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Maturation Promoting Factor: Properties and Regulation

by

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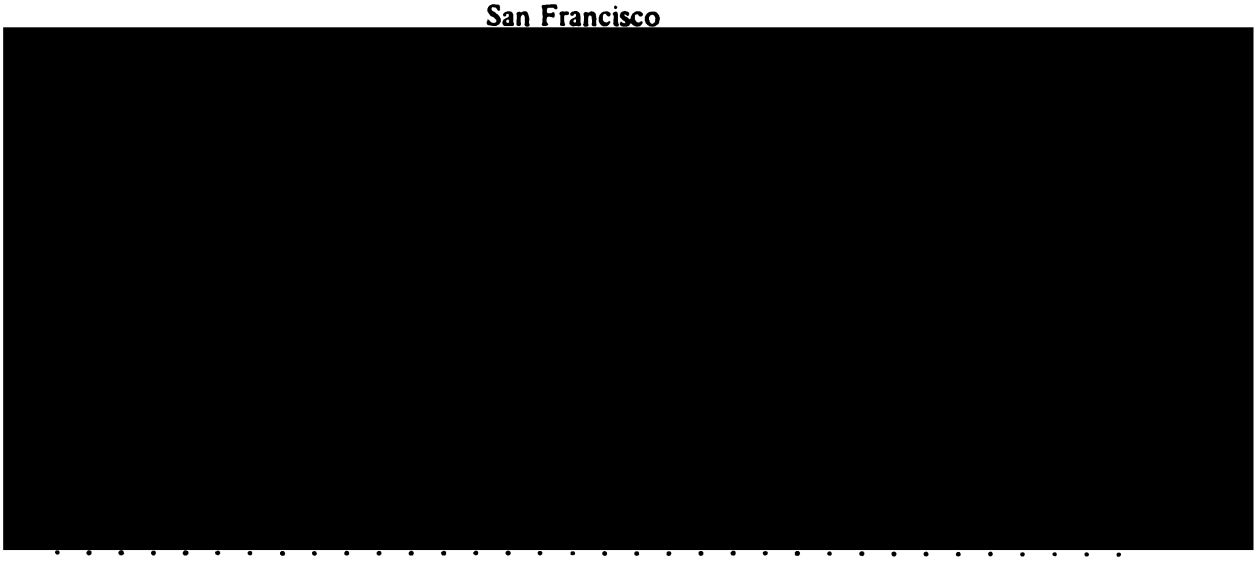

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To my parents,
Margaret Shadick Cyert and Richard Michael Cyert,
who got me into this mess by instilling in me a desire
for "higher learning",
and
to the World's Greatest Husband and World's Greatest Dog,
who got me through it.

"The prayer of the scientist if he prayed, which is not likely: Lord, grant that my discovery may increase knowledge and help other men. Failing that, Lord, grant that it will not lead to man's destruction. Failing that, Lord, grant that my article in...[*Cell*]...be published before the destruction takes place."

Walker Percy, from
Love in the Ruins

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Finally, after six and a half years at UCSF (who's counting?) I am both sad and happy to be leaving, and am reminded of a quote by that inspirational man of the mountains, Finnis Mitchell:

"Too many of us follow endless trails. Unless a trail leads us somewhere and ends, it is but a circle."

Maturation Promoting Factor: Properties and Regulation

Martha S. Cyert

Abstract

Maturation promoting factor (MPF) promotes the G2 to M phase transition in most, if not all, eukaryotic cells. We have studied the regulation of MPF activity in Xenopus laevis oocytes, and have explored its properties through the isolation of monoclonal antibodies that recognize MPF.

We describe a soluble, cell free, system from pre-meiotic Xenopus oocytes, that executes the post-translational activation of a precursor form of MPF. We have distinguished at least two components of this ATP-dependent reaction: a precursor to MPF, pre-MPF, that activates without added MPF, and an inhibitor of pre-MPF activation, INH, that renders the reaction MPF-dependent. We present evidence that INH is a phosphatase and that the activation of pre-MPF occurs via phosphorylation. INH activity itself seems also to be regulated by phosphorylation. We have directly examined the pattern of protein phosphorylation during the activation reaction and have found that a small number of phosphorylation changes mirror the appearance of MPF activity. This system makes possible for the first time a direct examination of the regulation of MPF activity during the cell cycle.

In an attempt to further characterize and purify active MPF from unfertilized Xenopus eggs, we have isolated five monoclonal antibodies that immunoprecipitate MPF activity, and inhibit the activity in solution. However, none of the antibodies reacts specifically with MPF, and we show that antibody binding is dependent on previous exposure of the preparation to ATPγS. This suggests that the antibodies specifically recognize thiophosphoproteins, although not all thiophosphorylated proteins in MPF are immunoprecipitated. One of the antibodies was used to

partially purify MPF by immunoadsorption chromatography. These experiments provide the first evidence that MPF from Xenopus is a phosphoprotein, and becomes thiophosphorylated upon exposure to ATPγS.

Additional results presented concern the preliminary characterization of MPF activity from the yeast Saccharomyces cerevisiae.

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Chapter 1

Introduction

One of the most fundamental cellular properties is the ability to proliferate. In order to reproduce, most cells must double their mass as well as duplicate and partition essential cellular organelles. While these growth and division activities must be coordinated, we consider only the latter processes as true cell cycle events. The eukaryotic cell cycle is conventionally described in terms of the major nuclear events, DNA synthesis (S-phase) and mitosis (M-phase), that duplicate and segregate the genetic material. These two landmark events are punctuated in most cells by time "gaps" labelled G1 (after mitosis) and G2 (after DNA synthesis). G1 is the most variable stage of the cell cycle in terms of its length, and seems to function as a growth period before the cell becomes committed to a round of cell division. Yeast cells, for example, must attain a critical size before beginning S phase, and growth conditions that result in different growth rates affect the length of the G1 period accordingly (Hartwell and Unger, 1977; Carter and Jagadish, 1978; Lord and Wheals, 1980; Nasmyth et al., 1979). In addition to the stages of the vegetative cell cycle, most types of cells can enter a distinct non-proliferating or resting state referred to as G_0. Under poor growth conditions, or at high density, cells enter G_0 after mitosis, and fail to initiate further rounds of cell division. When conditions become more favorable, cells can exit G_0, re-enter G1, and resume cell division, but once they have entered G_0, they must complete a specific series of events in order to become physiologically eqivalent to cells that have just completed division (reviewed in Pardee et al., 1986). In higher organisms, many stem cells are maintained in G_0 until they recieve the appropriate signals to proliferate and/or differentiate.

The regulatory mechanisms that control cell cycle events are complex, and operate at many different levels. First there is control by factors extrinsic to the cell, such as growth factors in the case of animal cells. These controls generally govern the entry of the cell from G_0 into G1, although some growth factors are also required for completion of G1. There has been extensive study of various growth factors and their effects on different cell types, and a review of that literature is beyond the scope of this introduction. Therefore their

role will be mentioned in the briefest possible manner. Second, there are intrinsic or cytoplasmic controls that govern the progress of the cell through the various stages of the vegetative cycle. Much less is known about these cytoplasmic signals, but two types of experiments have revealed their existence. The fusion of cells that differ in their cell cycle stage (Rao and Johnson, 1970; Kaufman and Wille, 1975), and the microinjection of nuclei and other cellular components into amphibian eggs (Gurdon, 1978; Karsenti et al., 1984), have demonstrated the importance of the cytoplasm in determining the timing of cell cycle events. In particular these approaches, as well as genetic studies in yeast, have suggested that inducers of S phase and M phase appear at appropriate times in the cell cycle.

Entry into S phase

An animal cell can enter the G1 phase either following mitosis and cytokinesis, or from the G_0 state after stimulation by growth factors. For mouse 3T3 cells, in the presence of the necessary nutrients and a single growth factor (IGF 1 or hyperphysiological concentrations of insulin), cells advance through G1 to a control point known as the restriction point (R-point) (Campisi and Pardee, 1984; Leof et al., 1984). R is a point of commitment for the cell to the next round of cell division. After the R-point, in the absence of any growth factors or transcription the cell will carry out the processes of DNA synthesis and nuclear and cellular division (Yang and Pardee, 1986). Concentrations of cycloheximide that only partially inhibit protein synthesis, and have no effect on other stages of the cell cycle, prevent the cell from passing the R point, so it has been suggested that transit through R is regulated by the accumulation of an unstable protein (Rossow et al., 1979; Campisi et al., 1982). A protein with this characteristic has been identified by two-dimensional gel electrophoresis, but its biological activity has not been tested (Croy and Pardee, 1983). Two hours after R, DNA synthesis begins. S-phase must be triggered by a positive cytoplasmic signal, because the nucleus of a G1 cell initiates DNA synthesis when fused with an S-phase cell (Rao and Johnson, 1970). The molecular nature of this

signal is unknown, although it seems plausible that it is a component of the DNA synthesis machinery. G1 cells are not advanced into S-phase when fused with G2 cells, so the induction signal seems to be present only during S.

In the budding yeast, S. cerevesiae, and the fission yeast S. pombe, studies of cell cycle regulation using the techniques of classical genetics has lead to the definition of a control point at the G1/S boundary termed "start" that has similarities to the R point defined in animal cells. Start is the earliest defined event of the cell cycle, and as such can be considered the point of initiation of the cell cycle (Pringle and Hartwell, 1981). After passing start the cell is committed to completing one round of cell division, and is not able to undergo the alternative fate of conjugation until cell division has been completed (Hartwell, 1974). Pheremones that induce the mating response arrest cells at start in preparation for conjugation (Bucking-Throm et al., 1973). Start is transversed only when a critical cell size, determined by the specific growth conditions used, is reached (Hartwell and Unger, 1977; Carter and Jagadish, 1978; Lord and Wheals, 1980). The products of the cdc28 gene in cerevesiae, and the cdc2 gene in pombe are required for start, and temperature sensitive mutations in these genes result in cell cycle arrest at the non-permissive temperature (Reed, 1980; Nurse and Bisset, 1981). These proteins are functionally related, as the $cdc28^{+}$ protein from cerevesiae permits growth of $cdc2^{ts}$ pombe strains at high temperature (Beach et al., 1982). Both proteins are phosphorylated and have kinase activity (Reed et al., 1985; Simanis and Nurse, 1986), and the two are 62% homologous by amino acid sequence (Lorincz and Reed, 1984; Hindley and Phear, 1984). The kinase activity of the cdc28 protein is assayed in vitro using a 40 kd substrate protein that co-immunoprecipitates with the cdc28 protein from crude extracts. Results obtained using this assay suggest that the cdc28 protein is active in dividing cells and not in cells that are in stationary phase or arrested with mating pheremones (Mendenhall et al., 1987). The kinase activity of the cdc2 protein, measured with casein as a substrate, also declines as cells enter stationary phase, and phosphorylation of the cdc2 protein decreases

concomitantly (Simanis and Nurse, 1986). These results suggest that the activity of these kinases is regulated, possibly through phosphorylation; the availability of their substrates may also be regulated through the cell cycle. We will discuss *cdc2 /cdc28* again, as there is evidence that this kinase is also involved in the regulation of entry into M-phase.

Entry into M-phase

Evidence from a number of different orgnaisms indicates that mitosis inducing factors accumulate in cells toward the end of G2. In the slime mold Physarum plasmodium the existence of such factors was shown by fusing plasmodia from different stages of the cell cycle. A plasmodium in late G2 can advance the time of mitosis of another plasmodium from an earlier stage when the two plasmodia are fused (Rusch et al., 1966). This mitosis-inducing activity is retained in extracts of G2 plasmodia, and is proteinacious in nature (Loidl and Grobner, 1982). Similarly, interphase animal cells, when fused to M-phase cells, respond to M-phase inducing factors by undergoing condensation of their chromatin into chromosomes, an event that is characteristic of prophase (Rao and Johnson, 1970).

In fission yeast, activities involved in the control of the G2 to M transition were identified by isolating mutants that were advanced in the entry into M, and thereby divided at a smaller size. Mutations were isolated in two genes; recessive, loss of function mutations defined an inhibitor of mitosis, wee1, and dominant mutations were isolated in the cdc2 gene (Nurse and Thuriaux, 1980). Temperature-sensitive mutations in cdc2, as discussed previously, arrest the cell cycle at start, but also in G2, indicating that this protein functions at two different times in the cell cycle (Nurse and Bisset, 1981). There is some evidence that its homologue, cdc28, is also necessary for mitosis in S. cerevesiae (Piggot et al., 1982). Also implicated in the control of mitosis in S. pombe was another temperature-sensitive mutation, cdc25. cdc25 mutants arrest in G2 at the non-permissive temperature, but a wee1 mutation can suppress this defect, suggesting that the two genes might interact in some way (Fantes,

1979). The cdc25 and wee1 genes were recently cloned, and overexpression studies revealed that they code respectively for an inducer and an inhibitor of mitosis (Russell and Nurse, 1986; Russell and Nurse, 1987a). Furthermore another gene has been identified, nim1, which induces mitosis by negatively regulating wee1 (Russell and Nurse, 1987b). wee1 and nim1 both have strong homology to protein kinases, and cdc2, as discussed previously, has been shown to be a phosphorylated kinase. One tentative model, then, for how these proteins interact, is that nim1 negatively regulates wee1 via phosphorylation, wee1 negatively regulates cdc2 kinase activity by phosphorylation, and that cdc25 stimulates cdc2 activity (by unknown mechanism). This model must be directly tested by investigating the biochemical activities of the proteins involved. One major caveat is that to date, cdc2 kinase activity has not been shown to vary during the cell cycle (Simanis and Nurse, 1986). Still, the activity has been assayed in vitro with a non-physiological substrate (casein), and subtle in vivo variations in activity or specificity of the kinase would probably not have been detected. The described model for the control of mitosis by a phosphorylation cascade is strikingly similar to models that have come from studies of higher eukaryotes that we will now discuss.

Many of our ideas of how the transition to M-phase is effected have come from the study of eggs and oocytes, particularly those of Xenopus laevis. The early embryo of Xenopus, as well as other species, exhibits a simplified cell cycle different from that of most somatic cells. The egg does not increase in mass or engage in transcription during its early development, and carries out a series of rapid, biphasic cell cycles consisting of only M and S phases (Graham and Morgan, 1966). The study of cell cycle regulation in these cells allows us to distinguish between events required for cell growth and those necessary for cell division.

An activity first identified in unfertilized frog eggs, maturation promoting factor (MPF), is now known to promote the G2 to M phase transition in many, if not all, eukaryotic cells. Originally MPF was described as an activity in unfertilized eggs cytoplasm that when injected into a premeiotic oocyte, would induce meiosis

("maturation") in the recipient independent of the normal stimulus by progesterone (Masui and Markert, 1971; Smith and Ecker, 1971). Unfertilized frog eggs are naturally arrested at metaphase of the second meiotic division, and immature or pre-meiotic oocytes are arrested in G2, so in terms of the cell cycle, MPF is a component of M-phase cytoplasm that can induce a G2 cell to enter M. When the oocyte undergoes the first meiotic division, it generates its own MPF, up to one hundred times more than the amount of MPF required to induce maturation (Gerhart et al., 1984). After the first meiotic division is complete, the oocyte proceeds into meiotic interphase during which MPF activity is undetectable, then MPF levels rise again as the oocyte enters the second meiotic division. At metaphase of the second meiotic division, when MPF levels are high, the cell cycle of the oocyte is naturally arrested, and remains at this point until fertilization. MPF activity in Xenopus is strictly cytoplasmic, as an oocyte produces as much MPF activity as a nucleated oocyte does (Wasserman and Masui, 1975). The induction of oocyte maturation is the principle assay for MPF.

MPF plays the same role in mitosis as it does in meiosis. MPF activity oscillates in the mitotic divisions of cleaving embryos as it does during meiosis in oocytes (Wasserman and Smith, 1978; Gerhart et al., 1984), and MPF can initiate mitotic events. 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 et al., 1985; Lohka and Maller, 1985).

MPF activity has been demonstrated 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 cdc mutants of budding yeast (Weintraub et al., 1982; Tachibana et al., 1987; chapter 4). In every case MPF activity can be assayed by the ability to induce meiosis in Xenopus oocytes, and is detected only in M phase cells, not in interphase cells. This implies that the protein and its activity have been strongly conserved through evolution.

All this evidence strongly indicates that MPF plays a direct role in the regulation of the G2 to M phase transition, and yet there is still very little known about the molecular nature of MPF, its mode of action, or how its own activity is regulated through the cell cycle. MPF is a protein, and has been partially purified from unfertilized eggs of Xenopus laevis (Wu and Gerhart, 1980; Gerhart et al., 1985; Nguyen-gia et al., 1986), and from mitotically arrested HeLa cells (Adlakha et al., 1985). In extracts from both sources, MPF activity is unstable, but can be preserved by the inclusion of phosphatase inhibitors and ATP or ATPγS. Thus, MPF may be a phosphoprotein, and its phosphorylation state may affect its activity. For Xenopus MPF there is more direct evidence that it is a phosphoprotein. A monoclonal antibody that specifically binds to some thiophosphoproteins can immunoprecipitate and inactivate MPF acitivity from preparations enriched in MPF that have been incubated with ATPγS (Cyert and Kirschner, manuscript in preparation; chapter 3). Therefore, MPF must have a phosphorylation site that becomes modified upon exposure to the sulphur-containing ATP analog. The antibody is not specific for MPF, as it recognizes many thiophosphorylated proteins in the partially purified MPF. The molecular weight of MPF is estimated to be 100 kd from HeLa cells (Adlakha et al., 1985), and from Xenopus eggs (Gerhart et al., 1985) on the basis of gel filtration.

The mechanism by which MPF induces M phase events is unknown, but 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 post-translationally. A mass of circumstantial evidence has been used to argure that the induction of M-phase is regulated by phosphorylation, and that MPF is a kinase that acts by initiating a phosphorylation cascade. Three types of evidence have led to the idea that MPF acts through phosphorylyation. First their are observations of MPF itself. As mentioned above, MPF is probably a phosphoprotein, and in vivo changes in protein phosphorylation correlate with changes in MPF activity (Capony et al., 1986; Maller et al., 1977). Also, partially purified preparations of MPF contain both kinases and phosphatases,

and one kinase activity may co-purify with MPF (Gerhart et al., 1985). Second, there are experiments that suggest that particular M-phase events are controlled by phosphorylation. Nuclear envelope breakdown is thought to be mediated by phosphorylation of the lamin proteins. 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). Similarly, chromosome condensation is thought to involve phosphorylation of histones (Bradbury et al., 1974; Gurley et al., 1978; Ajiro et al., 1983). Thirdly, many other M-phase specific phosphorylations and kinases have been described, although their physiological significance is unknown (Karsenti et al., 1987; Lohka et al., 1987; Davis et al., 1983; Sahasrabudde et al., 1984; Halleck et al., 1984).

The mechanism that governs the activity of MPF itself through the cell cycle is also in question. As described earlier, MPF activity oscillates with the cell cycle, but these fluctuations could represent either cycles of synthesis of MPF polypeptides followed by their degradation, or periodic activation and inactivation of continuosly existing MPF protein. The study of MPF activity in the oocyte shows that MPF activity can be regulated post-translationally- possibly through phosphorylation. In the absence of protein synthesis, in response to injection of a small amount of MPF, an oocyte can generate its full complement of MPF activity (Wasserman and Masui, 1975; Gerhart et al., 1984). This suggests that the oocyte contains a store of MPF in an inactive form that can be activated post-translationally. This MPF-dependent activation can be reproduced in vitro in a soluble, ATP dependent reaction (Cyert and Kirschner, in press; see chapter 2). Fractionation by ammonium sulfate revealed that at least two components are involved in this reaction. One fraction, designated pre-MPF, is capable of producing MPF activity spontaneously when incubated with ATP, and undergoes a change in apparent molecular weight concomitant with activation. Another fraction, designated INH, inhibits the spontaneous activation of pre-

MPF in an MPF-dependent manner. INH activity is inhibited by some general phosphatase inhibitors, suggesting that it may be a phosphatase, and that the molecular mechanism of pre-MPF activation is phosphorylation. Furthermore the activity of INH itself may be regulated by phosphorylation, because a specific inhibitor of protein phosphatase type 1, inhibitor 1, seems to stimulate INH activity. Thus, one possible model to explain the regulation of MPF activity in the oocyte , is that MPF exists in an equilibrium between active (phospho) and inactive (dephospho) forms. The inactivation reaction is catalyzed by INH, which itself may be regulated by phosphorylation, its inactivation being catalyzed by protein phosphatase type 1. Activation (phosphorylation) of pre-MPF may be autocatalytic (as proposed by Gerhart et al., 1985), such that when INH is present, the addition of a small amount of MPF is capable of activating much more pre-MPF, and when INH is removed (by ammonium sulfate fractionation), activation occurs spontaneously. A direct examination of the pattern of protein phosphorylation in extracts during pre-MPF activation shows that a small number of specific changes do accompany the appearance of MPF activity.

In the oocyte, the transition between pre-MPF and MPF is controlled exogenously by progesterone. Addition of progesterone causes a drop in the concentration of cAMP, which leads to inactivation of the cAMP dependent protein kinase, and somehow, in a pathway requiring protein synthesis, to production of MPF activity (reviewed in Maller, 1983). In other systems it is known that inhibitor 1 must be phosphorylated by cAMP dependent protein kinase to be active (reviewed in Ballou and Fischer, 1986), so it is tempting to speculate that progesterone acts through an inactivation of inhibitor 1, which would lead to activation of phosphatase 1, inactivation of INH, and activation of pre-MPF (see Maller,1983, and chapter 2). However, this model fails to explain the requirement for protein synthesis.

Though post-translational regulation of MPF activity clearly occurs in the oocyte, the question remains as to whether the oscillation of MPF activity in embryos is also controlled post-translationally. The oocyte is unique in its responsiveness to

progesterone, but downstream events that lead to pre-MPF activation could be identical in cycling embryos and oocytes. There is some preliminary evidence that INH activity exists in eggs and embryos (Mark Solomon, unpublished), but attempts to demonstrate the presence of an inactive MPF precursor in the embryo have failed.

