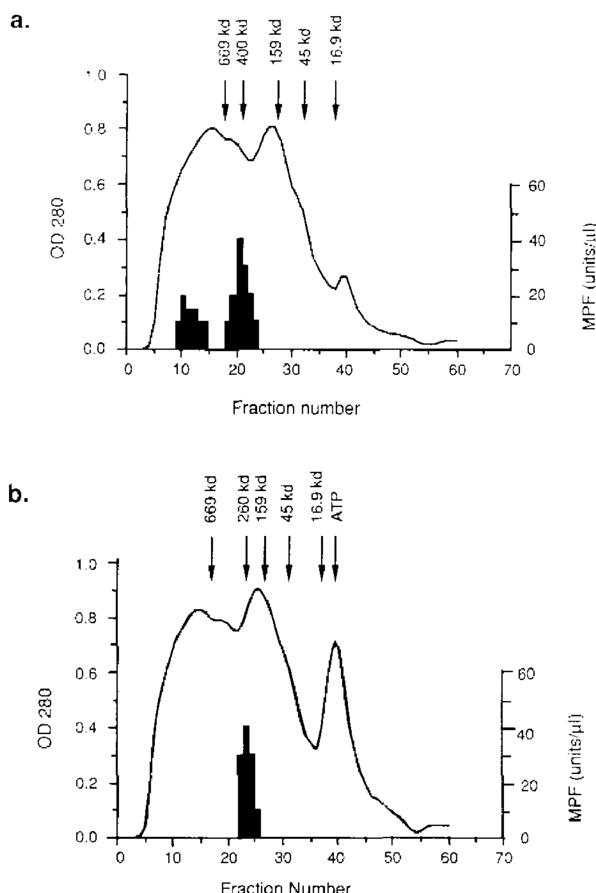

Figure 8. Separation of Pre-MPF and MPF Activities on Biosil TSK400 Column

In both panels, the curve represents the OD 280 and the bar graph represents MPF activity. Arrows show the position of molecular weight markers and estimated molecular weight of each activity. (a) A pre-MPF fraction was separated on the column, and fractions were incubated with an ATP-regenerating system and partially purified MPF and were assayed for MPF activity (see Experimental Procedures). (b) A pre-MPF fraction was incubated with an ATP-regenerating system to allow conversion to active MPF, and then separated on the column (see Experimental Procedures). Fractions were assayed for MPF activity.

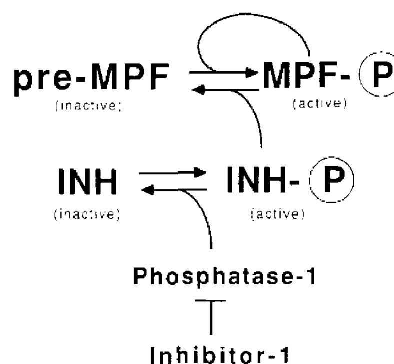

Figure 9. Model for Role of Pre-MPF and INH In Vivo

See text for explanation.

that which occurs in vivo. In vivo, the decrease is most likely due to specific, regulated inactivation of MPF (perhaps via dephosphorylation), whereas the slow decline in MPF that we observe in vitro may reflect a nonspecific loss of enzymatic activity. The egg contains a potent activity for inactivating MPF in interphase (Gerhart et al., 1984). However, INH activity does not seem to be able to inactivate MPF, only to block or delay its activation. Whether INH is distinct from the inactivating activity in the egg or whether it fails to inactivate MPF in vitro because its activity is weakened by unfavorable buffer conditions remains for further study.

Indirect evidence points to phosphorylation as the means both for controlling MPF activity and for regulating downstream events such as nuclear envelope assembly and disassembly. We asked whether specific phosphorylation changes occur concomitantly with MPF activation. Previously it was shown that most phosphoproteins in the egg increased their extent of labeling during M phase because of increased phosphate turnover, and that there are a few phosphoproteins that are specific to M phase (Karsenti et al., 1987; Lohka et al., 1987). In the partially fractionated system, we were able to label most proteins to a steady-state level of phosphorylation before MPF activity appeared. We found a small number of changes that correlated with MPF activity; therefore, we conclude that the degree of phosphorylation of most proteins is not altered during pre-MPF activation. Analysis by one- and two-dimensional gel electrophoresis revealed 92 kd and 140 kd proteins whose phosphorylation consistently increased when MPF activity first appeared. Either of these proteins could potentially be part of MPF itself, or targets of MPF-induced phosphorylation. Previous studies have focused on phosphorylation changes in vivo through the Xenopus cell cycle, and in vitro in extracts of Xenopus eggs that do or do not contain MPF activity. These studies have identified several proteins, whose phosphorylation correlates with the presence of MPF activity, with molecular weights of 116 kd, 46 kd, and 42 kd (Karsenti et al., 1987; Lohka et al., 1987). We failed to detect changes in proteins of these molecular weights. This discrepancy may be due to differences in labeling or in electrophoresis conditions. Alternatively, it is possible that these proteins or kinases that phosphorylate them have been fractionated away in our partially purified system.