How is MPF activity destroyed in the oocyte or embryo? Both egg and oocyte contain a potent interphase activity that destroys MPF activity, as do mammalian cells in G1(Gerhart et al., 1984; Adlakha et al., 1983), but it is not known if this destruction represents inactivation or actual degradation. If MPF activity is inactivated at the end of mitosis, it is possible that INH is responsible, although there could also be a distinct inactivator.

There are obvious similarities that emerge between the models for regulation of cdc2 activity at G2/M in S. pombe and the regulation of MPF activity in Xenopus (Hayles and Nurse, 1986). Unfortunately, mitotic pombe extracts do not induce maturation of Xenopus oocytes, so a direct functional homology between the two proteins seems unlikely (M. Solomon, unpublished). It has recently been shown that a human homologue to cdc2 exists, although the function of this protein is not known (Lee and Nurse1987; Hanks, 1987). An antibody that recognizes cdc28 in cerevesiae, cdc2 in pombe, and the cdc2 human homologue reacts with a protein in Xenopus extracts, and this protein does not co-purify with MPF activity (M. Solomon, unpublished). Also different alleles of cdc28 that differ in their degree of temperature sensitivity show no corresponding variation in MPF activity (Mark Solomon, unpublished). MPF and cdc2/cdc28 are distinct functions.

Comparison of the cell cycle in different systems

The overall view of cell cycle regulation that emerges from the study of Xenopus embryos is quite different than that suggested by experiments with animal cells and yeast. The frog embryo has a rapid biphasic cell cycle that seems to be run by a cytoplasmic clock, or oscillator that is independent of many of the actual events of the cell cycle. In the absence of a nucleus, centriole, or microtubules, the egg exhibits a periodic cortical contraction called the surface

contraction wave that occurs with normal cell cycle timing (Hara et al., 1980). MPF also continues to cycle in these eggs (Gerhart et al., 1984), so the disruption of specific cell cycle events like mitosis, cytokinesis, or DNA replication, does not inhibit this fundamental cell cycle timer. Cycles of MPF activity have never been uncoupled from this clock, so MPF must be tightly coupled to the oscillator, or possibly part of it. In cycloheximide-arrested embryos, cycles of MPF addition coupled to spontaneous decay of MPF activity is enough to produce a biphasic cell cycle, implying that the presence and absence of MPF is sufficient to produce both M and S phases (Newport and Kirschner, 1984). In vivo, cycling requires protein synthesis, but it is not known what component(s) must be synthesized (Wagenaar and Mazia, 1978; Miake-Lye et al., 1983). One candidate is a class of proteins called cyclins, whose levels accumulate during each cell cycle and are destroyed at mitosis (Evans et al., 1983). Expression of clam cyclinA was shown to induce maturation of Xenopus oocytes, so 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 each cell cycle, or by providing a new kinase activity to activate pre-MPF. Alternatively, MPF itself may need to be synthesized each cell cycle.

In contrast, the yeast cell cycle has been described by several parallel pathways of interdependent steps (Pringle and Hartwell, 1981). The cell cycle can be arrested either by mutations that prevent the execution of particular functions, or by the use of drugs that inhibit a particular event, for example hydroxyurea to inhibit DNA synthesis, or benamyl to inhibit mitosis. The transit of cultured animal cells through the cell cycle is also arrested at particular stages by similar drug treatments.

Is cell cycle regulation fundamentally different in these different systems? The frog embryo is unusual in that it contains stockpiles of many components, and does not increase its mass, so perhaps the mechanisms of the cycles are actually very similar (for example they both seem to use MPF to induce mitosis), but the frog embryo lacks homeostatic controls that the other, growing, cells must

impose on the timing of their cell cycles. In Xenopus after twelve rapid biphasic cell divisions have been completed, the embryo undergoes a transition, known as the midblastula transition. After this transition the cell cycle lengthens and acquires a G1 and a G2 phase, presumably because the supply of some necessary component(s) present in the unfertilized egg becomes limiting (Newport and Kirschner, 1982). Post-MBT, the cell cycle can be arrested by drug treatments such as hydroxyurea which inhibits DNA synthesis (John Newport, unpublished). Thus, the embryonic cell cycle can apparently be modified to produce a more typical somatic cycle.

On the other hand, a cytoplasmic oscillator may exist in yeast and animal cells in culture, but its physical manifestations may not be as easily observed as the surface contraction waves of amphibian eggs. Under specific conditions, there is some evidence that in these other systems particular events can be uncoupled from cell cycle timing. For example one cdc mutant of S. cerevesiae, cdc4, when shifted to high temperature, arrests the processes of DNA synthesis and spindle pole body duplication, but the cells continue periodically to produce anucleate buds (Pringle and Hartwell, 1981). Thus a cell cycle timer, as manifest by periodic budding, may be uncoupled from the control of nuclear events. Animal cells in culture, when arrested with a temperature sensitive mutation (tsBN2), or with DNA synthesis inhibitors and caffeine, undergo premature chromosome condensation; thus entry into M-phase under these conditions is uncoupled from the completion of DNA synthesis (Schlegel and Pardee, 1986). These observations may indicate that in some cases a cell cycle timer can function independently of cell cycle events in yeast and animal cells, as has been observed for Xenopus eggs, and that cell cycle control mechanisms in these different systems may in fact be similar.

Chapter 2

Regulation of MPF activity in vitro

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Abstract

We have developed a soluble, cell free, system from pre-meiotic Xenopus oocytes, that executes the post-translational activation of a precursor form of MPF. We have distinguished at least two components of this ATP-dependent reaction. One component, pre-MPF, is a precursor to MPF that activates independently of added MPF. Upon activation its apparent molecular weight changes from 400 kd to 260 kd. Another component, INH, is an inhibitor of pre-MPF activation that confers MPF-dependence on the reaction. We present experimental evidence that suggests 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 that a small number of phosphorylation changes occur concomitantly with MPF activation. This system makes possible for the first time 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, which 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 principle 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 in yeast (Weintraub et al., 1982; Tachibana et al., 1987; Mark Solomon, unpublished). 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 et al., 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, mode of action, or the regulation of its activity through the cell cycle. MPF has been partially purified from unfertilized eggs of Xenopus 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 can immunoprecipitate and inactivate the activity of partially purified MPF that has been incubated with ATPγS (Cyert and Kirschner, manuscript in preparation). Therefore, MPF must have a phosphorylation site that becomes modified upon exposure to the sulphur-containing ATP analog. Many proteins, however, 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 post-translationally, 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 exisisting 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 post-translationally. 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 pre-existing 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 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 post-translational 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.

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 assayed for production of MPF. Aliquots were withdrawn from the reaction mixture at 15 minute intervals, diluted, and immediately injected into Xenopus oocytes to assay for MPF activity. After 2 hours, 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 2- 1, MPF activity remained undetectable (< 15 units/μl) in the extract for the first 15 minutes and then rose quickly to a peak of 90 units/μl at 45 minutes. Activity then declined slowly over the next 90 minutes.

There are two strict critieria for MPF that distingish it from a variety of upstream effectors that can induce maturation, but do not directly promote M-phase. These critieria 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 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 2- 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/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. The appearance of MPF is 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 ooctye. The disappearance of MPF activity is slower in the extract than in the oocyte, possibly due to the use of a buffer that was developed to prevent MPF inactivation (Wu and Gerhart, 1980). After extended incubation of the extract, when MPF activity

has 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 two hours (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 x g for one hour also produced MPF activity when incubated at room temperature with MPF and an ATP regenerating system (figure 2-2). This result indicated that all the components that were neccesary 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 ulikely 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 how much MPF was added at the start (figure 2-2). If no MPF was added, some, but not all, extracts produced MPF spontaneously in 2 1/2 to 3 hours. 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 ten minutes. 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. Likewise, 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 lowered to 5 mM for Mg^{++} and EGTA, and 1 mM for DTT with little loss of activity. Other buffers could be substituted for β-glycerophosphate, but another phosphatase inhibitor, such as 5mM NaF, had to be included. Mn^{++} could partially substitute for Mg^{++}. Treatment of the extract at 50° C for 5 minutes 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 all of 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 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 four fold purification of the pre-MPF activity. After activation, such a fraction typically attained an activity of one unit / 55ng. The same fraction of an unfertilized egg extract usually has a comparable activity (one unit/ 35-50 ng).

In contrast to the crude extract and the intact oocyte, the pre-MPF fraction rapidly generated MPF activity (by 10 minutes) even without the addition of active MPF at the start of the reaction (fig. 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, and incubated at room temperature with an ATP-

regenerating system. These reactions were assayed for MPF activity at 20 minutes, by which time a mixture of the 0-33% fraction with buffer containing BSA was fully active (table 2-1). All the reactions had generated some MPF activity by 20 minutes 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 2-4, when pre-MPF and INH were combined, a two hour 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 had been 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 minutes to fully activate pre-MPF, and then mixed with either the 45-55% fraction, or buffer containing BSA, neither mixture showed a significant decrease in activity over the next 60 minutes (figure 2-5). INH activity in the 45 - 55% fraction is somewhat heat labile. Although little activity was lost after incubation at 50° C for 15 minutes, a 15 minute 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 pre-MPF. The kinetics of the reaction were slower in the presence of ATPγS, but the extent of activation was comparable.

Suprisingly, INH did not delay the activation reaction in the presence of ATPγS (figure 2-6). 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 2-2, 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 $t_{1/2}$ of 140 minutes to 25 minutes, 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, 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. Suprisingly, inhibitor-1 completely inhibited the activation reaction (table 2-2). 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 2-1 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 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 spontaeous 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 ADP. To assure 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/pmole +/- 15%) did not vary during the course of the reaction.

The pattern of protein phosphorlyation in a mixture of the pre-MPF and INH fractions incubated at room temperature for 180 minutes with the [γ^{32}P]-ATP regenerating system is shown in figure 2-7. 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 minutes. MPF activity was first detected at 140 minutes. At least three specific changes (labelled A, B, and C) in the pattern of protein phosphorylation paralleled the appearance of MPF. Bands A (140 kd) and C (92 kd) both showed an increase in phosphorylation that was greater than that seen for the average protein between 120 and 140 minutes. This increase in intensity could be due to increased phosphorlation of an existing phosphoprotein, or to the phosphorylation of a new protein that co-migrates with an existing phosphoprotein in the reaction. At this same time band B (115 kd) seemed to disappear. Although other subtle changes may be difficult to detect, it is clear that most proteins do not undergo radical changes in their rate or extent of phosphorylation when MPF activity appears.

A similar analysis was performed by labelling 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 (fig. 2-8). In this case MPF was first detected at 20 minutes and peaked at 50 minutes. The phosphorylation of bands A and C increased during this interval, and band B disappeared. Thus specific phosphorylation changes correlated with pre-MPF activation under two different conditions. When pre-MPF is incubated with the radioactive ATP-regenerating system in the absence of the INH fraction, bands A, B, and C become labelled (fig 2-9), indicating that all three proteins are present in the pre-MPF.

We extended our analysis of phosphorylation changes during the activation reaction to non-equilibrium two-dimensional gel electrophoresis (figs 2-10 to 2-15) so that it would be possible to see phosphorylation changes that were masked in the one-dimensional gels by co-migrating proteins. The phosphorylation patterns at several time points of the three reactions described above were compared. Figure 2-10 shows the pattern of phosphorylated proteins in a reaction containing pre-MPF and INH before MPF appears (60 minutes). Figure 2-11 shows a gel of material containing MPF activity, taken at 60 minutes 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 10 after MPF activity had appeared (180 minutes), as shown in figure 2-12. We also examined a gel of activated pre-MPF in the absence of INH (fig. 2-13). As found in the one-dimensionsal 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 labelled in figures 2-10 to 2-15, 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, that have maximal levels of MPF activity (fig. 2-14), but not in eggs arrested post-DNA synthesis with cycloheximide, that have no measurable MPF activity (fig. 2-15). This 92kD 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 pre-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, comparing their biophysical properties might provide information about the basis for the activation reaction. Pre-MPF fractions were analyzed by gel filtration on a TSK-400 column both before and after activation (fig. 2-16). The pre-MPF containing fraction was eluted in the absense of ATP, fractions were collected, and each fraction was then activated to generate MPF by incubation with ATP and a small amount of partially purified MPF. (The inclusion of active MPF insured uniform kinetics of activation in such dilute samples.) After 20 minutes, each fraction was 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) (fig. 2-16a). 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 (fig 2-16b). In this case, MPF activity was recovered at 260 kD (36% of the input). There was

20% overlap in the position of MPF and pre-MPF elution. This molecular weight for activated MPF differs somewhat from the previous estimate of 100 kd for thiophosphorylated MPF purified from Xenopus eggs (Gerhart et al., 1985). This discrepancy could reflect the different sizing resins used (Sephacryl 200, Biosil TSK400), or a real difference between the two preparations; for example our sample was not thiophosphorylated.

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 post-translationally. 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 sytem reproduces the MPF amplification observed in the intact oocyte. Post-translational acitivation 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, and 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 in vivo (see figure 2-17). MPF exists in an active (phosphorylated) and an inactive (dephosphorylated) form. In the oocyte, these two forms are in equilibrium. The dephosphorylation reaction is catalyzed by INH activity and the phosphorylation reaction is catalyzed by MPF itself (as suggested by Gerhart et al., 1985). The dephosphorylating, or inactivating 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 which 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 preceeded 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 that which occurs in vivo. In vivo, the decrease is most likely due to a specific, regulated inactivation of MPF (perhaps via dephosphorylation), whereas the slow decline in MPF that we observe in vitro may reflect a non-specific 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 both the means for controlling MPF activity and for regulating downstream events, such as nuclear envelope assembly and disasssembly. We asked whether specific phosphorylation changes occur concommitantly with MPF activation. Previously it was shown that most phosphoproteins in the egg increased their extent of labelling during M-phase due to 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 phosphorlyation 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 Xenoupus 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 fail to detect changes in proteins of these molecular weights. This discrepancy may be due to differences in labelling or 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 seems more likely that this apparent change in molecular weight is due to a change in MPF conformation, its composition, or its interaction with other components. This alteration in chromatographic properties on activation could be exploited for purification of MPF. A particular fractionation technique could be applied to pre-MPF, then after the preparatation has been activated the same technique could be reapplied. If active MPF fractionates differently from pre-MPF, a large purification might be achieved.

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 decrease 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 in a process requireng protein synthesis (reviewed in Maller,1983; Ozon et al., 1987). Other authors have suggested models to explain the relationship between changes in protein phosphorylation and the production of MPF (Maller, 1983; Ozon et al., 1987). Based on our observations, we offer the following as one possible mechanism of progesterone action. 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 (see figure 2-17). 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 auto-activation 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 in 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 obviously 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). We have failed, however, to produce MPF activity from 0-33% ammonium sulfate fractions of interphase egg extracts by incubating them with ATP.

Post-translational regulation of MPF activity is not sufficient to explain mitotic cycling in vivo (see Gerhart et al., 1985). The mitotic cycle in the frog as well as 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 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 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 the direct analysis and assay of these components possible.

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 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 (Wu and Gerhart, unpublished). 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: Xenopus laevis females were anaesthetized with 0.3% 3-amino benzoic acid ethyl ester (tricaine) prior to surgical removal of a section of ovary. Ovary was maintained in a modified Barth's saline developed by Gurdon and Laskey (1970), which was further modified by adding 10 mM Hepes, ph 7.4 instead of Tris as the buffer (MBSH). Stage 6 oocytes were hand-dissected 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 1/2 to 2 hours after injection and dissected to determine whether or not the germinal vescicle had dissolved. A dilution that induced 50% GVBD was said to have an MPF concentration of 1 unit/50 nl. To induce oocyte maturation with progesterone, oocytes were incubated with 1 µg/ml progesterone (Sigma Chemical Co.).

Low speed extracts: Ovary was incubated in MBSH containing10mg/ml collagenase (type IA, Sigma Chemical Co.), which had been passed over a column of P6-DG resin (Biorad Co.), for 2 to 4 hours 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 1X protease inhibitors (25 μg/ml leupeptin and aprotinin, 1 mM Benzamidine HCl, 10 μg/ml pepstatin, 0.5 mM PMSF). The oocytes were transferred to an eppindorf tube, spun at 35 x g for 1 min, and excess buffer was removed. The oocytes were lysed by pipetting up and down using a P200 Pipetman, and were centrifuged at 12,000 x g for 5 minutes 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 one volume of EB with 10 mM DTT and 1X protease inhibitors by repeated pipetting through a 10 ml pipette, and centrifuged at 160,000 x g for one hour 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 adding one half volume of a saturated solution of ammonium sulfate in EB to the extract, incubating on ice for 30 minutes, and resuspending the pellet collected at 20,000 x g for 15 minutes at 4° C to an approximate protein concentration of 15 mg/ml. (Protein determination was by Bradford reagent from Biorad Co., using BSA as a standard.) The rest of the extract was adjusted to an ammonium sulfate concentration of 45%, and the resulting precipitate 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 1/10 X protease inhibitors at 4° C before use. For activation, ammonium sulfate cuts were incubated at room temperature with 1mM ATP (Sigma), 50 μg/ml creatine phosphokinase, and 10 mM creatine phosphate.

Labelling with $^{32}PO_4$: Creatine phosphate was labelled with ^{32}P by an exchange reaction to which 5 mCi carrier-free $^{32}PO_4$ (aqueous solution, New England Nuclear) was added (Floyd and Traugh, 1979). The reaction mixture, containing 400 nmoles of creatine phosphate at a specific activity of 4 x 10^6 cpm/pmole, 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 unlabelled 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/pmole,10 mM ADP and 0.5 mg/ml creatine phosphokinase were incubated at room temperature for 5 minutes and then 1 part of this reaction was added to 10 parts of the extract to be labelled.

Analysis of specific activity in $^{32}PO_4$-labelled reactions: Aliquots of activation reactions incubated with ^{32}P-labelled creatine phosphate were incubated on ice with 12 % perchloric acid to precipitate all proteins; samples were spun at 12,000 x g for 30 minutes, and the supernatants were collected and neutralized with KOH. The samples were separated by TLC on a PEI cellulose plate and developed in 1M 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 using firefly lucifierase (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), using 5- 15% acylamide gradient gels with a pH of 9.2. (Acrylamide from Biorad Co.) 2-D NEPHGE gels were prepared and run according to O'Farrell et al. (1977) using ampholines from LKB Co., and a 8.5% acrylamide gel, pH 9.2, for the second dimension. Samples for 2-D gels were lyophilized and resuspended in isoelectric focusing lysis buffer (O'Farrell et al., 1977). $^{32}PO_4$ labelled egg samples were prepared according to Karsenti et al. (1987).

Gel filtration: 0 - 33% ammonium sulfate fractions were applied to a Biosil TSK400 HPLC size exclusion column (7.5 mm x 300 mm; Biorad, Richmond, CA) equilibrated in EB plus 1 mM DTT and 1/10 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 units/μl) at room temperature for 20 minutes, 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 minutes, 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.

Acknowledgements

We are grateful to James Maller for helpful discussions and his gifts of phosphatase inhibitor-1, and S6 kinases I and II. We thank Louise Evans for technical assistance. We would also like to thank the following people for helpful discussions and critical reading of the manuscript: Tina Lee, Andrew Murray, Talma Scherson, Mark Solomon, and Gary Ward. We thank John Gerhart for his enthusiasm, encouragement, and thoughtful suggestions throughout the course of this work. This work was supported by a grant from NIGMS and the American Cancer Society.

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

Table 2-1 Fractionation of inhibitor (INH) activity. 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 minutes each reaction was assayed for MPF as described in Experimental Procedures.

Table 2-2 Effect of phosphatase inhibitors on INH activity. 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. $t_{1/2}$ 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.

Table 2

Effect of phosphatase inhibitors on INH activity

reaction	$t_{1/2}$ (min)
pre-MPF	< 10
pre-MPF + INH (1:2)	140
" + NaF	25
" + Pyrophosphate	140
" + inhibitor 1	> 180
" + inhibitor 1 + MPF	45
" + MPF	45
pre-MPF + inhibitor 1	< 10
pre-MPF + NaF	<10
pre-MPF + INH (1:1)	20
" + inhibitor 1	45

Figure legends

Figure 2-1 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 Materials and Methods) was incubated at room temperature. At 0 minutes, an ATP regenerating system (50 μg/ml creatine phosphokinase and 10 mM creatine phosphate) and partially purified MPF (final concentration 20 units/μl) were added, and at the indicated times, aliquots of the extract were injected into oocytes to assay MPF activity as described in Materials and Methods. The number of units of MPF in 500 nl of extract (the estimated volume of extract equivalent to one oocyte) is plotted. The dotted line, plotted as MPF units/oocyte, represents data obtained by Gerhart et al. (1984). They injected oocytes with 5 units MPF (at time 0) and made small extracts at the indicated time points that were assayed for MPF activity.

Figure 2-2 Amplification of MPF activity in a high speed extract of X. laevis oocytes. A high speed extract of oocytes, as described in Experimental procedures, was incubated at room temperature. At 0 minutes 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 units/μl ("10"), 2.5 units/μl ("2.5"), or .63 units/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.

Figure 2-3 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 minutes 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

units/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 2-4 Reconstitution of MPF-dependent activation of MPF. The 0-33% and 45-55% ammonium sulfate fractions of a X. laevis oocyte extract were incubated together at a 1:1 voume ratio at room temperature with 1 mM ATP, 50 μg/ml creatine phosphokinase, and 20 mM creatine phosphate, with ("pre-MPF + INH + MPF") or without ("pre-MPF + INH") 5 units/μl partially purified MPF. Aliquots of the reactions were taken at the indicated times and injected into oocytes to assay MPF activity.

Figure 2-5 INH does not inactivate MPF. Pre-MPF was activated by a 20 minute incubation with ATP, creatine phosphate, and creatine phosphokinase as described. At 20 minutes, the reaction was split and mixed with an equal volume of INH fraction or EB containing 10 mg/ml BSA and incubated at room temperature. Both samples were subsequently assayed for MPF activity at the indicated times.

Figure 2-6 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.

Figure 2-7 One-dimensional gel analysis of phosphorylation changes associated with pre-MPF activation. Ammonium sulfate fractions of oocyte extracts were incubated with 1mM [γ-^{32}P]ATP, 50 μg/ml creatine phosphokinase, and 20 mM creatine $^{32}PO_4$. Two aliqouts 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 was loaded on the gel. Bands that showed consistent alterations at the time of appearance of MPF activity are labelled A (140 kd), B (115 kd), and C (92 kd). MPF activity in each sample is shown as number of units/μl. Shown is the 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).

Figure 2-8 One-dimensional gel analysis of phosphorylation changes associated with pre-MPF activation. Gel of a reaction that contained the pre-MPF and INH fractions (1:1 mixture) and partially purified MPF (5 units/μl) added at 0 minutes. (Conditions are as described for figure 2-7).

Figure 2-9 One-dimensional gel analysis of phosphorylation changes associated with pre-MPF activation. Gel of a reaction that contained the pre-MPF fraction. (Conditions are as described for figure 2-8).

Figure 2-10 Two-dimensional gel analysis of phosphorylation changes asssociated with pre-MPF activation. Aliquots of reactions were frozen, lyopholized, resuspended in isoelectric focusing lysis buffer, and run on NEPHGE gels. The acidic side of the gel is to the right. ^{32}P labelling is as described for figure 2-7 A gel of the 60 minute time point of a reaction containing the pre-MPF and INH fractions (1:1 mixture by volume) is shown. This sample had <10 units/μl MPF activity. In figures 2-10 to 2-15 the 92 kd protein whose phosphorylation correlates with MPF activity is labelled with arrows. Other phosphorylation differences (that are not labelled), did not consistently correlate with MPF activity in all samples.

Figure 2-11 Two-dimensional gel analysis of phosphorylation changes asssociated with pre-MPF activation. A gel of the 60 minute time point of a reaction containing the pre-MPF and INH fractions (1:1 mixture) and partially purified MPF (5units/μl) added at time 0. This sample had 90 units/μl MPF activity. Conditions were as described for figure 2-10.

Figure 2-12 Two-dimensional gel analysis of phosphorylation changes asssociated with pre-MPF activation. A gel of the 180 minute time point from the reaction described in figure 2-10. This sample had 60 units/μl MPF activity. Conditions were as described for figure 2-10.

Figure 2-13 Two-dimensional gel analysis of phosphorylation changes asssociated with pre-MPF activation. A gel of the 40 minute time point of a reaction containing the pre-MPF fraction. This sample had 120 units/μl MPF activity. Conditions were as described for figure 2-10.

Figure 2-14 Two-dimensional gel analysis of $^{32}PO_4$-labelled unfertilized eggs. Sample was prepared as described in Experimental Procedures.

Figure 2-15 Two-dimensional gel analysis of $^{32}PO_4$-labelled eggs arrested in interphase with cycloheximide. Sample was prepared as described in Experimental Procedures.

Figure 2-16 Separation of pre-MPF and MPF activities on Biosil TSK 400 column. In both panels the curve represents the absorbance at λ 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 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 2-17 Model for role of pre-MPF and INH in vivo. See text for explanation.

Figure 2-1

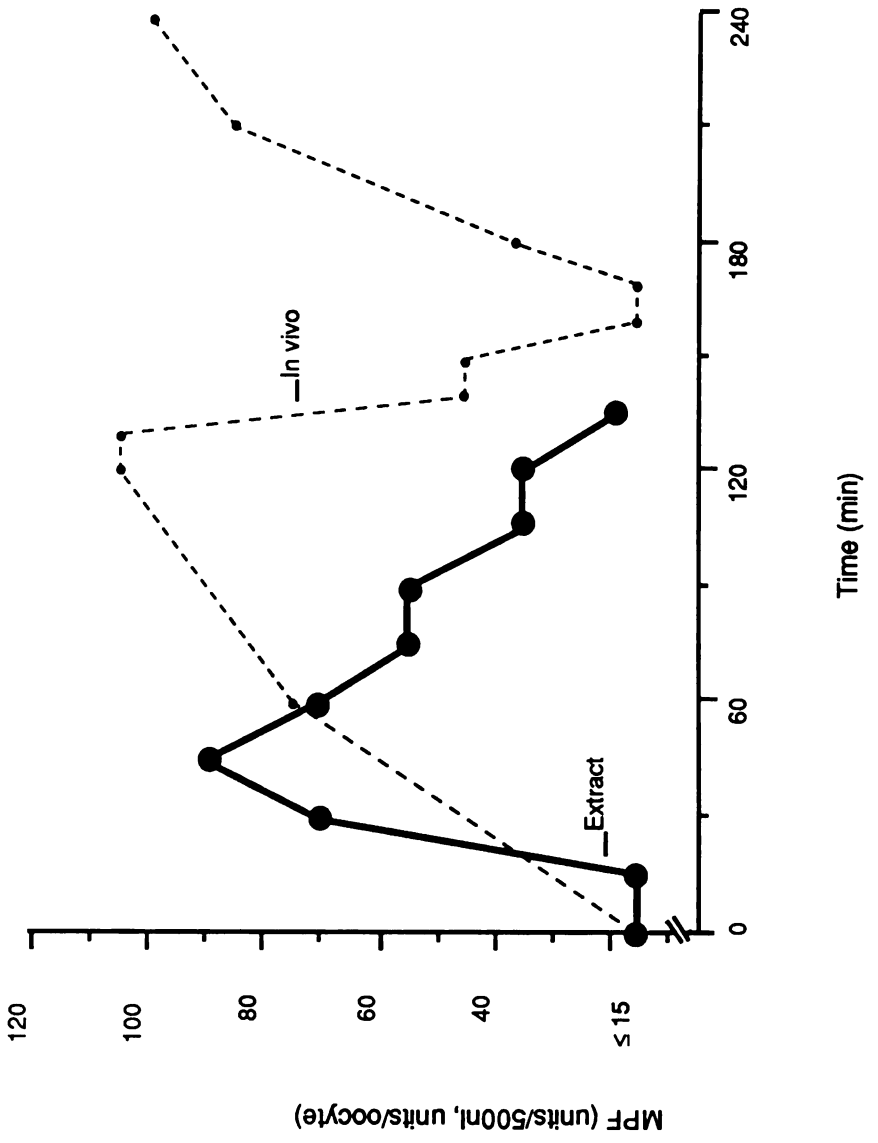

MPF (units/500nl, units/oocyte)
120
100
80
60
40
≤ 15
0
60
120
180
240
Time (min)
In vivo
Extract

Figure 2-2

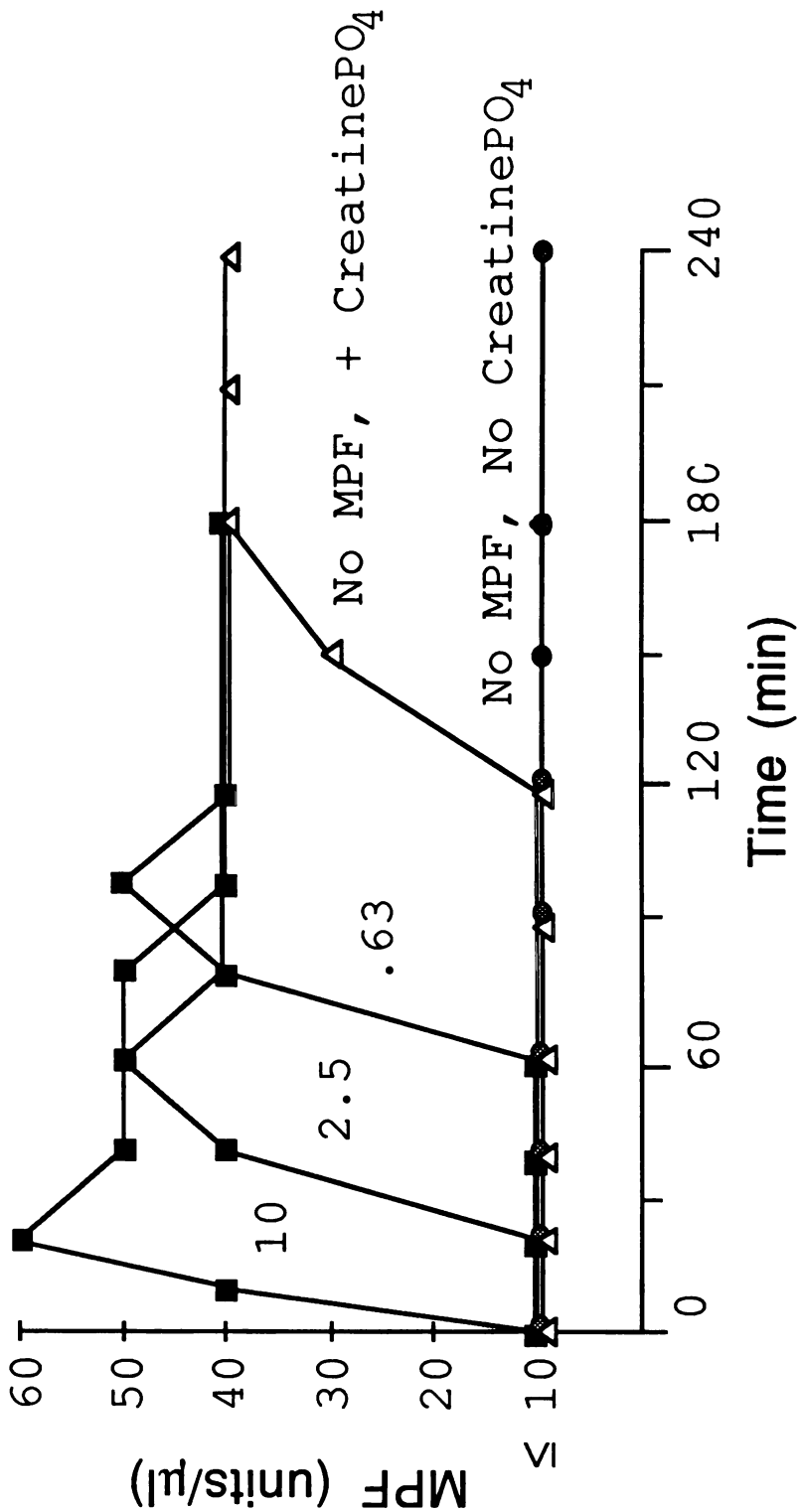

MPF (units/μl)
60
50
40
30
20
10
≤
0
60
120
180
240
Time (min)
No MPF, No CreatinePO4
No MPF, + CreatinePO4
10
2.5
.63

Figure 2-3

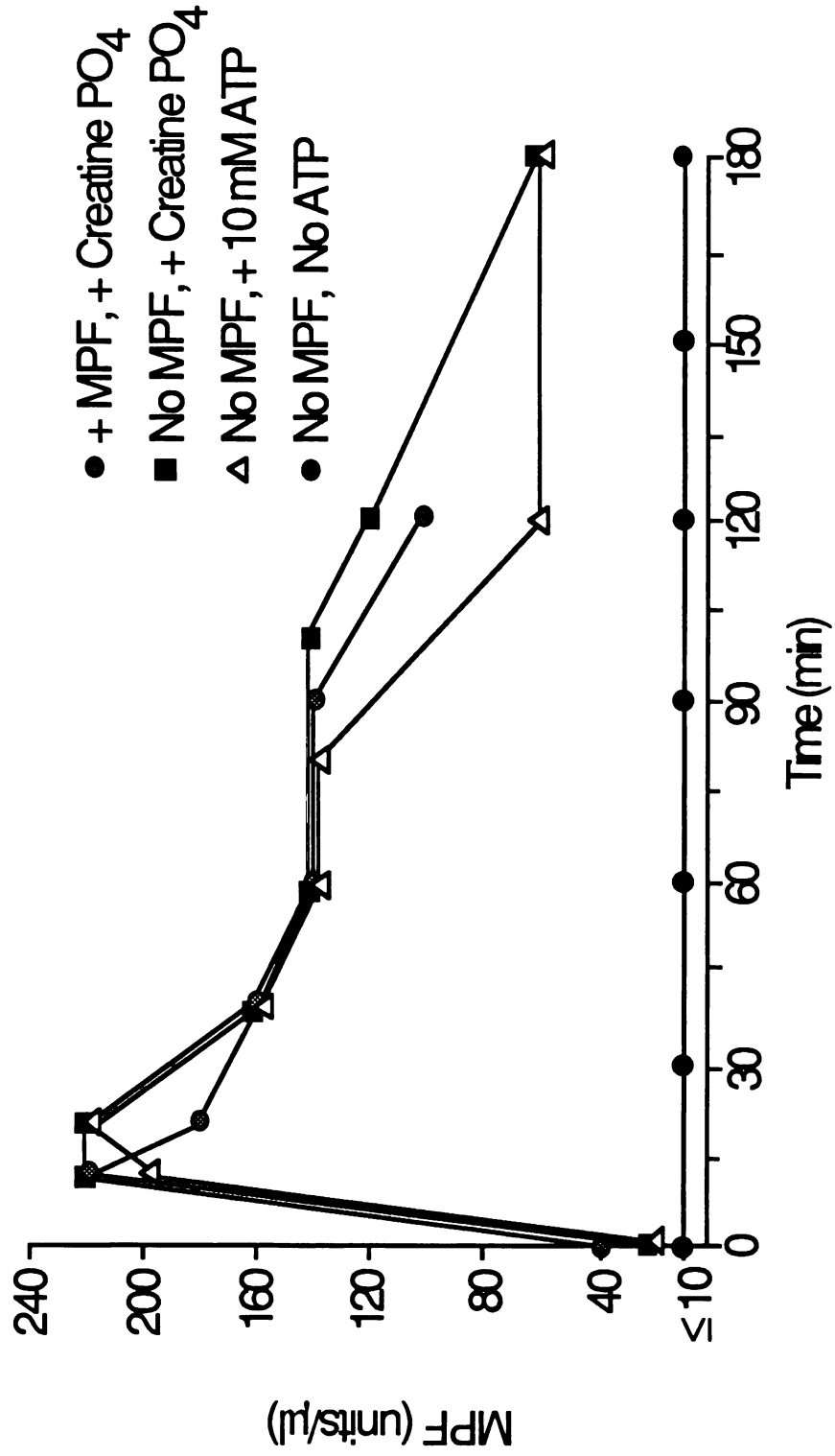

+ MPF, + Creatine PO_4
No MPF, + Creatine PO_4
No MPF, + 10 mM ATP
No MPF, No ATP
MPF (units/μl)
240
200
160
120
80
40
≤10
0
30
60
90
120
150
180
Time (min)

Figure 2-4

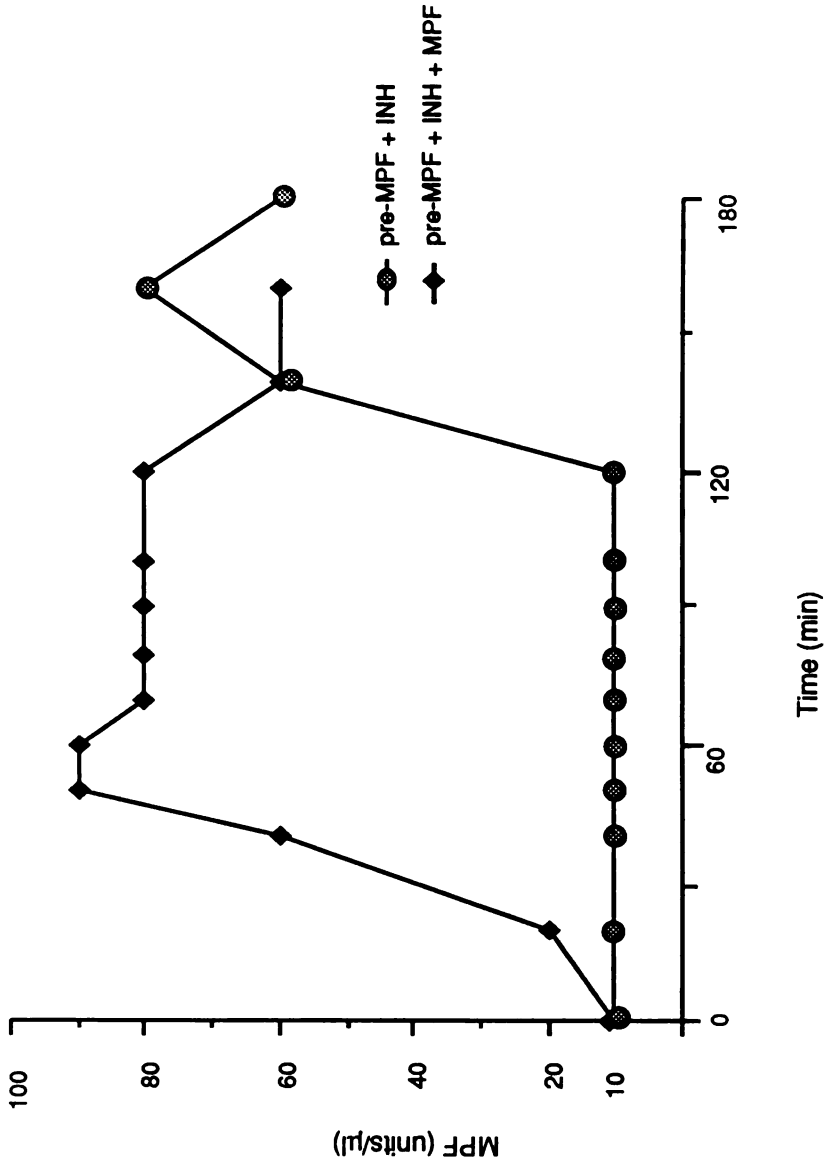

pre-MPF + INH
pre-MPF + INH + MPF
MPF (units/µl)
100
80
60
40
20
10
0
60
120
180
Time (min)

Figure 2-5

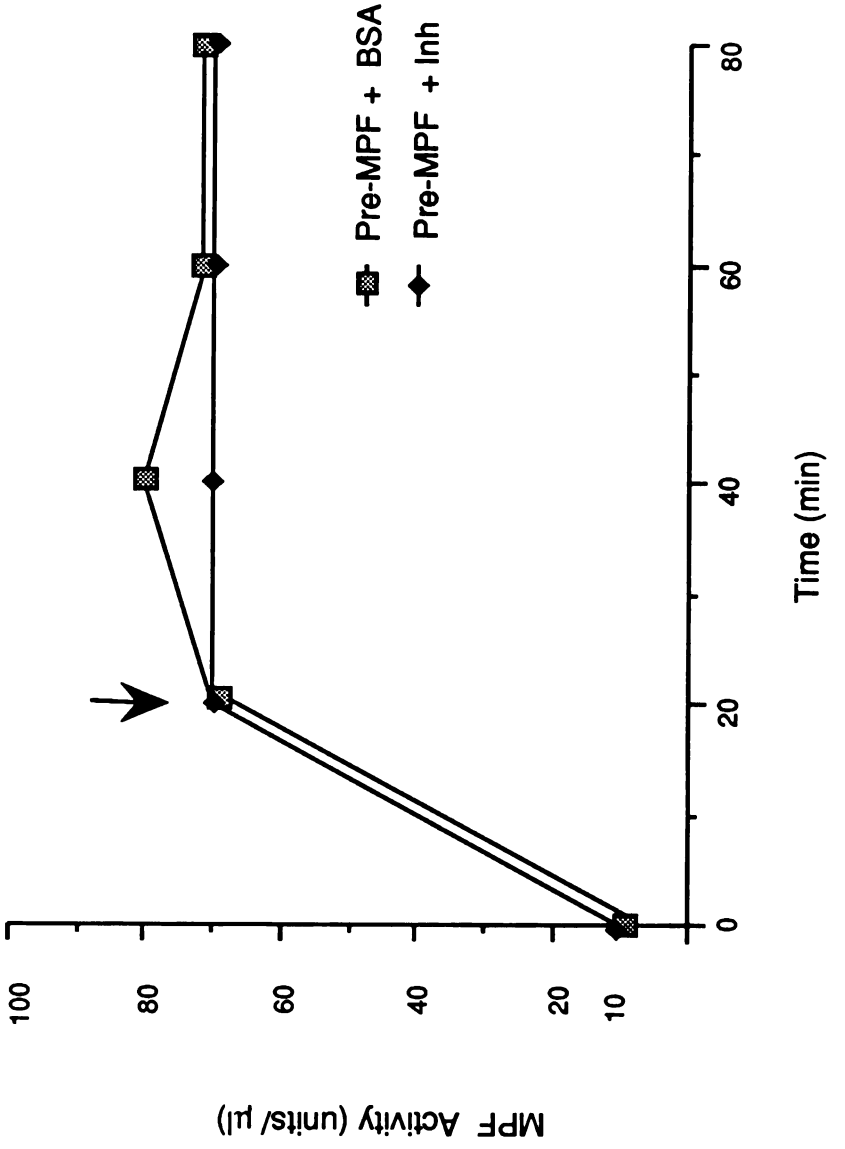

Pre-MPF + BSA
Pre-MPF + Inh
MPF Activity (units/ µl)
100
80
60
40
20
10
0
20
40
60
80
Time (min)