Although phosphorylation may be the basis for the activation of pre-MPF, other changes may occur as a consequence. We fractionated pre-MPF on a gel filtration column and found that pre-MPF elutes with a molecular weight of 400 kd, whereas the active MPF generated from it elutes at 260 kd. This shift in molecular weight on activation cannot be due solely to a change in phosphorylation (unless phosphorylation produces an affinity for the TSK resin). It could be due to a conformational change in pre-MPF, dissociation in pre-MPF into subunits, or a change in the interaction of pre-MPF with other components. The alteration in chromatographic properties on activation could be exploited for purification of MPF.

In the oocyte, the transition between pre-MPF and MPF is subject to exogenous regulation by progesterone, which is secreted by the follicle cells. Progesterone causes a de-

crease in the concentration of cyclic AMP in the oocyte, resulting in a decrease in the activity of the cAMP-dependent protein kinase, which somehow leads to production of MPF by a process requiring protein synthesis (reviewed in Maller, 1983; Ozon et al., 1987). It has been proposed that progesterone induces maturation by effecting specific phosphorylation changes (Maller, 1983; Ozon et al., 1987). Based on our observations, we suggest one possible model for the regulation of MPF by progesterone (Figure 9). Progesterone could induce MPF activity in the oocyte by inactivating INH. This would then allow pre-MPF to autoactivate. We have some evidence that the activity of INH can be regulated. When protein phosphatase-1 is specifically inhibited in extracts by phosphatase inhibitor-1, the spontaneous activation of pre-MPF is delayed. This suggests that phosphatase-1 may directly or indirectly control the activity of INH. Direct control of INH by phosphatase-1 could occur if INH is itself phosphorylated and only active in the phosphorylated state. Inhibitor-1 does not affect the pre-MPF fraction, suggesting that regulation by phosphatase-1 does not occur via pre-MPF but through INH or something else in the same ammonium sulfate fraction. Even in the presence of inhibitor-1, MPF can still accelerate pre-MPF activation. These in vitro results are consistent with observations that inhibitor-1 and inhibitor-2 both delay progesterone-induced maturation in Xenopus oocytes, but not MPF-induced maturation (Huchon et al., 1981; Foulkes and Maller, 1982). It is possible that a progesterone-induced decrease in cAMP results in an increase in phosphatase-1 activity that in turn leads to a decrease in INH activity, and subsequent autoactivation of MPF. In this regard, it is interesting that inhibitor-1, which is widely distributed in different cells, is activated by cAMP-dependent protein kinase (reviewed by Ballou and Fischer, 1986). Therefore, a decrease in cAMP could lead to an increase in phosphatase-1 activity through the inactivation of inhibitor-1. However, this sequence does not explain the requirement for protein synthesis in the progesterone-induced pathway. Further purification and characterization of the components of pre-MPF activation are necessary to understand this complex regulatory pathway.

All evidence to date suggests that the meiotic cell cycle is merely a modification of the mitotic cycle. Most of the major substrates for control are the same, such as the nuclear membrane, microtubules, and chromosomes. The major regulatory factor, MPF, seems to be functionally identical. For this reason we believe that pre-MPF and INH will be features of MPF regulation in the mitotic cycle as well as in the oocyte. Preliminary experiments from our laboratory have identified an activity in eggs that seems to correspond to INH (Mark Solomon, unpublished data). We have failed, however, to produce MPF activity from 0%–33% ammonium sulfate fractions of interphase egg extracts by incubating them with ATP.

Posttranslational regulation of MPF activity is not sufficient to explain mitotic cycling in vivo. The mitotic cycle in the frog as well as in many other organisms requires protein synthesis (Wagenaar and Mazia, 1978; Miake-Lye et al., 1983). It is not known what component(s) must be synthesized during each cell cycle. One candidate is a class of proteins called cyclins, whose levels accumulate during each cell cycle and are destroyed at each mitosis (Evans et al., 1983). Expression of clam cyclin A was shown to induce maturation of Xenopus oocytes, and it has been suggested that cyclins may participate in the regulation of MPF (Swenson et al., 1986; Murray, 1987). Cyclins could function, for example, by inhibiting INH in each cell cycle, or by providing a new kinase activity to activate pre-MPF.