Figure 2-6

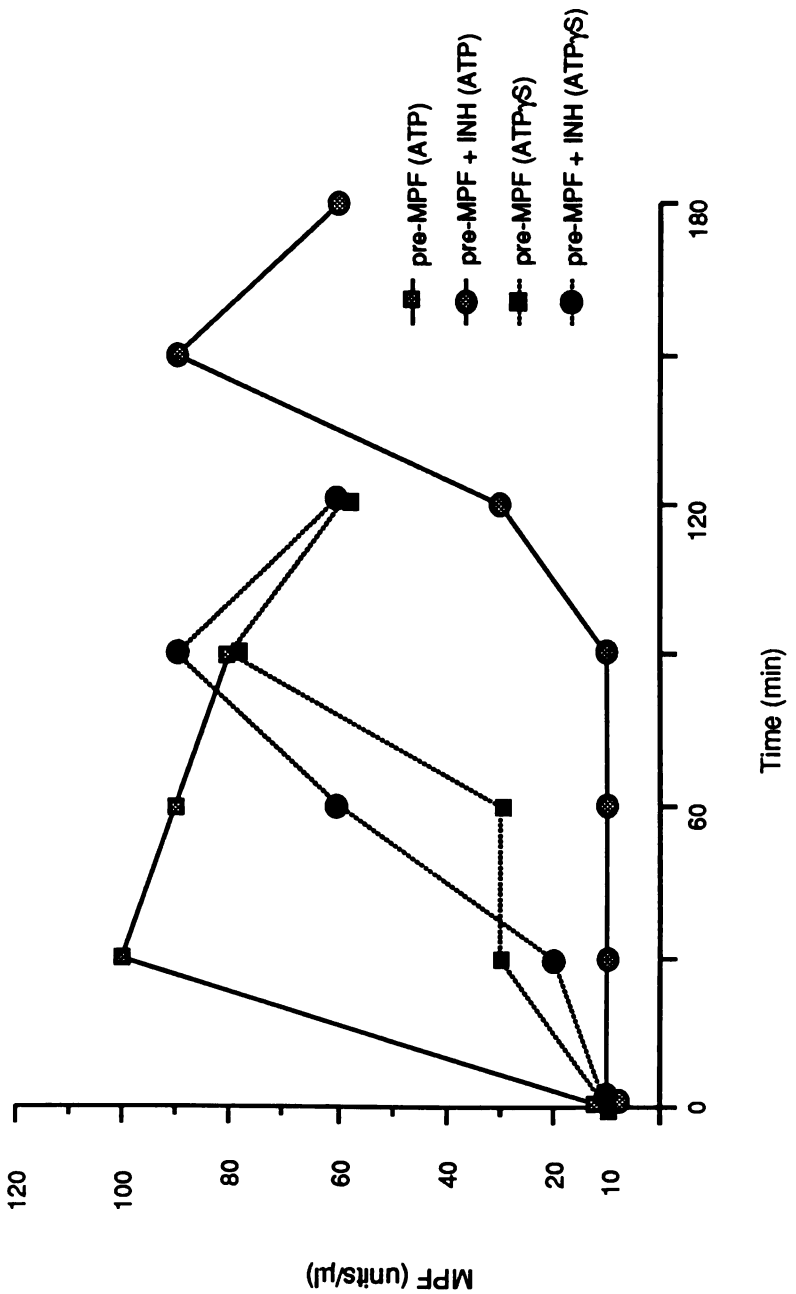

pre-MPF (ATP)
pre-MPF + INH (ATP)
pre-MPF (ATPγS)
pre-MPF + INH (ATPγS)
MPF (units/µl)
120
100
80
60
40
20
10
0
60
120
180
Time (min)

Figure 2-7

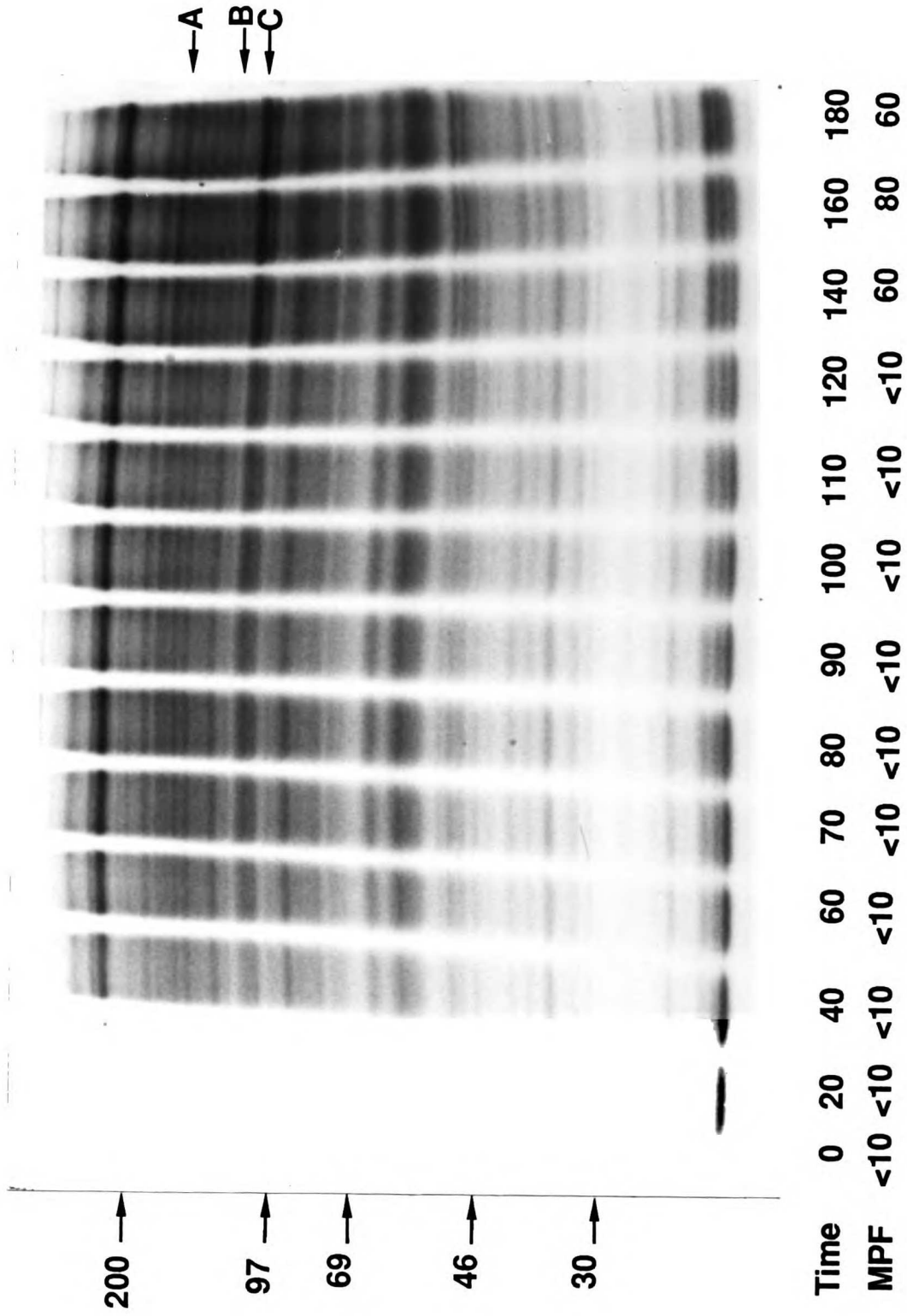

A
B
C
200
97
69
46
30
Time 0 20 40 60 70 80 90 100 110 120 140 160 180 60
MPF <10 <10 <10 <10 <10 <10 <10 <10 <10 <10 60 80 60

Figure 2-8

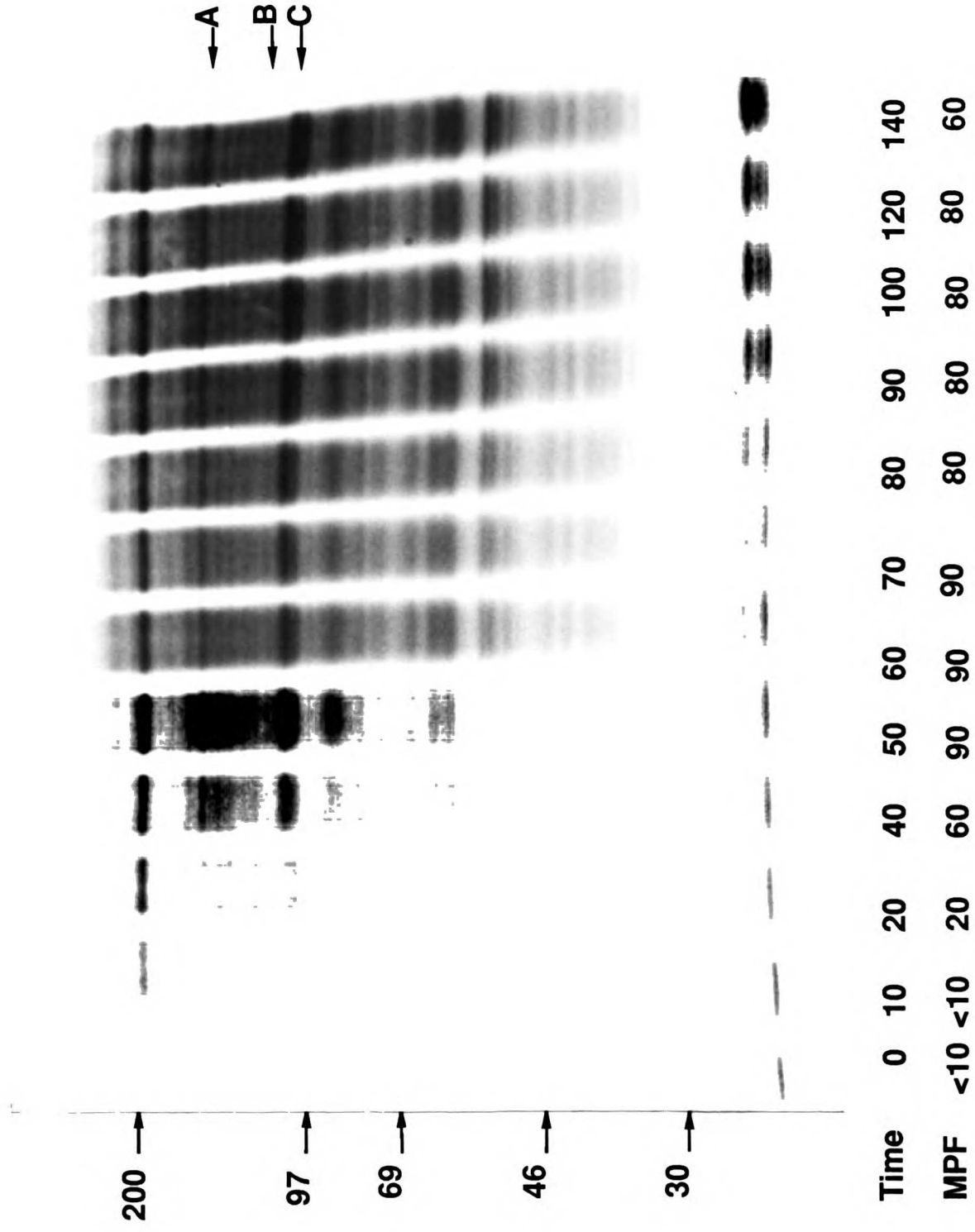

A
B
C
200
97
69
46
30
Time
MPF
0 10 20 40 50 60 70 80 90 100 120 140
<10 <10 20 60 90 90 90 80 80 80 80 60

Figure 2-9

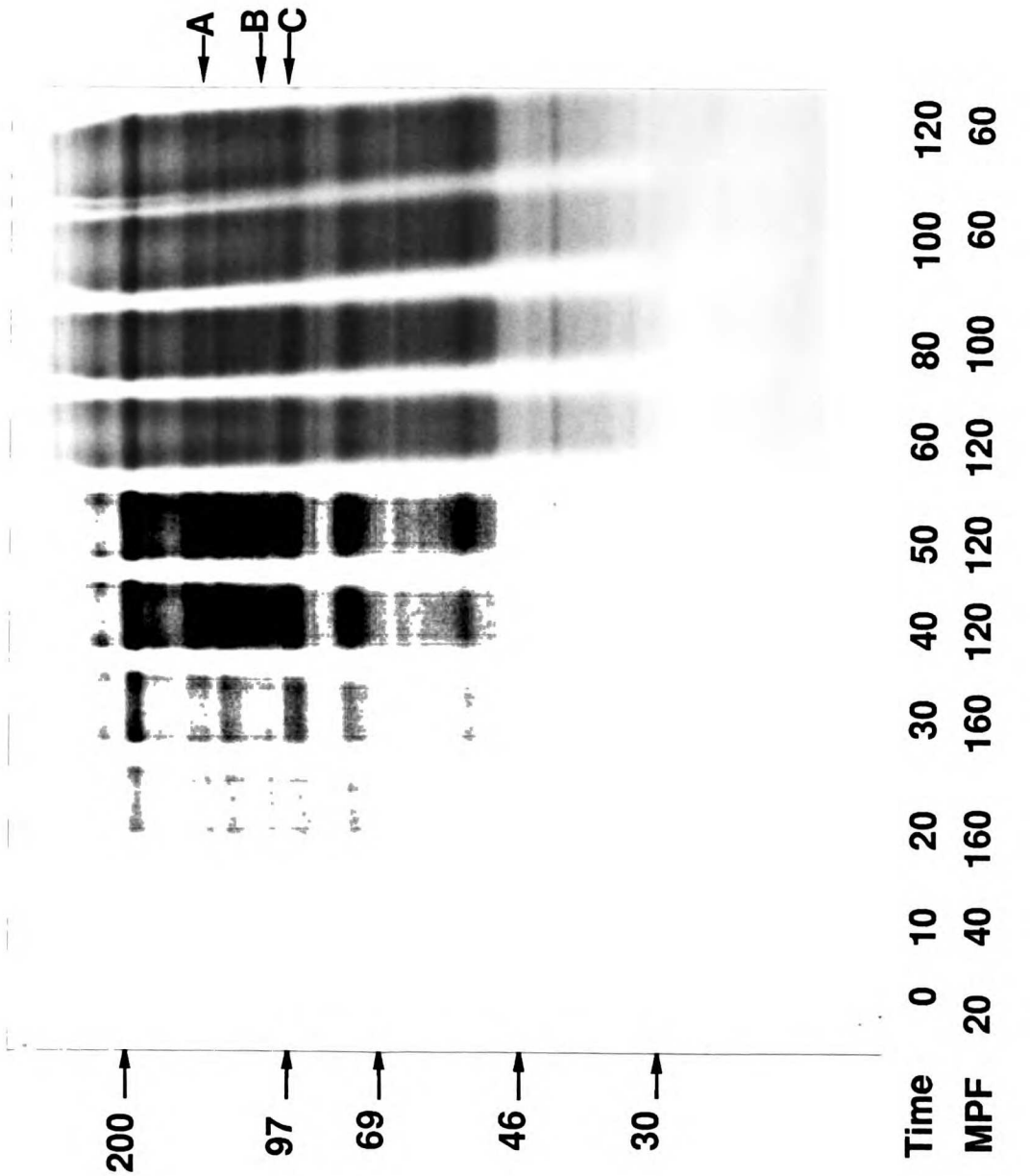
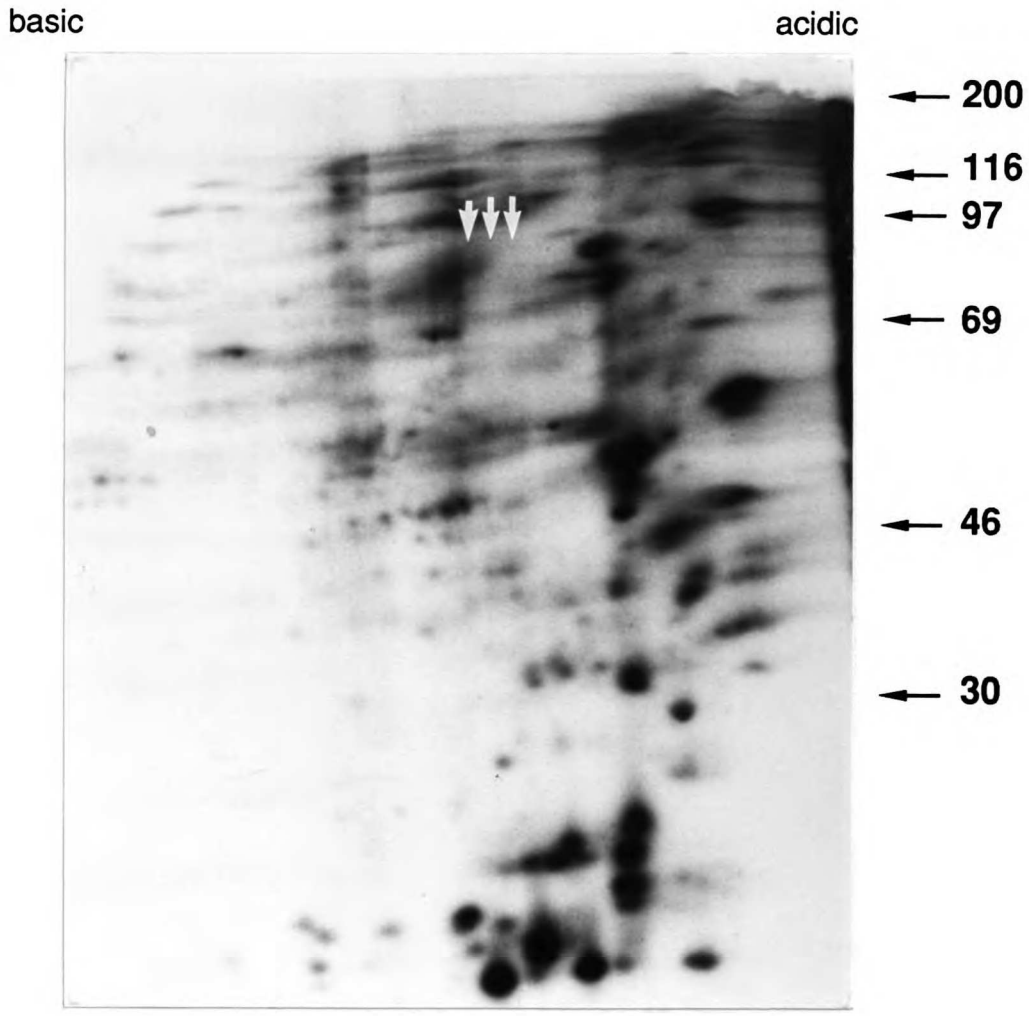
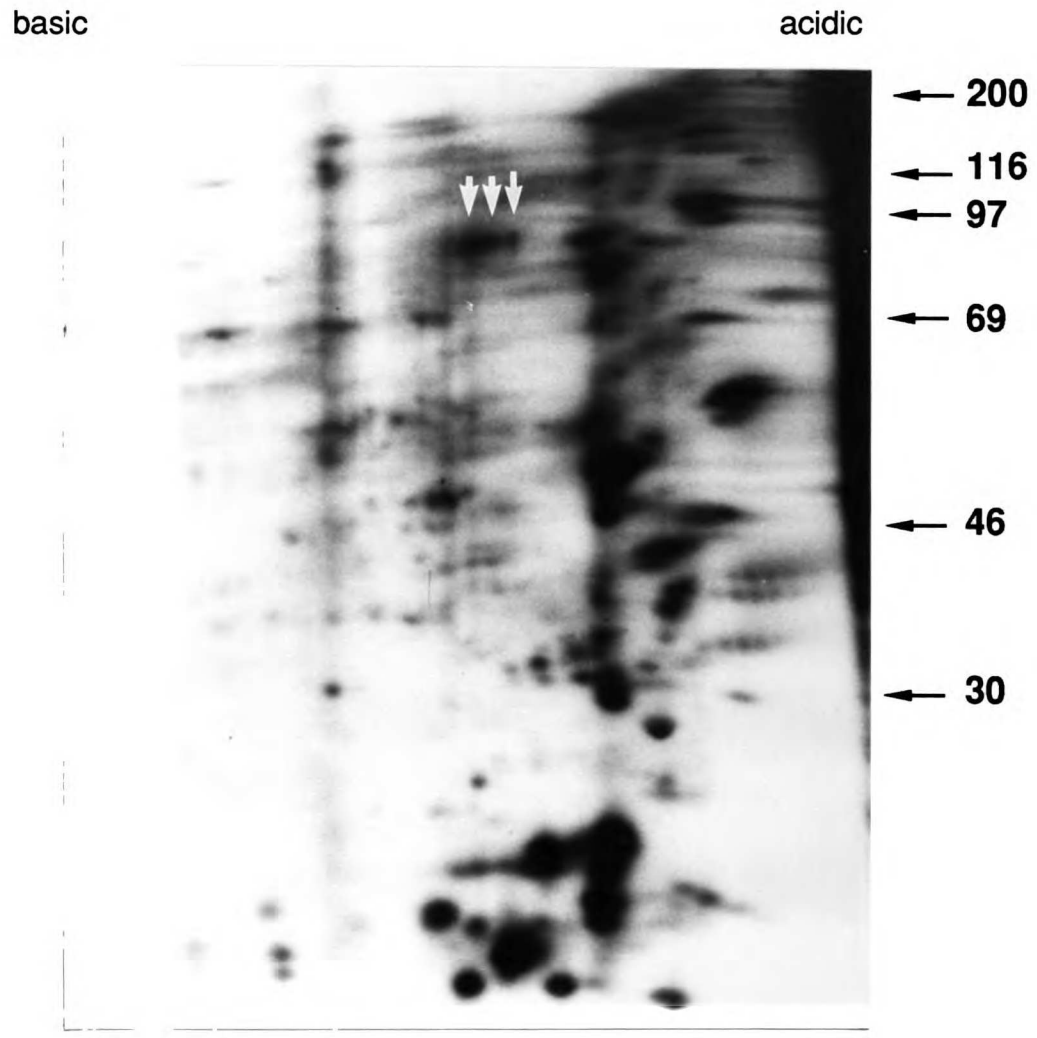

A
B
C
200
97
69
46
30
Time 0 10 20 30 40 50 60 80 100 120 60
MPF 20 40 160 160 120 120 120 100 60 60

Figure 2-10



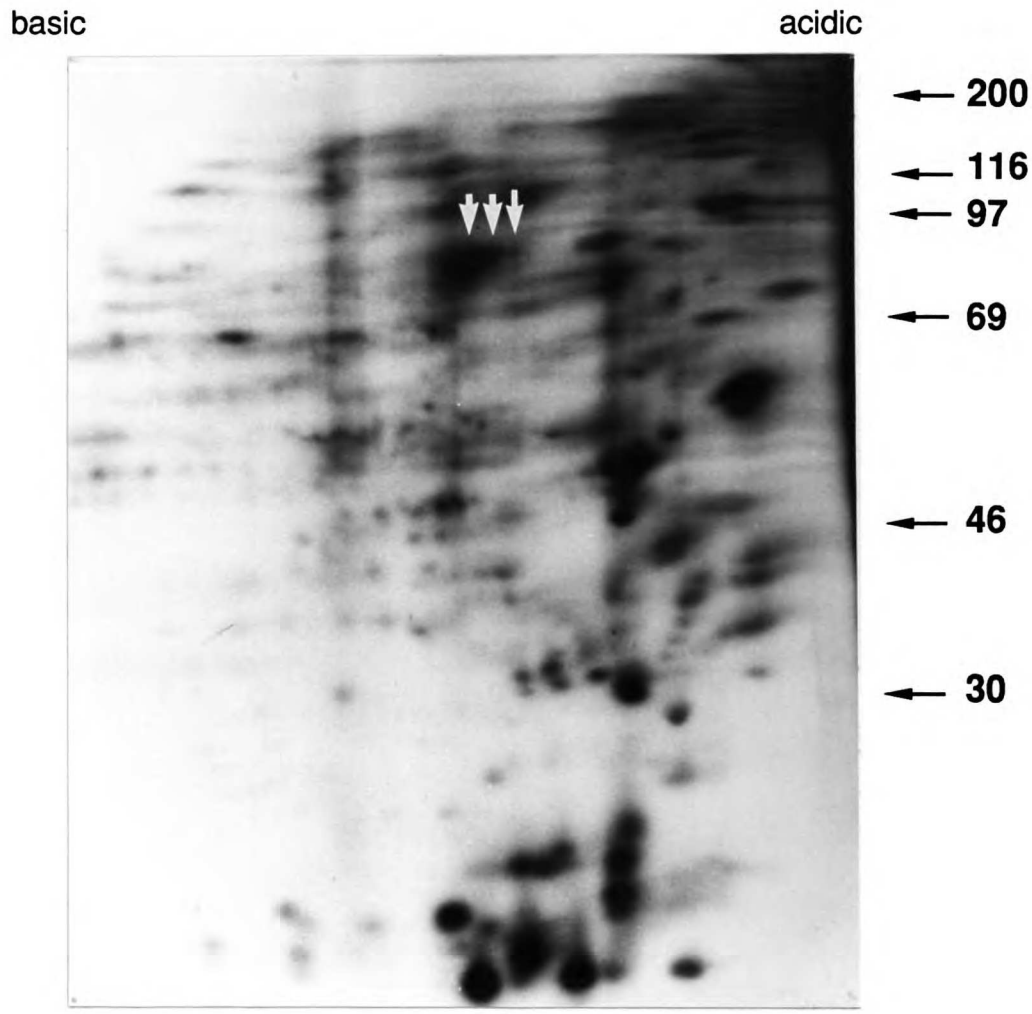
MPF : < 10 units / μl

Figure 2-11



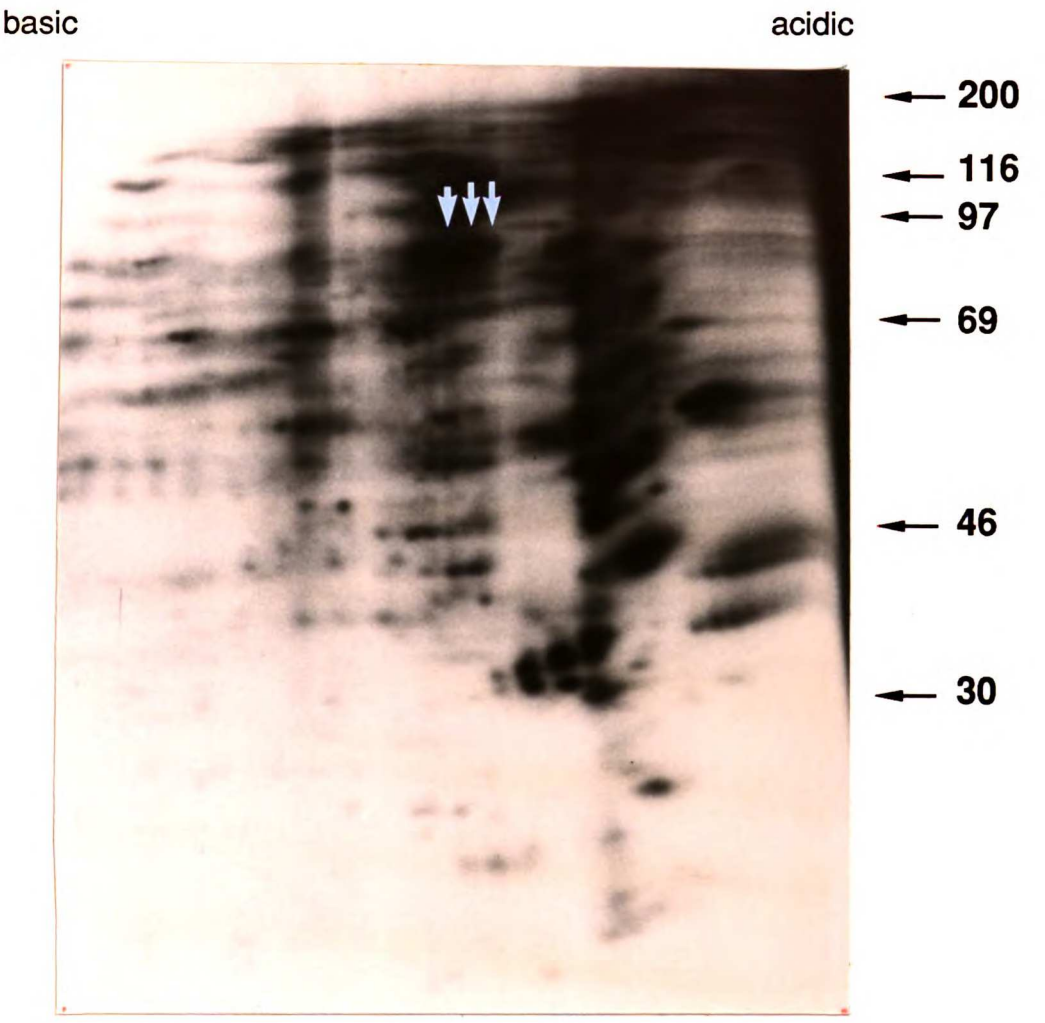
MPF : 90 units / μl

Figure 2-12



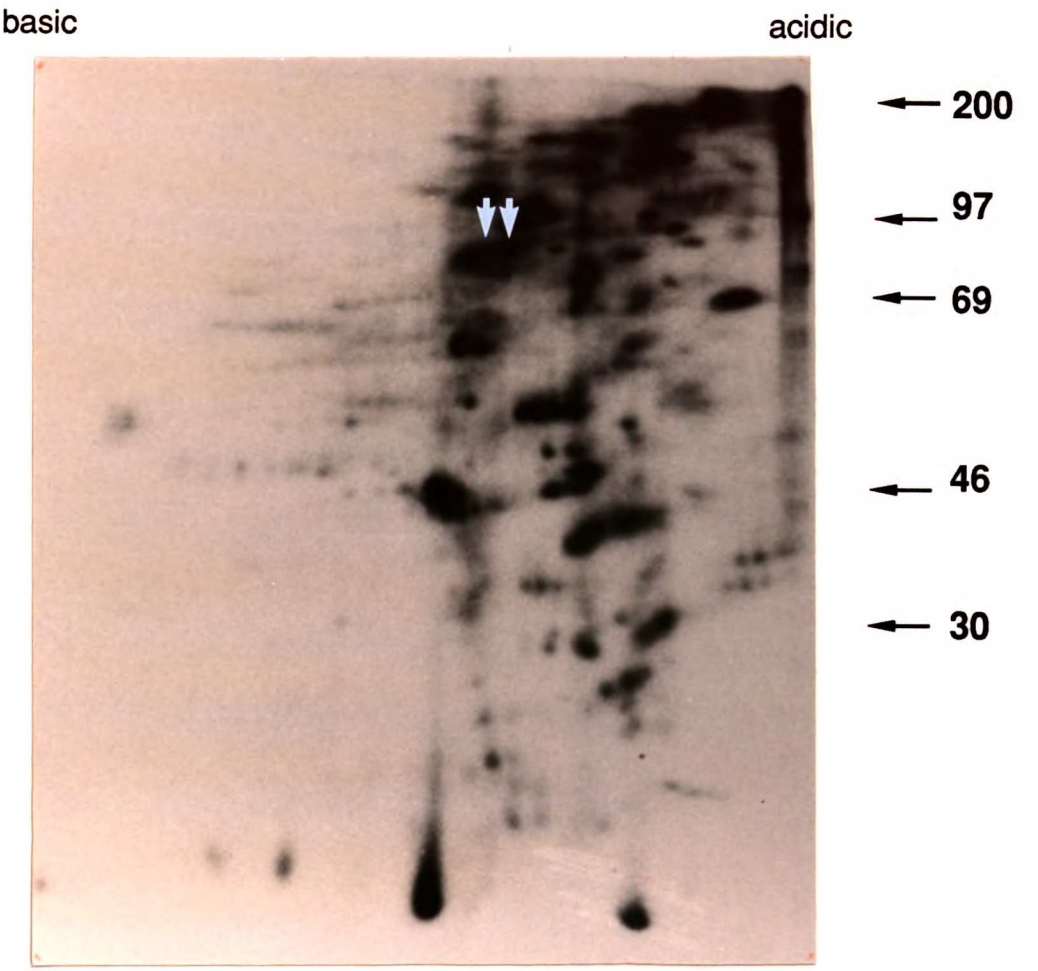
MPF : 60 units / μl

Figure 2-13



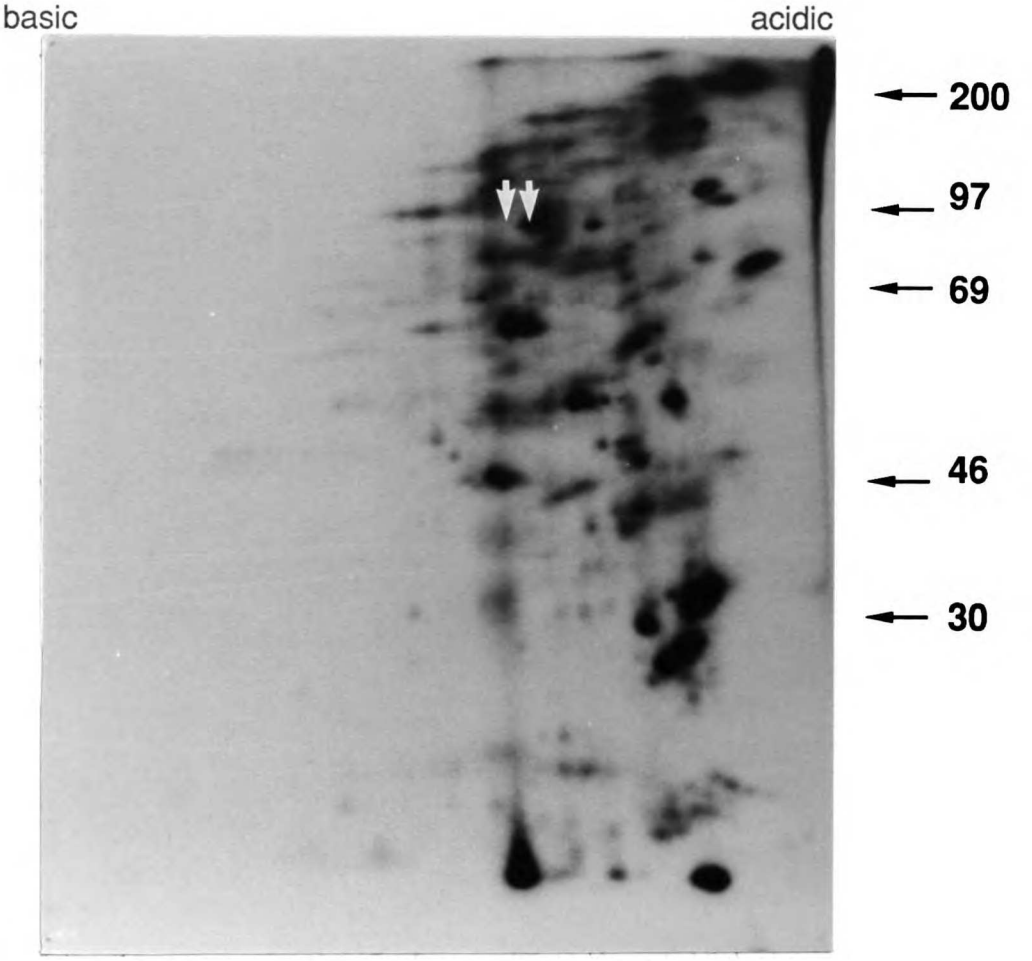
MPF : 120 units / μl

Figure 2-14



unfertilized egg (MPF +)

Figure 2-15



cycloheximide-arrested egg (MPF -)

Figure 2-16a

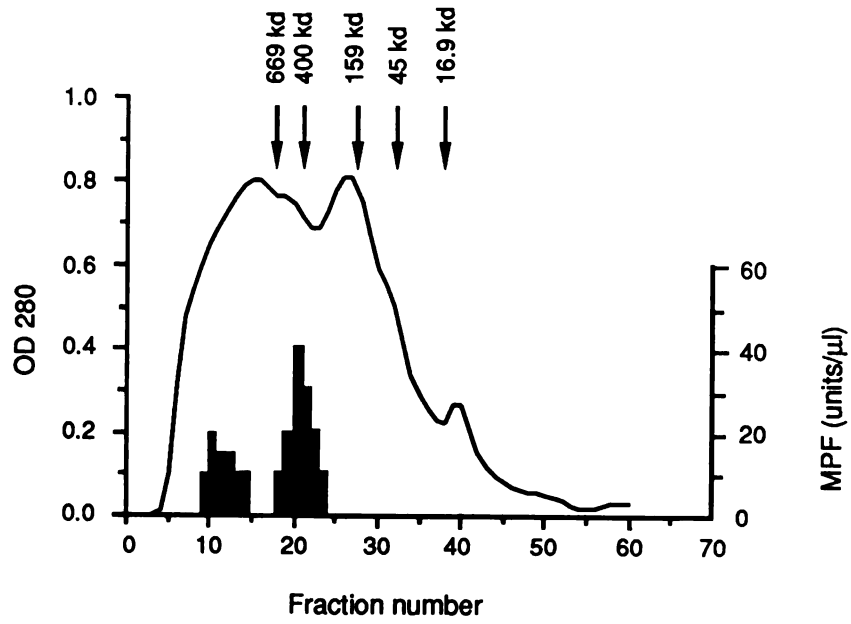

669 kd
400 kd
159 kd
45 kd
16.9 kd
1.0
0.8
0.6
0.4
0.2
0.0
OD 280
60
40
20
0
MPF (units/µl)
0
10
20
30
40
50
60
70
Fraction number

Figure 2-16b

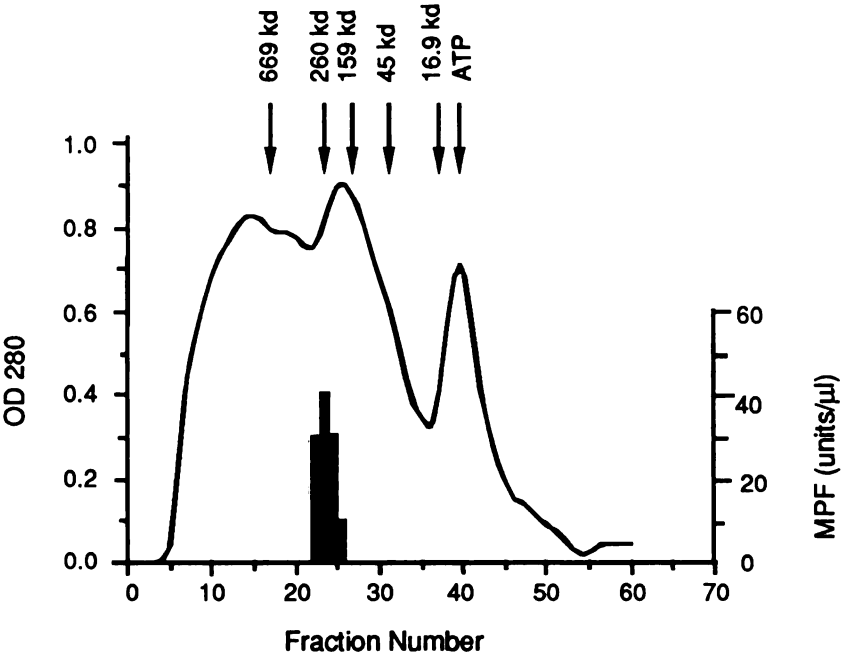
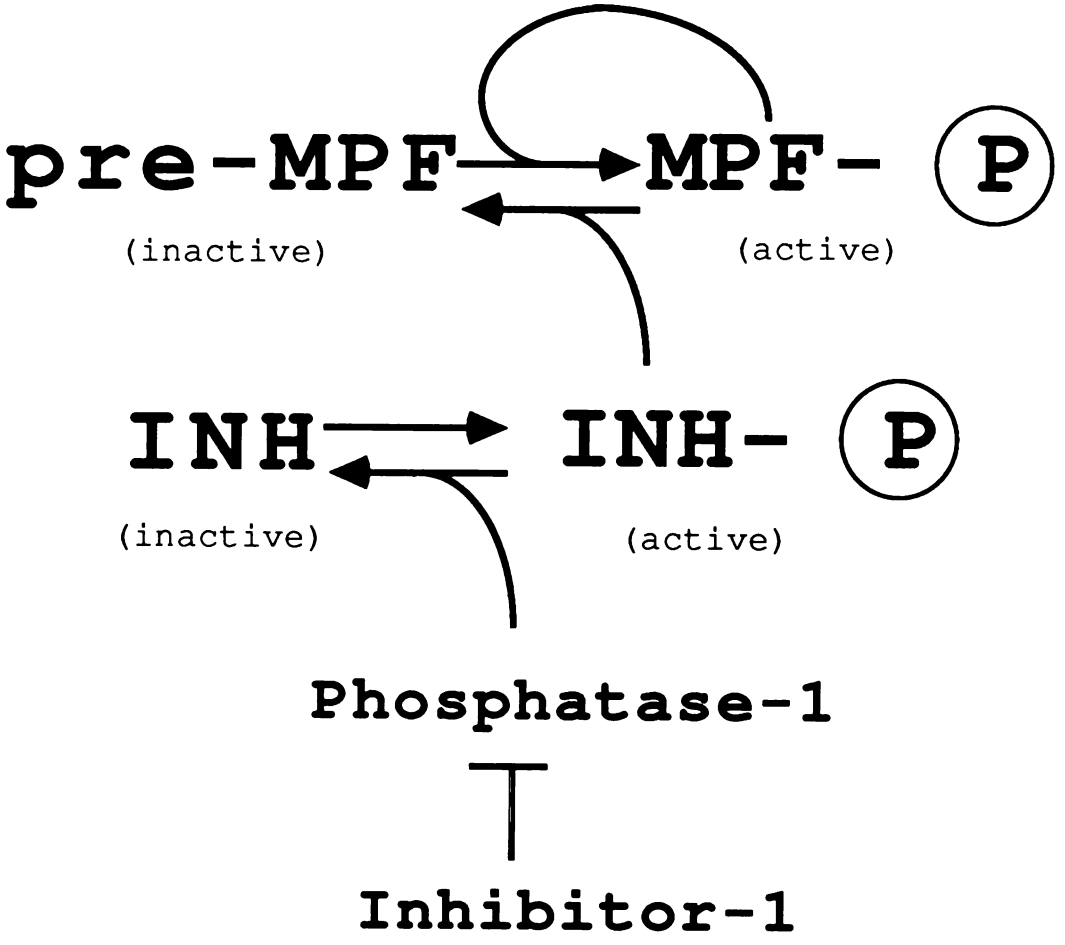

669 kd
260 kd
159 kd
45 kd
16.9 kd
ATP
OD 280
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
Fraction Number
60
40
20
0
MPF (units/µl)

Figure 2-17


pre-MPF
MPF-
P
(inactive)
(active)
INH
INH-
P
(inactive)
(active)
Phosphatase-1
Inhibitor-1

Chapter 3

Isolation and Characterization of Monoclonal Antibodies that Recognize MPF

Abstract

Maturation promoting factor, (MPF), is a crucial regulatory component of the eukaryotic cell cycle. Though it is ubiquitous, MPF has been difficult to purify to homogeneity, and little is known about its physical properties or composition. In an attempt to further characterize and purify this protein, we have isolated five monoclonal antibodies that immunoprecipitate MPF activity, and inhibit the activity in solution. However, all the antibodies, recognize many proteins in partially purified MPF. We have shown that antibody binding is dependent on previous exposure of the preparation to ATPγS. This suggests that the antibodies specifically recognize thiophosphoproteins, although not all thiophosphorylated proteins in MPF are immunoadsorbd. Using one antibody, MPF was partially purified by immunoadsorption chromatography. These experiments provide the first evidence that MPF from Xenopus is a phosphoprotein that becomes thiophosphorylated upon addition of ATPγS.

Maturation Promoting Factor, or MPF, promotes the G2 to M-phase transition in many, perhaps all, eukaryotic cells. MPF was first defined as a component of unfertilized frog egg cytoplasm, which, when injected into oocytes, caused the recipient to undergo the meiotic divisions, or "mature" (Masui and Markert, 1971; Smith and Ecker, 1971). This ability to induce oocyte maturation is used to assay MPF activity. MPF activity oscillates during the meiotic and mitotic cell cycles, appearing at the end of G2, peaking during M-phase, and then dropping to undetectable levels in interphase cells. (Gerhart et al., 1984; Wasserman and Smith, 1978) The unfertilized amphibian egg is naturally arrested at second meiotic metaphase, and is therefore a good source of MPF, whereas the oocyte, arrested in G2, contains no detectable MPF activity.