In summary, we have shown that MPF exists as an inactive precursor in oocytes that can be activated in vitro by the addition of ATP, and that this activation is accompanied by a change in its apparent molecular weight by gel filtration from 400 to 260 kd. We have defined a component in crude extracts, INH, that inhibits this reaction. INH may be a phosphatase, and there is indirect evidence that it itself may be regulated by phosphorylation and dephosphorylation. The activation of MPF is accompanied by a few specific changes in protein phosphorylation that can be correlated with the activation of MPF. These changes may represent crucial regulatory steps in the induction of M phase. Pre-MPF and INH may be general features of the regulation of MPF in all cell cycles, and the in vitro system we have described makes possible the direct analysis and assay of these components.

Experimental Procedures

Xenopus

Adult Xenopus laevis females and males were obtained from Xenopus One (Ann Arbor, MI) or were raised in the laboratory of Dr. J. Gerhart (University of California, Berkeley). Females were injected with 25 units of gestyl (Diosynth Inc., Chicago, Ill) 2 days before the removal of ovary to be used either in MPF assays or for making extracts.

MPF

MPF was partially purified from extracts of unfertilized eggs, as described by Wu and Gerhart (1980), with the following modifications also suggested by Wu and Gerhart. Phenyl agarose (Sigma Chemical Co.) was used in place of pentyl agarose, and the arginine agarose step was omitted and replaced with a fractional precipitation with 7.5% polyethylene glycol 6000 (MCB reagents).

MPF Assay

X. laevis females were anesthetized with 3% 3-aminobenzoic acid ethyl ester (tricaine) prior to surgical removal of a section of ovary. Ovary was maintained in a modifed Barth's saline developed by Gurdon and Laskey (1970), which was further modified by addition of 10 mM HEPES (pH 7.4) instead of Tris as the buffer (MBSH). Stage 6 oocytes were dissected by hand from the surrounding follicle. To assay a sample for MPF activity, 50 nl of appropriate dilutions of the sample was injected into several oocytes. The oocytes were fixed with 10% trichloroacetic acid 1.5 to 2 hr after injection and were dissected to determine whether or not the germinal vesicle had dissolved. A dilution that induced 50% GVBD was said to have an MPF concentration of 1 U/50 nl. To induce oocyte maturation with progesterone, oocytes were incubated with 1 μg/ml of progesterone (Sigma Chemical Co.).

Low-Speed Extracts

Ovary was incubated in MBSH containing 10 mg/ml of collagenase (type IA, Sigma Chemical Co.), which had been passed over a column of P6-DG resin (Bio-Rad Co.), for 2 to 4 hr at room temperature with gentle agitation. Stage 6 oocytes were selected and rinsed extensively with MBSH. Oocytes were rinsed with extraction buffer (EB) developed by Wu and Gerhart (1980) (80 mM Naβ-glycerophosphate, 20 mM NaEGTA, 15 mM $MgCl_2$ [pH 7.3]) plus 10 mM DTT, 20 μg/ml cytochalasin B, and 1× protease inhibitors (25 μg/ml leupeptin and aprotinin, 1 mM benzamidine HCl, 10 μg/ml pepstatin, 0.5 mM PMSF). The co-

cytes were transferred to an Eppendorf tube and spun at 35 × g for 1 min, and excess buffer was removed. The oocytes were lysed by pipetting up and down with a P200 Pipetman, and were centrifuged at 12,000 × g for 5 min at 4°C; the cytoplasmic layer was removed. For activation, creatine phosphokinase was added to 50 μg/ml, and creatine phosphate was added to 10 mM (both from Sigma Chemical Co.).