MPF seems to be a highly conserved component of M-phase 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 budding yeast (Weintraub et al., 1982; Tachibana et al., 1987; chapter 4) all contain MPF activity during M-phase, as assayed by the induction of Xenopus oocyte maturation. Because of its generality, it has been suggested that MPF be referred to as an "M-phase promoting factor", rather than simply a maturation promoting factor (Gerhart et al., 1984).

There is still very little known about MPF on the molecular level. In vitro systems, using extracts of Xenopus eggs, have been developed to study the biochemical effects of MPF addition (Miake-Lye et al., 1985; Lokha and Maller, 1985). When MPF activity is present in these systems, mitotic events such as nuclear envelope breakdown, chromosome condensation, and spindle formation are induced, and when MPF activity decays, nuclei reform and chromosomes decondense, indicating that the transition to S-phase has occurred. These events can be induced in the absence of protein synthesis, indicating that control is at the post-translational level. It is thought that MPF may act through phosphorylation to control the activity of a host of enzymes which then execute specific mitotic events. Though the evidence that supports this idea is largely

circumstantial, one mitotic event, nuclear envelope breakdown, does seem to be regulated by phosphorylation. In vivo and in vitro, the lamin proteins, major structural proteins underlying the nuclear envelope, become hyperphosphorylated during mitosis (Gerace and Blobel, 1980; Miake-Lye et al., 1985), and dephosphorylation seems to be necessary for the nucleus to reform (Burke and Gerace, 1986; Lokha and Maller, 1987).

Phosphorylation has also been implicated as the mechanism of regulation of MPF itself. In oocytes, MPF exists as an inactive precursor that is activated in an ATP-dependent reaction (Cyert and Kirschner, in press; chapter 1). This activation is inhibited by a distinct component that renders the reaction MPF-dependent. This second component appears to be a phosphatase, suggesting that MPF is a phosphoprotein, and that its activity is controlled by competing phosphorylation and dephosphorylation reactions. In extracts, MPF activity is stabilized by phosphatase inhibitors and ATPγS, again indicating that phosphorylation is key to its activity (Wu and Gerhart, 1980). ATPγS is an ATP analog that can substitute for ATP in most phosphorylation reactions, producing thiophosphorylated substrates that are resistant to the action of protein phosphatases (Eckstein, 1985).

MPF has been partially purified from unfertilized eggs of Xenopus laevis (Wu and Gerhart, 1980; Gerhart et al., 1985; Nguyen-gia et al., 1986), and from mitotically arrested HeLa cells (Adlakha et al., 1985), and it behaves as a protein of 100 kd. As MPF has not yet been purified to homogeneity from any source, we decided to take a new approach to its characterization, and attempted to isolate monoclonal antibodies that recognize MPF. Our goal was to define the molecular components of MPF, and potentially to develop a purification for MPF based on immunoadsorption chromatography. Here we report the isolation of several monoclonal antibodies that immunoprecipitate and inhibit MPF activity. All the antibodies described recognize many polypeptides in partially purified MPF preparations, and therefore have limited utility for MPF purification. We investigated the basis for this broad range of specificity further, and found that prior incubation of the preparation with ATPγS is

necessary for binding to the antibodies. The antibodies, therefore, seem to specifically recognize thiophosphoproteins, although we show that not all thiophosphoproteins are recognized. One of the antibodies was used to further purify MPF. Because of the specificity of this antibody, we can conclude that active MPF is a phosphoprotein, strengthening the view that its activity is regulated by phosphorylation.

Results

Isolation of antibodies that recognize MPF

We developed an assay to identify antibodies that react directly or indirectly with MPF. This assay relies on the ability of antibodies to deplete partially purified MPF preparations of activity, but does not require the inactivation of MPF. The mouse antibodies to be tested are bound to a secondary antibody that is coupled to beads. The beads are then washed, incubated with partially purified MPF, and the supernatant from this immunoadsorption is assayed to quantitate MPF activity. One unit of MPF is defined as the amount of material, when injected in a 50 nl volume, that will induce 50 % oocyte maturation (Wu and Gerhart, 1980). A decrease in the activity of this supernatant indicates the presence of antibodies against MPF. As shown in table 3-1A, there is little non-specific loss of MPF activity during these manipulations (14%). This assay was used to screen both polyclonal sera from mice injected with partially purified MPF, and culture supernatants from hybridomas secreting monoclonal antibodies.

Two of five sera from mice injected with partially purified MPF removed MPF activity from the supernatant (data not shown). The spleen from one of these mice was fused to the hybridoma cell line Sp2/0-Ag14 (Shulman, 1978), and nine supernatants from the resulting 230 cultures were found to deplete MPF activity in the assay described. Five of these cultures were successfully subcloned to produce stable, antibody-producing cell lines. The ability of culture supernatants from these cell lines to deplete MPF activity when immobilized on beads via binding to a secondary antibody is

shown in table 3-1A. The antibodies decreased MPF activity by 40 to 50 %, whereas a control antibody isolated from the same fusion (5E5) produced a 14 % decrease. The variation in percentage decrease of MPF may be due to different antibody concentrations in the culture supernatants. Each monoclonal antibody reacted with anti-γ_1 antibodies in Ouchterlony immunodiffusion assays and gave single light and heavy chain bands on SDS PAGE under reducing conditions.

One of these antibodies, 3F3/2, was characterized further. 3F3/2 IgG was partially purified from ascites fluid, and chemically coupled to sepharose beads. These beads were more convenient, gave more reproducible results in immunoadsorption experiments, and were more effective for depletion of MPF activity than the antibody beads described above; this was probably because the 3F3/2-sepharose beads had a higher concentration of 3F3/2 IgG molecules than the beads used in table 3-1A. The use of antibody-sepharose beads to deplete MPF activity is shown in table 3-1B. MPF (200 units/μl) was mixed with an equal volume of buffer, sepharose coupled to (control) monoclonal 5E5 (5E5-sepharose), or 3F3/2-sepharose. There was no loss of activity due to non-specific binding of MPF to the affinity matrix under the conditions used. However, 85% of the MPF activity was bound by 3F3/2 sepharose. Since none of the antibodies we isolated had been selected to inactivate MPF, we tested the ability of ascites fluid containing 3F3/2 (approximately 20 mg/ml IgG) to inhibit MPF activity in solution (table 3-1C). MPF (220 units/μl) mixed 3:1 by volume with a control ascites (5E5) had a activity of 160 units/μl, whereas MPF incubated with 3F3/2 had only 38 % of this activity (60 units/μl), indicating that there was some inhibition by the antibody. When one fifth as much antibody was used, the sample retained 64% of the MPF activity.

Antibodies recognize thiophosphorylated proteins

All five antibodies recognized an identical pattern of polypeptides in partially purified MPF by western blotting (fig. 3-1). Several major bands, ranging in molecular weight from 68 to 230 kd

were recognized. Surprisingly, there was no detectable binding to crude extracts of unfertilized eggs, even after long exposures of the western blot to film (fig.1, panel 5'). Since the partially purified MPF had been enriched only twenty fold, there should have been a weak but discernable reaction with the crude extract compared to equal amounts of protein from the enriched fraction. The only possible explanation for this unusual observation was that the antibody detected an actual difference between proteins in the crude extract and in partially purified MPF. The one major modification that should have occurred to proteins during the purification procedure was thiophosphorylation, due to the addition of ATPγS to the preparation in the purification steps after the crude extract stage. Thiophosphoproteins are resistant to the action of phosphatases, and it is this protection from phosphatases that is thought to stabilize MPF activity. It is difficult to prepare MPF from Xenopus eggs without the addition of ATPγS, and when such preparations are made, they generally have lower activity than those that contain ATPγS.

To test the role of ATPγS in antibody recognition, we tested the ability of the antibodies to react with partially purified MPF that had not been exposed to ATPγS (fig. 3-2). As shown for 3F3/2, there was no reaction on a western blot with MPF made in the absence of ATPγS , and no significant precipitation of MPF activity by the antibody without exposure to ATPγS (table 3-1D). When this MPF was incubated with ATPγS, the antibody gradually reacted with more proteins on a western blot (fig. 3-2). Similar results were obtained with all the other anti-MPF antibodies (data not shown). The antibodies, therefore, seemed to specifically recognize thiophosphoproteins.

To determine if monoclonal antibody 3F3/2 recognized all thiophosphorylated proteins in MPF, or a subset of them, we labelled partially purified MPF with ATPγ^{35}S, and compared the pattern of proteins immunoadsorbd by the antibody with the pattern of overall incorporation of ATPγ^{35}S. As shown in figure 3-3, most, but not all, thiophosphorylated proteins were precipitated by the antibody. For example, there are three prominent thiophosphorylated

polypeptides, approximately 250, 225, and 200kd, that do not seem to bind to the antibody.

Although the antibodies we isolated showed a fairly broad specificity of binding to proteins in partially purified MPF, we explored the use of one of the antibodies, 3F3/2, as an affinity matrix for MPF purification. Preliminary experiments indicated that low pH, high pH, and 0.5 M sodium thiophosphate could release proteins that were bound to the antibody, whereas phosphoserine, phosphothreonine, ATP, NaCl, and $MgCl_2$ could not. Of the three treatments that did elute proteins from the antibody, high pH was the least detrimental to MPF activity. As shown in figure 3-4, we recovered MPF activity from 3F3/2-sepharose using a buffer containing 0.25 M carbonate, pH 10.4, and achieved a three-fold purification. The antibody precipitates approximately 18 % of the protein and 90% of the activity in partially purified MPF, so theoretically a five-fold purification could be achieved by this procedure, but some activity is lost due to exposure of the MPF to high pH. The overall recovery of activity was approximately 20 %.

Discussion

We have described the isolation of antibodies that recognize thiophosphorylated MPF from Xenopus eggs. These are currently the only existing antibodies known to react with MPF. The antibodies inhibit MPF activity in solution and immunoadsorb the activity. Furthermore, we showed that MPF activity can be recovered after binding to at least one of the antibodies. The fact that the antibodies both deplete and directly inhibit MPF activity suggests that the antibodies bind directly to MPF, rather than either binding to a protein that associates with MPF, or to an accessory factor that affects the activity of the MPF protein. The antibodies were not specific for MPF since they reacted with many polypeptides in the MPF preparation; binding was dependent on thiophosphorylation of the antigen via ATPγS. It has been suggested previously, based on the observations that MPF activity is enhanced by ATP and stabilized by phosphatase inhibitors, that MPF is a phosphoprotein whose

activity is affected by its phosphorylation state (Wu and Gerhart, 1980). The findings described in this report, however, provide the only direct evidence that active MPF from Xenopus eggs is phosphorylated, and becomes thiophosphorylated in the presence of ATPγS. Recent experiments with Xenopus oocytes support the idea that MPF activition requires phosphorylation (Cyert and Kirschner, in press; chapter 1).

Not all thiophosphorylated proteins in the enriched MPF fraction react with the antibody. This raises the possibility that the antigenic determinant is more specific than thiophosphate, and that proteins that bind to the antibody share a common site of (thio)phosphorylation. A monoclonal antibody has been described that seems to recognize a phosphorylation site that is shared by several proteins, and in that case the modification is specific to M-phase (Davis et al., 1983). The antibody that we describe does not seem to be M-phase specific, since extracts of Xenopus eggs arrested in interphase and incubated with ATPγS react with the antibody (data not shown). Still, the proteins that react with the antibody might all be substrates of a particular kinase with a characteristic site of thiophosphorylation. Alternatively, some thiophosphoproteins may be sterically hindered from binding to the antibody, or have local charges that interfere with antibody binding. For example, antibodies that bind phosphotyrosine quantitatively fail to bind all proteins containing phosphotyrosine in a cell (Frackelton et al., 1983).

Because of the broad specificity of the antibody, it did not prove to be particularly useful for the characterization or purification of MPF at this stage, since we achieved only a three fold purification with poor recovery of activity. Still, we have demonstrated that MPF activity can be recovered after binding to monoclonal antibody 3F3/2, and at a more advanced stage of MPF purification, this affinity step may prove to be useful.

The antibody 3F3/2 reacts with thiophosphorylated proteins from other organisms. It has been used with spindle preparations from diatoms (Wordeman and Cande, 1987) and sea urchins (Kuriyama, 1987), bovine brain microtubules (data not shown), and

kangaroo rat cells in culture (personal communication, Tim Mitchison). The antibody has proved to be a useful tool in these other systems for specifically localizing a subset of proteins that become thiophosphorylated upon exposure to ATPγS. It has been used to identify proteins by indirect immunoflourescence and western blotting, and could potentially be used as a purification step for these thiophosphorylated proteins.

Experimental Procedures

MPF and MPF assays. MPF was partially purified from extracts of unfertilized eggs as described by Wu and Gerhart (1980), with the following modifications (Wu and Gerhart, unpublished): The ammonium sulfate pellet was resuspended with buffer containing 0.3 mM ATPγS; phenyl agarose (Sigma Chemical Co.) was used in place of pentyl agarose; and the arganine agarose step was omitted and replaced with a fractional precipitation with 7.5% polyethylene glycol 6000 (MCB reagents). To prepare MPF in the absence of ATPγS, extracts were precipitated with 33% ammonium sulfate, resuspended without any nucleotide, and then precipitated with 7.5 % polyethylene glycol. To assay MPF, Xenopus laevis females (Xenopus One, Ann Arbor, MI), primed two days earlier with 25 units of pregnant mare's serum gonadotropin (Diosynth, Inc., Chicago, Ill.), were anaesthetized with 0.3% 3-amino benzoic acid ethyl ester (tricaine, Sigma Chemical Co., St. Louis, MO) prior to surgical removal of a section of ovary. Ovary was maintained in a modified Barth's saline developed by Gurdon and Laskey (1970), which was further modified by adding 10 mM NaHepes, pH 7.4 instead of Tris as the buffer. Stage 6 oocytes were hand-dissected 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 1/2 to 2 hours after injection and 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 unit/50 nl (Wu and Gerhart, 1980).

Mice and immunizations. Female 8-week-old BALB/c mice from Simonsen (Gilroy, CA) were immunized intraperitoneally and in their footpads with 100 μg phenyl-agarose-purified MPF emulsified in complete Freund adjuvant. Over the next six months the mice were injected intraperitoneally four more times with this antigen emulsified in incomplete Freund adjuvant. Three days before fusion, one mouse was injected intravenously with 100 μg of antigen.

Hybridoma and monoclonal antibody production. Spleen cells from the immunized mouse and Sp2/0-Ag14 (Shulman, 1978) cells were fused according to the protocol devised by Fazekas de St. Groth and Sheidegger (1980). The fusion was plated out in five 96-well microtiter dishes, and two weeks later, supernatants were withdrawn and tested for immunoadsorption of MPF as described below. Supernatants that tested positive were rescreened, and positive clones were transferred to 24 well dishes. The supernatants were tested again at this stage, and positive cultures were cloned by limiting dilution (Mishell, 1980) to establish permanent cell lines. Cells were maintained in RPMI 1640 plus 10% heat inactivated fetal calf serum, 50 μg/ml gentamycin, 1 mM Na pyruvate, 50 μM β-mercaptoethanol, and 10 mM Hepes, pH 7.4. Cloned hybridoma cells producing anti-MPF antibody were grown as an ascites in BALB/c mice (Mishell, 1980), and antibody was purified by precipitation with 50% ammonium sulfate. Ammonium sulfate fractions were coupled to CNBr-activated sepharose 4B (Pharmacia, Uppsala, Sweden) at a concentration of 2.5 mg IgG/ ml of packed gel (Pharmacia manual). IgG concentration was determined by comparing samples of purified ascites to an IgG standard by densitometry of coomassie stained SDS PAGE gels.

Immunoadsorption of MPF. To screen polyclonal and monoclonal antibodies, 2 μl of serum or 50 μl of culture supernatant was incubated with 100 μl of a suspension of polyacrylamide beads coupled to a rabbit anti-mouse immunoglobulin antibody (#170-5104, Biorad, Richmond, CA)) for two hours at 4° C. The beads were washed twice with MPF extraction buffer (Wu and Gerhart, 1980) (EB:15 mM $MgCl_2$, 80 mM β-glycerophosphate, 20 mM NaEGTA, pH 7.3) and once with EB containing 1mM DTT, 0.3 mM ATPγS (Boehringer Mannhein), and protease inhibitors (25 μg/ml leupeptin, and aprotinin, 0.5 mM PMSF, 1mM Benzamidine HCL, 10 μg/ml pepstatin). The beads were pelleted by a five minute spin in an eppindorf centrifuge, all buffer was removed, and 5μl of partially purified MPF (diluted to approximately 100 units/μl) was added. After a two-hour incubation with agitation at 4° C, the supernatant was removed and assayed for MPF activity. When monoclonal

antibodies coupled to sepharose were used for immunoadsorption, the following modifications were used: the antibody-sepharose matrix was stored with 0.5 mg/ml β–lactoglobulin, washed twice with EB containing 0.5 M NaCl, and once with EB containing 0.2 M NaCl, 1mM DTT, protease inhibitors, and 0.3 mM ATPγS before adding MPF which had been adjusted to a final concentration of 0.2 M NaCl. These conditions minimized non-specific binding of protein to the matrix.

Purification of MPF using 3F3/2-sepharose. Typically, 20 µl of a 50 % suspension of 3F3/2-sepharose (prepared as described above) was incubated with 10 µl of partially purified MPF (400 units/µl) for two hours at 4° C. The sepharose beads were then washed three times with EB plus 0.2 M NaCl, and twice with 1/2 strength EB. 200 µl of elution buffer (250 mM sodium carbonate, 10 mM EGTA, 7.5 mM MgCl2, 5mM NaF, 1mM DTT, protease inhibitors, 0.5 mg/ml β–lactoglobulin, pH 10.4) was added to the beads, and they were agitated at 4° C for 20 minutes. The supernatant was removed, neutralized, and an equal volume of saturated ammonium sulfate (at room temperature) in EB was added. After two hours on ice, the precipitated proteins were collected by a 15 minute centrifugation in an eppindorf centrifuge, and resuspended in 10 µl water. This eluted sample was dialyzed against EB plus 1mM DTT, protease inhibitors and 0.3 mM ATPγS before assay. It was impossible to quantitate how much protein was bound to the 3F3/2-sepharose beads or eluted from the beads by a standard protein assay because of the presence of the carrier protein, β–lactoglobulin. Therefore the protein content of various samples was compared by densitometry of silver-stained samples (Merril et al., 1981) after SDS PAGE.

Labelling of MPF with ATPγ-^{35}S. ATPγ-^{35}S (NEN) was diluted to a specific activity of 50 µCi/ mM and added to MPF made without ATPγS to a final concentration of 1mM. After a one hour incubation at 4° C, the labelled MPF was immunoadsorbed with 3F3/2-sepharose as described above.

Electrophoresis and western blotting. One dimensional electrophoresis was carried out essentially according to Laemmli

(1970), using gels with a pH of 9.2. Western blotting was performed according to Burnette (1981), except that the following buffer was used for transfer from gel to nitrocellulose: 0.15 M glycine, 0.02% SDS. Nitrocellulose filters were incubated with a 200 fold dilution of 3F3/2 ascites, and ^{125}I-labelled (Hunter and Greenwood, 1962) secondary antibody (goat anti-mouse immunglobulins, #0611-3261, Cappell, Malvern, PA). ^{35}S labelled gels were flourographed with Enhance (NEN) before autoradiography.

Table 3-1. Effect of monoclonal antibodies on MPF activity. Immunoadsorptions in A were performed as described in Experimental Procedures, with 100 µl of culture supernatant. For immunoadsorptions in B, equal volumes of MPF (200 units/µl) and a 50% (v/v) suspension of antibody sepharose, prepared as described, were incubated at 4° C for 2 hours. (Final concentration of IgG was approximately 0.6 mg/ml) For inhibition (C), one volume of ascities fluid, undiluted (20 mg/ml IgG) or diluted five fold with PBS (4 mg/ml IgG), was mixed with three volumes of MPF (220 units/µl). After a two-hour incubation at 0° C, the samples were assayed for MPF. Immunoadsorptions in D were performed as for B, except that the MPF used (120 units/µl) was made without exposure to ATPγS, as described.

Table 3-1

A. Immunoadsorption of MPF activity with culture supernatants bound to beads

	MPF	% activity
MPF + buffer	80 units/μl	100
" + 5E5	70 units/μl	86
" + 3F3/2	40 units/μl	50
" + 2A3/11	40 units/μl	50
" + 3F6/25	50 units/μl	63
" + 5B4/33	40 units/μl	50
" + 2B9/44	40 units/μl	50

B. Immunoadsorption of MPF activity with 3F3/2-sepharose

	MPF	% activity
MPF + Buffer	100 units/μl	100
" + 5E5-sepharose	100 units/μl	100
" + 3F3/2-sepharose	15 units/μl	15

C. Inhibition of MPF activity

	MPF	% activity
MPF + 5E5 (5 mg/ml)	160 units/μl	100
" + 3F3/2 "	60 units/μl	46
" + 5E5 (1 mg/ml)	160 units/μl	100
" + 3F3/2 "	100 units/μl	63

D. Immunoadsorption of MPF made without ATPγS

	MPF	% activity
MPF + 5E5-sepharose	60 units/μl	100
" + 3F3/2-sepharose	50 units/μl	83

Figure legends

Figure 3-1. Binding of monoclonal antibodies to Xenopus egg crude extracts and partially purified MPF. Crude extract (C) and partially purified MPF (P) were electrophoresed on a 8.5% polyacrylamide gel and western blotted with culture supernatants from cell lines 2B9/44 (1), 5B4/33 (2), 3F6/25 (3), 2A3/11 (4), and 3F3/2 (5). Panel 5' shows a ten times longer exposure of the blot shown in 5. Molecular weight estimates are expressed in kilodaltons.

Figure 3-2. Binding of antibody to partially purified MPF incubated with ATPγS. MPF (160 units/μl) was partially purified without ATPγS as described in Experimental Procedures, and incubated at room temperature with 5 mM ATP or ATPγS for the times indicated. Samples were electrophoresed on a 5-15% gradient gel and western blotted with monoclonal 3F3/2. A coomassie stained lane of the gel before transfer to nitrocellulose is shown at right (C). Molecular weight estimates are expressed in kilodaltons.

Figure 3-3. Antibody does not bind to all thiophosphorylated proteins in MPF. MPF partially purified without ATPγS was labelled with ATP-γ-^{35}S and immunoadsorbed with 3F3/2-sepharose as described in Experimental Procedures. Shown are the total (T) labelling pattern of MPF, as well as the pellet (P) and supernatant (S) from the immunoadsorption. Samples were electrophoresed on a 7% gel which was fluorographed before autoradiography. Molecular weight estimates are expressed in kilodaltons. (Positions of molecular wight markers are not shown.)

Figure 3-4. Purification of MPF with 3F3/2-sepharose. MPF was bound to and eluted from 3F3/2-sepharose as described in Experimental Procedures. Equal amounts of MPF activity before immunoadsorption (I, input) and after elution (E) were electrophoresed on a 7% gel and silver stained. The input contains 3 times more protein than the eluted sample as estimated by densitometry of both samples. Molecular weight estimates are expressed in kilodaltons.

Figure 3-1

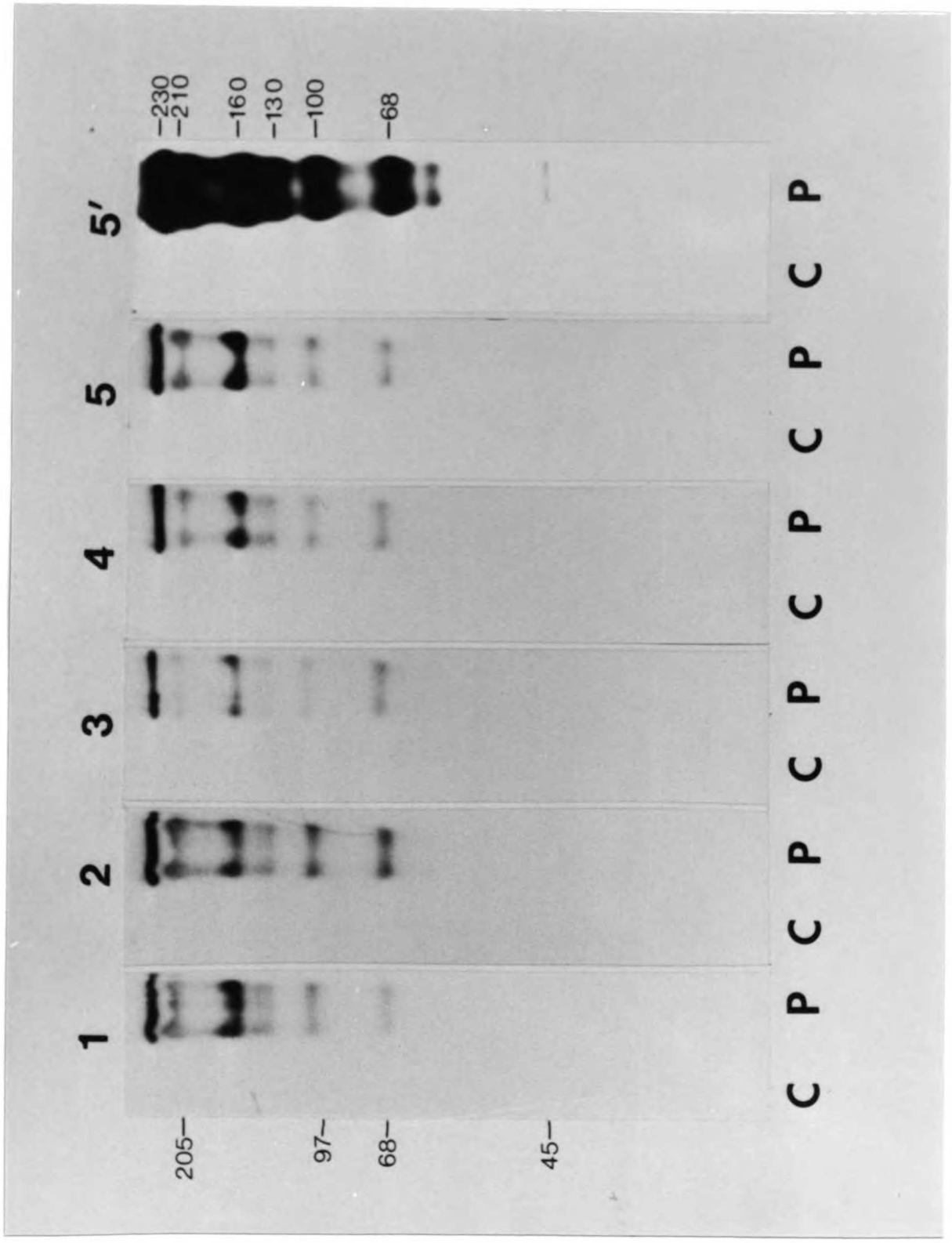
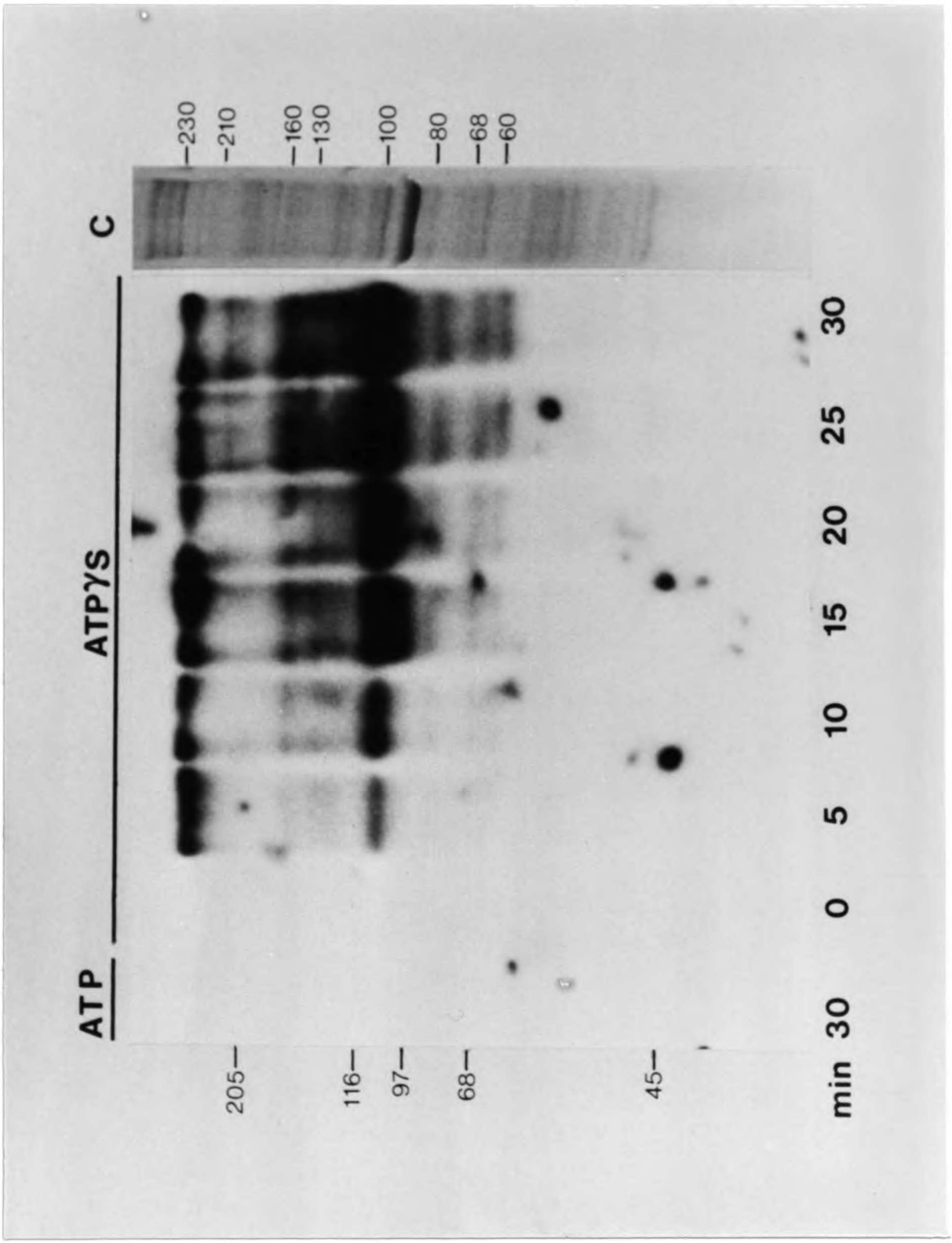


Figure 3-2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ATPγS
C
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230
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80
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Figure 3-3

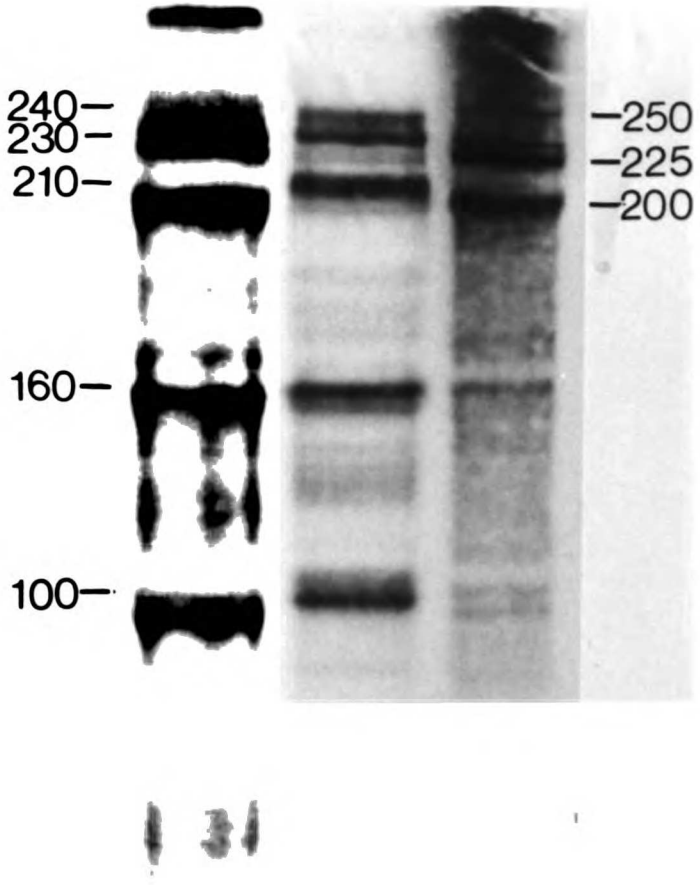
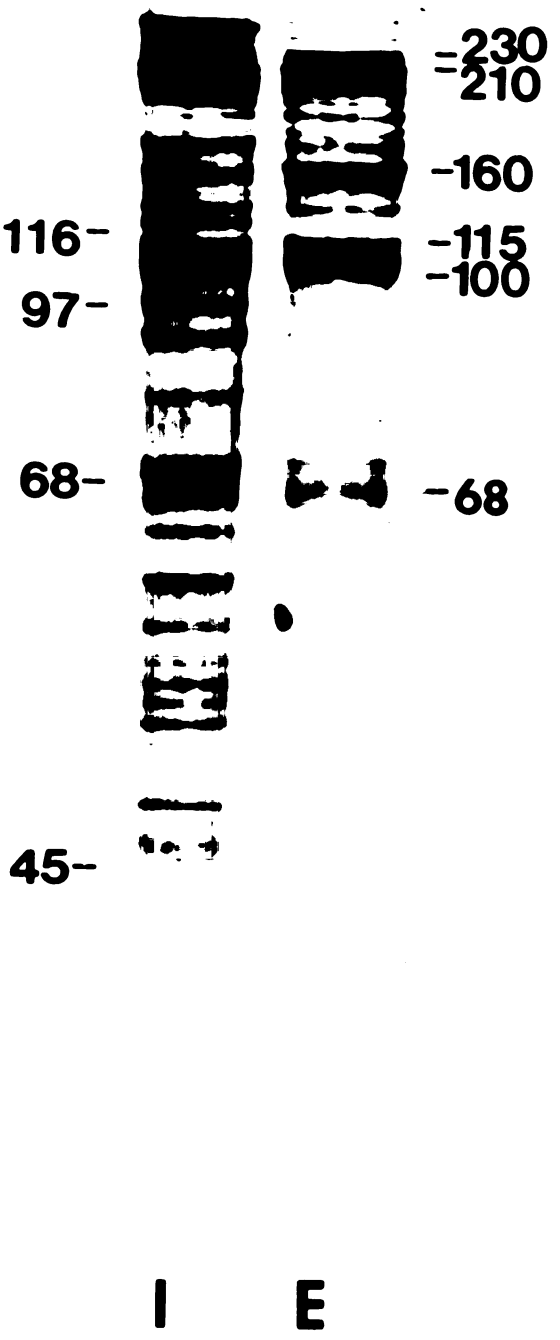


Figure 3-4



Chapter 4

Preliminary studies on MPF in the Yeast Saccharomyces cerevesiae

Introduction

The generality of maturation-promoting factor (MPF) as an inducer of M-phase has been appreciated for more than ten years. Although it was originally defined as an activity that would induce meiosis in amphibian oocytes (Masui and Markert, 1971; Smith and Ecker, 1971) , MPF has been found in M-phase cytoplasm from oocytes and/or embryos of starfish (Kishimoto and Kanatani, 1976), sea cucumbers (Kishimoto et al., 1982), surf clams (Kishimoto et al., 1984), and mice (Sorensen et al., 1985), as well as in mammalian tissue culture cells (Sunkara et al., 1979; Nelkin et al., 1980). MPF activity has never been found in interphase cells. MPF from one species will often induce M-phase in another (Kishimoto et al., 1982), and this remarkable observation implies that it has been highly conserved through evolution. In 1982, a publication by Weintraub et al. reported the existence of MPF activity in the budding yeast Saccaromyces cerevisiae, as assayed by the induction of Xenopus oocyte maturation. The authors employed several temperature-sensitive mutations known to arrest the yeast cell cycle to suggest that this activity existed in yeast only close to M-phase, as one would expect for MPF.

Cell division cycle, or cdc, mutations, which now define more than 50 genetic loci, were originally isolated using several criteria (reviewed in Pringle and Hartwell, 1981). Mutagenized cultures that did not grow at high temperature were screened for their morphology under non-permissive conditions. Only cultures that showed a uniform morphology at high temperature were characterized further and designated as cdc mutants. During the cell cycle of S. cerevisiae, a bud emerges on the surface of the "mother" cell, and grows progressively larger, until it reaches the same size as its mother, and the two cells separate. Thus the size of its bud can be used to estimate the position of a cell in the cell cycle. The terminal morphologies of the cdc mutants, therefore, were used to determine the stage of the cell cycle at which their growth was arrested.

Weintraub et al. (1982) tested extracts of five different cdc mutants, arrested at high temperature, for MPF activity. They found no activity in extracts of *cdc4*, a mutation that arrests the cell cycle in G1 (before DNA synthesis), nor in extracts of *cdc14* and *cdc15*. Both of these mutations arrest the cell cycle after DNA synthesis in what is generally considered a G2-like stage, but this designation is somewhoat ambiguous, since the cells contain fully elongated spindles. Weintraub et al. found some activity in extracts of *cdc16*, and more in extracts of *cdc 20*; both of these strains appear to arrest the cell cycle slightly earlier than *cdc14* and *cdc15*, also in "G2", but with shorter spindles. Extracts of *cdc20* cultures that were grown at low temperature, and therefore were not synchronized with respect to the cell cycle, did not have MPF activity. These observations clearly suggested that yeast MPF activity was present during a particular window of time, not throughout the entire cell cycle.

In 1983, we began a preliminary characterization of MPF from S. cerevisiae. We wanted to confirm the results of Weintraub et al., as well as to extend their observations using more stringent criteria to test for the presence of MPF activity in mitotic yeast extracts. In addition to MPF, there are many components, such as the inhibitor and the regulatory subunit of cyclic AMP-dependent protein kinase, that will induce maturation when injected into Xenopus oocytes (reviewed in Maller, 1983). However, MPF is unique in its ability to induce breakdown of the oocyte nucleus (germinal vesicle) in the absence of protein synthesis (Wasserman and Masui, 1975). Similarly, Miake-Lye et al. (1983) showed that MPF induces nuclear envelope breakdown and chromosome condensation of embryonic nuclei the absence of protein synthesis. We wanted to eliminate the possibility that the activity that Weintraub et al. observed in S. cerevisiae was due to a component other than MPF, by determining the effect of mitotic yeast extracts on oocytes and embryos in the absence of protein synthesis.

We were attracted by the potential of yeast as a source of material for the purification of MPF. Efforts to purify MPF from unfertilized Xenopus eggs and mitotically arrested HeLa cells have been only partially successful (Wu and Gerhart, 1980; Nguyen-gia et

al., 1986; Adlakha et al., 1985). One limitation in using either of these systems for purification is that it is relatively difficult to collect large amounts of MPF-containing material. If we confirmed that yeast extracts could be used as a source of MPF activity, this species would be attractive as a starting point for purification because it would be relatively easy to collect large amounts of MPF-containing material. Still, a potential disadvantage of this approach was that the reported MPF activity in crude yeast extracts (1200 ng/unit MPF) was at least six-fold lower than that found in crude extracts of Xenopus eggs (200 ng/unit MPF). We attempted some preliminary purification steps with mitotic yeast extracts to explore the feasibility of this approach to MPF characterization.

Results

Induction of meiosis in Xenopus oocytes by injection of yeast extracts. We observed that crude extracts prepared from *cdc23*, *cdc20* , or *cdc 16* cells synchronized at M phase by incubation at the non-permissive temperature induced the breakdown of the germinal vesicle (GVBD) when injected into immature Xenopus oocytes. These results essentially confirm those of Weintraub et al. (1982), although those authors did not test *cdc23*. Extracts were made in one of two ways, either by vortexing with glass beads or passing frozen cells through an eaton cell with a hydrolic press. Similar activities were achieved with both methods, for example when extracts of *cdc23* made by either method were injected into oocytes, on average approximately 1200 ng of protein in a volume of 50 nl induced 50% GVBD (1200 ng/unit MPF). This value is similar to that reported by Weintraub et al for *cdc20*. (Activity was somewhat variable, and ranged from as little as 800 ng to as much as 1600 ng per unit of MPF.) When extracts from the three mutants were made in parallel, *cdc20* and *cdc23* had comparable levels of activity, whereas *cdc16* had less (table 4-1). Weintraub et al. also found that extracts of *cdc16* had less activity than those of *cdc20*.

We showed that an extract of *cdc23* induced GVBD in oocytes that had been previously treated with cycloheximide to inhibit

protein synthesis. When 100 nl of a crude extract was injected into untreated oocytes, 10/19 oocytes (50%) were induced to mature. When the same extract was injected into oocytes that had been soaked for one hour in 100 μg/ml cycloheximide, 7/20 (35%) matured.

Purification attempts with yeast MPF. We found that supernatants from precipitation of *cdc23* crude extracts with protamine sulfate (90 mg added/g of protein) had approximately twice the activity of the initial extract. Before precipitation, on average 1200 ng protein/ 50 nl induced 50% GVBD, and after precipitation, on average 600 ng protein/ 50 nl induced 50% GVBD. Protamine sulfate is strongly acidic, and precipitates nucleic acids and some proteins from crude extracts. In the yeast extracts, 25 - 30% of the total protein was precipitated.

Other purification steps we tried met with less success. Three different attempts to bind crude extract to a DEAE cellulose (DE 52) column in half strength EB buffer gave three different results, ranging from no binding of activity to good binding of activity with little recovery after elution with 0.5 M KCl. Activity seemed to bind well to both CM cellulose (cellex CM; Biorad) and phosphocellulose (Whatman) in EB buffer, but little or no activity could be recovered, even when the resin was washed with 1 M KCL. We made several attempts to fractionate the crude extract with ammonium sulfate. We always recovered a low percentage of activity overall (10-20%), and the activity that was recovered was spread thoughout several ammonium sulfate fractions. In three different fractionations of *cdc23* extracts after protamine precipitation, 1-6% activity was recovered in the 0-35% fraction, 1.5-5% in the 35-45% fraction, and 0-10% in the 45-55% fraction. Although the recovery of activity was low, it was possible to achieve a two to three-fold purification and a substantial concentration of activity by using the 0-35% ammonium sulfate fraction. We used this type of preparation to examine the effect of yeast MPF on nuclei in embryos that had been arrested in interphase with cycloheximide.

Induction of embryonic nuclear breakdown with yeast MPF. Miake-Lye et al. (1983) showed that cycloheximide arrested developing Xenopus embryos after S-phase, and that MPF injected into coenocytic cycloheximide-arrested embryos caused the nuclei to breakdown, and chromatin to condense. We followed their protocol exactly, and showed that yeast MPF also induced embryonic nuclei to breakdown (table 4-2). When diluted two-fold with EB buffer, this preparation induced 100% GVBD in oocytes and 88% embryonic nuclear breakdown. Larger dilutions resulted in a decrease in activity as measured by both assays. Miake-Lye et al. found, using partially purified MPF from Xenopus eggs, that the response of embryonic nuclei in this assay is graded, and proportional to the concentration of MPF, in contrast to the sharp threshold response to MPF that the oocyte nucleus displays. Our results with yeast MPF in the two assays are comparable to those of Miake-Lye et al. for frog MPF.