High-Speed Extracts

Whole ovaries from several frogs were removed and incubated overnight at 16°C with collagenase and with gentle agitation. Oocytes were rinsed extensively with 100 mM NaCl, and most of the small oocytes (stage 4 and smaller) were removed with a reverse flow column. The oocytes were placed in a glass column, with 100 mM NaCl flowing into the bottom of the column. The smaller oocytes floated out the top of the column, while the larger, heavier oocytes were retained. Oocytes were rinsed with EB, lysed in 1 volume of EB with 10 mM DTT and 1× protease inhibitors by repeated pipetting through a 10 ml pipette, and centrifuged at 160,000 × g for 1 hr at 4°C. The cytoplasmic layer (usually 6–8 mg/ml protein) was removed and incubated with creatine phosphokinase and creatine phosphate for activation. Ammonium sulfate fractionation was carried out by addition of 0.5 volume of a saturated solution of ammonium sulfate in EB to the extract, incubation on ice for 30 min, and resuspension of the pellet collected at 200,000 × g for 15 min at 4°C to an approximate protein concentration of 15 mg/ml. (Protein determination was by Bradford reagent from Bio-Rad Co., with BSA used as a standard.) The rest of the extract was adjusted to an ammonium sulfate concentration of 45%, and the resulting precipitate was collected and discarded. The extract was then adjusted to 55% ammonium sulfate, and the resulting precipitate was collected and resuspended to the same volume used to resuspend the 0%–33% pellet. The protein concentration of the 45%–55% fraction was 12–15 mg/ml. Resuspended fractions were dialyzed against EB with 1 mM DTT and 0.1× protease inhibitors at 4°C before use. For activation, ammonium sulfate cuts were incubated at room temperature with 1 mM ATP (Sigma), 50 μg/ml creatine phosphokinase, and 10 mM creatine phosphate.

Labeling with $^{32}PO_4$

Creatine phosphate was labeled with ^{32}P by an exchange reaction to which 5 mCi of carrier-free $^{32}PO_4$ (aqueous solution; New England Nuclear) was added (Floyd and Traugh, 1979). The reaction mixture, containing 400 nmol of creatine phosphate at a specific activity of 4×10^6 cpm/pmol, was separated by TLC on a PEI cellulose plate (EM Science) developed in 0.25 M LiCl. The overall yield of $^{32}PO_4$ incorporated into creatine phosphate was 40%. The creatine $^{32}PO_4$ was eluted from the TLC plate with 0.25 M ammonium acetate, lyophilized, and diluted with unlabeled creatine phosphate to achieve the desired specific activity. For analysis of the extracts, 200 mM creatine $^{32}PO_4$, at a specific activity of 200 cpm/pmol, 10 mM ADP, and 0.5 μg/ml creatine phosphokinase were incubated at room temperature for 5 min and then 1 part of this reaction was added to 10 parts of the extract to be labeled.

Analysis of Specific Activity in $^{32}PO_4$-Labeled Reactions

Aliquots of activation reactions incubated with ^{32}P-labeled creatine phosphate were incubated on ice with 12% perchloric acid to precipitate all proteins; samples were spun at 12,000 × g for 30 min, and the supernatants were collected and neutralized with KOH. The samples were separated by TLC on a PEI cellulose plate and developed in 1 M acetic acid:4 M LiCl, 8:2. The ATP spots were scraped from the plate and eluted with 0.7 M $MgCl_2$. An aliquot of the eluted sample was counted by liquid scintillation counting to determine the amount of radioactivity it contained. Another aliquot was assayed by using firefly luciferase (extract from Sigma Chemical Co.) according to Strehler (1968) to determine the concentration of ATP it contained.

Electrophoresis

One-dimensional electrophoresis was carried out essentially according to Laemmli (1970), by using 5%–15% acrylamide gradient gels with a pH of 9.2 (acrylamide from Bio-Rad Co.) 2D NEPHGE gells were prepared and run according to O'Farrell et al. (1977) by using ampholines from LKB Co. and a 8.5% acrylamide gel (pH 9.2) for the second dimension. Samples for 2D gels were lyophilized and resuspended in isoelectric focusing lysis buffer (O'Farrell et al., 1977). $^{32}PO_4$-labeled egg samples were prepared according to Karsenti et al. (1987).

Gel Filtration

The 0%–33% ammonium sulfate fractions were applied to a Biosil TSK400 HPLC size exclusion column (7.5 mm × 300 mm; Bio-Rad, Richmond, CA) equilibrated in EB plus 1 mM DTT and 0.1× protease inhibitors. Elution was at 0.5 ml/min, and 0.25 ml fractions were collected. When unactivated pre-MPF was applied to the column, the TSK fractions were incubated with 1 mM ATP, 10 mM creatine phosphate, 50 μg/ml creatine phosphokinase, and partially purified MPF (final concentration, 5 U/μl) at room temperature for 20 min, then assayed for MPF activity. For analysis of extracts after activation of pre-MPF, the column input was incubated with ATP, creatine phosphate, and creatine phosphokinase at room temperature for 20 min, then loaded onto the column. In this case, ATPγS (0.4 μM) was added to the fractions collected from the TSK column, and they were assayed for MPF activity.

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