Discussion

The preliminary results presented here confirm the published report by Weintraub et al. that extracts of S. cerevisiae arrested in M-phase have an activity that induces maturation in Xenopus oocytes. Weintraub et al. found activity in arrested cultures of *cdc20* and, to a lesser extent, *cdc16*. We also found that *cdc16* had less activity than *cdc20*, and in addition, that *cdc23* had levels of activity comparable to *cdc20*. We extended the previous results to show that mitotic yeast extracts can induce GVBD in oocytes whose ability to synthesize proteins has been severely inhibited, and can induce the breakdown of embryoic nuclei. These two activities are unique to MPF, and by these criteria, mitotic yeast cells contain MPF. We attempted to purifiy the MPF activity in these extracts, and found that protamine precipitation improved the activity two-fold from 1200 ng/unit MPF to 600 ng/unit MPF. Presumably protamine precipitates component(s) in the extract that inhibit MPF activity. Crude extracts of Xenopus eggs generally have higher activity (200 ng/unit MPF), but it is more difficult to generate large quantities of

extract from Xenopus eggs than from yeast, so yeast could be a good source of material for purification of MPF. Unfortunately, none of the preliminary fractionation techniques we tried were successful for purification of yeast MPF. Although the explanation for this is unclear, one possibility is that multiple components in the extract are required for MPF activity, and that the fractionation procedures separate these essential components. This possibility could be tested by reconstituting fractions to see if MPF activity is restored. Alternatively, MPF activity may be easily destroyed or inactivated during fractionation.

Since the work presented here was carried out (1983), the results of Weintraub et al. have been repeated by Tachibana et al. (1987), and Mark Solomon (unpublished). Tachibana et al. also showed that yeast extracts induced the maturation of Xenopus oocytes that were inactive for protein synthesis. Additionally, they reported that yeast MPF activity is heat labile, and associated with a protein having a 6S sedimentation coefficient. They do not report any purification of yeast MPF.

The study of yeast MPF remains an attractive approach to a difficult problem for two reasons. First, as mentioned above, for the convenience as a source of material for purification, and second because it may be possible with yeast to apply the tools of genetics and molecular biology to the study of MPF. If a mutation in yeast MPF could be isolated, then the gene could probably be cloned, which could be useful for producing large amounts of the gene product for biochemical studies, and to produce MPF-specific antibodies. One would expect a mutation in MPF to be lethal, so conditionally-lethal, for example temperature-sensitive, mutations could be screened for defects in MPF. One would expect a defect in MPF to cause cells to arrrest in G2, and never enter M-phase. The obvious strategy, therefore, would be to screen cdc mutants with this phenotype for defective MPF by testing the MPF from extracts made at the permissive temperature for increased heat lability.

One cdc mutant, *cdc28*, which is homologous to *cdc2* from S. pombe, encodes a protein kinase that seems to be a regulator of the cell cycle (reviewed in Hayles and Nurse, 1986). This gene product

acts at G1/S, and may also act at G2/M (Piggot et al., 1982). Though in some ways this protein is an attractive candidate, it most likely is not MPF. Extracts of *cdc28* alleles that differ in the degree of temperature sensitive kinase activity, show no corresponding variation in MPF activity (Mark Solomon, unpublished). Also, antibodies that react with *cdc28* protein cross react with a protein in frog extracts that fails to co-purify with MPF activity (Mark Solomon unpublished).

There are several potential pitfalls to the search for a MPF-defective mutant. MPF may be encoded by several different genes, i.e. the activity may require several different polypeptide chains that either assemble into one structure, or simply act in concert. In this case it is possible that mutations in all the chains could be isolated, but it would be difficult to know, for the purposes of the analysis, how many such mutations would need to be found, and how many gene products would have to be expressed together to reconstitute the activity. Or, MPF may be regulated post-translationally as it is in Xenopus oocytes (see Chapter 2), in which case mutants whose phenotype is a defect in MPF activity may have alterations in the MPF-activating enzyme rather than in a structural gene of MPF. The analysis of MPF-defective mutations could, therefore, be very confusing. Also, if MPF is regulated post-translationally, it may not be possible to reconstitute MPF activity from clone(s) of the structural gene(s) without the additional activity of the activating enzyme. If yeast MPF is to be pursued seriously, a combination of biochemical and genetic analyses will be the most likely to succeed in a characterization of this cell cycle regulator. The information gained from one approach would complement and clarify information gained from the other approach.

Experimental Procedures

Yeast strains and cell culture. The following strains, originally isolated by Hartwell, were obtained from the Yeast Stock Center, University of California, Berkeley:

281; MAT *a cdc16-1 ade1 ade2 ura1 his7 tyr1 lys2 gal1*
127; MAT*a cdc20-1 ade1 ade2 ura1 his7 tyr1 lys2 gal1*
9031; MAT*a cdc23-1 ade1 ade2 ura1 his7 tyr1 lys2 gal1*

Cells were cultured at 23 °C in YEPD (20 g/l glucose, 20 g/l Bacto peptone, 10 g/l Difco yeast extract) with continuous agitation to a concentration of 1 x 10^7 cells/ml. Cultures were then shifted to 36°C, and incubated for three hours, at which time more than 85% of the cell had arrested with the typical "dumbell" mitotic morphology.

Preparation of yeast extracts. Two different methods were used. For the eaton press method, cells were pelleted, washed once, and resuspended to a concentration of 1.2 x 10^9/ml in EB buffer (80 mM β-glycerophosphate, 15 mM $MgCl_2$, 20 mM NaEGTA, pH 7.3) containing 10 mM DTT, 1 mM ATP, 5% w/v glycerol, and protease inhibitors (1mM PMSF, 1 mM BenzamidineHCl, 2.5 μg/ml leupeptin and aprotinin, 1μg/ml pepstatin), and dripped into liquid nitrogen to form frozen pellets. The pellets were crushed in an eaton cell (pre-cooled in a dry-ice/ethanol bath) using a hydrolic press, and the resultant extract thawed and spun at 12,000 x g for 15 minutes to pellet large debris, then 45K for 1 hour in SW 50.1. The crude extract was typically 15 mg.ml protein. For the glass bead method, cells were pelleted, washed once, and resuspended in a minimal amount of EB containing 10 mM DTT, 1 mM ATP, and protease inhibitors. Two volumes of glass beads (VWR, diameter 0.45 - 0.50 mM) were added, and the mixture was vortexed at top speed for 30 seconds then cooled on ice for 30 seconds for a total of ten times (ten minutes). The lysed cells were centrifuged at 12,000 x g for 15 minutes; the supernatant ranged from 20 - 30 mg/ml.

Protamine precipitation. A 20 mg/ml solution of protamine sulfate in EB was added to crude extract to a final concentration of 90 mg protamine/mg protein. After 5 minutes on ice, the mixture was centrufuged at 23,000 x g for 15 minutes; the supernatant was retained and the pellet discarded. 25 -30 % of the protein was precipitated by this procedure.

Ammonium sulfate precipitation. Solid ammonium sulfate was slowly added to extract to the desired concentration. After 15 minutes on ice, precipitated protein was collected by centrifugation at 16,000 x g for 15 minutes, resuspended in EB containing 10 mM DTT, 1 mM ATP, and protease inhibitors, and dialyzed before assay.

Assay of MPF activty in oocytes. To assay MPF, Xenopus laevis females (Xenopus One, Ann Arbor, MI) were anaesthetized with 0.3% 3-amino benzoic acid ethyl ester (tricaine, Sigma Chemical Co., St. Louis, MO) prior to surgical removal of a section of ovary. Ovary was maintained in a modified Barth's saline developed by Gurdon and Laskey (1970), which was further modified by adding 10 mM NaHepes, pH 7.4 instead of Tris as the buffer. Stage 6 oocytes were hand-dissected 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 1/2 to 2 hours after injection and dissected to determine whether or not the germinal vescicle had dissolved. A dilution that induced 50% GVBD was said to have an MPF concentration of 1 unit/50 nl.

Induction of nuclear envelope breakdown in Xenopus embryos. Embryos were prepared and analyzed exactly as described by Miake-Lye et al. (1983).

Table 4-1: Comparison of MPF activity in different yeast strains

	protein injected (ng)			
cdc strain	575	860	1150	2300
cdc16	0	0	0	1
cdc23	2	6	10	10
cdc20	2	9	9	10

Table 4-1. Comparison of MPF activity in different yeast strains. Extracts were made by the glass bead method as described in experimental procedures. 50 nl of extract containing the stated amount of protein was injected into ten oocytes, and the number of oocytes that underwent GVBD is shown.

Table 4-2: Embryonic nuclear breakdown induced by yeast MPF

dilution	%GVBD	#nuclei brokendown	%breakdown
1/2	100	59/67	88
1/4	30	53/80	66
1/6	10	55/94	58

Table 4-2. Embryonic nuclear breakdown induced by yeast MPF. A 35% ammonium sulfate fraction of a *cdc23* extract (22 mg/ml) was diluted with EB containing10 mM DTT, protease inhibitors, and 0.3 mM ATPγS, injected into 10 Xenopus oocytes, and the % GVBD was scored. The same diluted fractions were injected into embryos arrested in interphase with cycloheximide (see Miake-Lye et al. 1983), and after 15 minutes at room temperature, the number of nuclei that had brokendown was scored by flourescence with Hoechst dye as described. The numbers presented are the sum of observations made on two embryos per dilution.

Chapter 5

Perspectives and Conclusions

MPF remains a tantalizing key to understanding the biochemical mechanisms of the cell cycle. The ultimate challenge is to purify this important regulator and to characterize it on the molecular level. While the studies described here fall short of unraveling all the mysteries of MPF, they do add significantly to our current knowledge of MPF's properties, and create some new approaches to its study. Most importantly, the development of an in vitro system that generates MPF activity from a precursor makes possible biochemical investigations of the regulation of MPF activity.

At the start of these investigations, in vivo observations had suggested the possibility that Xenopus oocytes contained a store of MPF in an inactive form which was transformed into active MPF by a post-translational mechanism. In response to the injection of a small amount of MPF, oocytes produce up to one hundred times more MPF, and this amplification of activity was found to occur even in oocytes that had been treated with cycloheximide to inhibit protein synthesis (Gerhart et al., 1984; Wasseran and Masui, 1975). Still, the possibility remained that a very small amount of protein synthesis was sufficient to produce this response. On the other hand, if amplification did occur post-translationally, there was no evidence to suggest any particular mechanism.

We developed an extract of oocytes that generates MPF activity in vitro in an MPF dependent reaction (chapter 2). MPF activity is still produced after the protein synthetic machinery has been fractionated away, proving conclusively that the activity is generated post-translationally. The reaction proceeds in a soluble system, and in the presence of protease inhibitors, making it unlikely that MPF is generated by release from an intracellular compartment, or through proteolysis. We fractionated this extract, and showed that there are at least two components involved in the activation of an MPF precursor. One fraction, designated pre-MPF, contains the MPF precursor, and generates MPF activity spontaneously in the presence of ATP; this suggests that the precursor may autoactivate. A change in the apparent molecular weight occurrs concomitantly with activation. Another fraction, designated INH, contains an inhibitor of pre-MPF activation that renders the reaction MPF-dependent. The

activity of the INH fraction is inhibited by some phosphatase inhibitors, suggesting that INH is a phosphatase, and that MPF activity is regulated by competing phosphorylation and dephosphorylation reactions. Furthermore, the addition of a specific inhibitor of protein phosphatase type 1, inhibitor 1, to the INH fraction increases its activity, suggesting that INH itself might be regulated through phosphorylation.

Further characterization of this reaction and its components is necessary to conclusively establish the mechanism of pre-MPF activation. It might be informative to add purified phosphatases to the pre-MPF fraction, to see if any of them mimic the action of the INH fraction on pre-MPF activation. INH activity must be purified, but in order to do so, a quantitative assay, based on the ability of INH to delay pre-MPF activation, must first be established. Preliminary results suggested that INH activity was somewhat heat stable, so heat treatment of the INH fraction may be a useful early purification step. Once the INH fraction has been further fractionated, for example by ion exchange, the position of INH activity relative to characterized phosphatase activities should be determined using established phosphatase assays. For example, type 1 phosphatase is assayed by measuring dephosphorylation of the β subunit of phosphorylase kinase, and type 2 phosphatases are assayed with the α subunit as substrate (reviewed in Ballou and Fischer, 1986). If INH activity seems to co-purify with a particular phosphatase activity, then it might be possible to use the standard assay for that phosphatase to purify INH. Any phosphatase assay would probably be more convenient for testing column fractions than an assay for INH activity. If purified INH does prove to be a phosphatase, this would strongly support the view that phosphorylation is the mechanism of pre-MPF activation.

The potential regulation of INH by phosphorylation is best addressed after further purification of INH. However, until then, the effect of purified phosphatase 1 on the INH fraction could be tested (one would predict that it would inactivate INH, since inhibitor 1 seems to stimulate INH). Also the effect of cAMP dependent protein kinase should be tested. Preliminary results indicate that when the

catalytic subunit of cA kinase is added to a mixture of pre-MPF and INH, the activation of pre-MPF is delayed. However, it has not been established whether this effect is mediated by the INH fraction, or if it is a direct effect on pre-MPF. cA kinase could conceivably stimulate INH by activating inhibitor 1, as it is known to do in other systems (reviewed in Ballou and Fischer, 1986), but it is not known if endogenous inhibitor 1 is present in the oocyte fractions. Also, the INH fraction could be incubated with ATPγS to allow the potential thiophosphorylaton of INH, and the effect of this fraction (after dialysis to eliminate the ATPγS) on pre-MPF activation could be tested. If phosphorylation activates INH, then one might expect thiophosphorylated INH to be more active than a non-thiophosphorylated preparation, since thiophosphoproteins are more resisitant to phosphatases (Eckstein, 1985).

The mechanism of pre-MPF activation can be conclusively established only by a direct comparison of purified pre-MPF, and purified active MPF. Pre-MPF purification is also necessary in order to stringently test its ability to auto-activate. Purification of pre-MPF and MPF will be discussed below.

The role of pre-MPF and INH in the control of MPF activity in embryos must be determined. Preliminary experiments have indicated the presence of INH type activity in eggs, but we will not know how meaningful this observation is until oocyte INH has been purified, or at least better characterized, and its properties can be compared to the embryonic activity. If INH is present in embryos, is it responsible for inactivation of MPF activity after mitosis? Under the conditions that have been used, oocyte INH cannot inactivate MPF. However, the buffer used was developed to stabilize MPF activity and inhibit the action of phosphatases (Wu and Gerhart, 1980), and may significantly inhibit INH activity. Therefore, activity of INH under diffferent buffer conditions should be explored.

Attempts to demonstrate the presence of an MPF precursor in eggs have failed to date, possibly because pre-MPF does not exist in embryos. Alternatively, the conditions that allow the activation of pre-MPF in oocyte extracts may not work in egg extracts; for example, interphase-arrested embyros may contain more MPF-

inactivating activity than immature oocytes do. Once pre-MPF has been purified, its presence in interphase embryos could be tested directly with specific antibodies. Until then, if a partial purification of pre-MPF is achieved, the same purification steps could be applied to extracts of embryos, and pre-MPF activity sought.

Many questions about MPF can be answered only after it has been purified to homogeneity. Our work has made possible new strategies for MPF purification. To date, MPF has been enriched up to 200 fold from unfertilized Xenoupus eggs (Wu and Gerhart, 1980; Nguyen-gia et al., 1986), but it has been refractory to further purification. One possible problem with the purifications that have been attempted, is that MPF may lose activity during isolation procedures. The demonstration of an inactive form of MPF in oocytes makes possible a different purification strategy. MPF could be purified from oocytes in its inactive form, which might be more stable to biochemical manipulations; the position of the pre-MPF could be determined by activating aliquots of column fractions. Also, pre-MPF seems to change its chromatographic properties after activation, (fig. 16, chapter 2) so it may be possible to apply the same fractionation technique to pre-MPF before and after activation, and achieve a substantial purification.

We have presented preliminary data suggesting that pre-MPF and MPF may be made up of components that partially separate on gel-filtration columns (appendix 1). If these observations are confirmed, a different strategy for MPF purification should be employed. Instead of attempting to maintain activity after fractionation, fractions should be reconstituted to test for activity. Individual components could be purified separately if they were assayed by adding them back to crude fractions containing other, essential components. If MPF is made up of separable components, this could explain the tendency to lose all MPF activity during fractionation.

The components of pre-MPF activation that we have defined could also be used to develop a more sensitive assay for MPF purification than the oocyte maturation assay currently used. It may be possible to add dilute MPF containing fractions to concentrated

pre-MPF and INH fractions, and assay the ability of the added MPF to accelerate the activation reaction. The oocyte maturation assay cannot detect MPF activity below an approximate concentration of 10 units/μl. In one crude extract of oocytes tested (fig. 2 chapter 2), a final concentraion of 0.6 units/μl MPF accelerated the time of pre-MPF activation from 150 minutes to 90 minutes. If half as much MPF (0.3 units/μl has a measurable effect, and three parts of an MPF-containing fraction can be added to one part concentrated pre-MPF, INH and an ATP-regenerating system for assay, a concentration of 0.4 units/μl MPF could be detected. This is more than 20 times the current sensitivity. The ability to detect such low concentrations of MPF activity could be very useful in assaying fractions from columns, where dilutions often occur. The sensitivity of this assay to various salt conditions would have to be determined.

The studies with monoclonal antibodies to MPF that we have reported (chapter 3) provide direct evidence that MPF is a phosphoprotein, adding still more weight to the hypothesis that MPF activity is controlled through phosphorylation. These studies also demonstrate the feasibility of isolating antibodies that recognize MPF for its characterization and purification. Althought the particular antibodies we isolated were not very useful for MPF purification, other, more specific antibodies could perhaps be isolated using a similar approach. Other immunization protocols could be tried. For example mice can be immuno-tolerized to one antigen and then stimulated with another. In this way specific antibodies have been raised to rare components of crude fractions (Lewis et al., 1987; Mathew and Patterson, 1983). Specifically, mice could first be tolerized to thiophosphoproteins, and then injected with partially purified MPF to prevent the isolation of the same type of antibodies that we characterized. Alternatively, mice could be tolerized to pre-MPF fractions, and then injected with activated pre-MPF to raise a specific response to active MPF. An antibody that recognized MPF specifically could be useful for the purification of MPF, the determination of its composition, and for establishing whether MPF polypeptides are presint throughout the cell cycle, or are periodically synthesized.

In conclusion, our findings have provided a few possible answers, and generated many more questions concerning MPF and its regulation. We hope that some of the strategies outlined here will eventurally lead to a more complete understanding of the biochemical nature of MPF, and hence the regulation of the eukaryotic cell cycle.

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Appendix I: Preliminary evidence indicating MPF may consist of multiple components

In analyzing the molecular weight of pre-MPF before and after activation by gel filtration (as described in chapter 2), we found in several cases that mixing equal volumes of some fractions from the gel filtration column produced MPF activity, when neither fraction had activity on its own (tables I-1,I-2 and I-3). The data presented here are far from complete--they only suggest that such a phenomenon might exist. I present these preliminary results because if these observations were confirmed, the impact on strategies for MPF purification would be significant. If mixing fractions reconstitutes MPF activity, this suggests that multiple separable components are required for activity. In this case a purification scheme should be devised to purify the components individually, but reconstituting them with crude fractions containing the other components for assay.

Briefly, as shown in table I-1, fractions 17 and 25 from a fractionation of pre-MPF had no activity, but when mixed together and activated, caused 2/5 oocytes to mature. In another experiment with pre-MPF, shown in table I-2, of fractions 20 - 24, only fraction 22 had pre-MPF activity on its own, but mixtures of fractions 21 + 23, and 20 + 24, had pre-MPF activity. Finally, an analysis of pre-MPF that was first activated to form MPF, then fractionated, shows that mixtures of fractions 22 + 26, and 22 + 27, had MPF activity, whereas 22 and 27 on their own had no activity, and 26 had very little activity.

Table 1. pre-MPF fraction mixing I

Fraction	# oocytes matured
17	0/5
19	0/5
20	0/5
21	1/5
22	2/5
23	2/5
24	1/5
25	0/5
17 + 25	2/5

Table I-1. Pre-MPF fraction mixing I. 330 µl of pre-MPF (260 units/µl when activated) was fractionated by HPLC on a TSK-400 column as described in Experimental procedures, chapter 2. Fractions were assayed immediately after fractionation; this data is presented in figure 16a, chapter 2. After approximately 4 hours at 0° C, fractions 17-25 were reassayed, as well as a 1:1 (by volume) mixture of fractions 17 and 25, as presented here. As described in chapter 2, fractions were mixed with ATP, creatine phosphate, creatine phosphokinase, and MPF (final concntration 5 units/µl), and after 20 minutes incubation at room temperature, 50 nl was injected into 5 oocytes. Two hours later the oocytes were fixed and examined to determine how many had matured.

Table 2. pre-MPF fraction mixing II

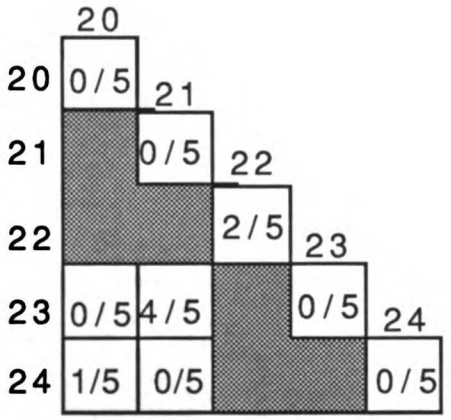

Table I-2. Pre-MPF fraction mixing II. 270 µl of pre-MPF (260 units/ µl when activated) was fractionated on a TSK-400 colunm as described. Aliquots of fractions 20 - 24 were frozen, then thawed and assayed for this analysis. The fractions, or 1:1 (by volume) mixtures of fractions (20 +23, 20 + 24, 21 + 23, 21 + 24), were incubated with ATP, creatine phosphate, creatine phosphokinase, and MPF (final concentration 5 units/µl) as described, and 100 nl was injected into 5 oocytes. After two hours, the oocytes were fixed, and the number of oocytes in each sample that had matured was scored, as presented here. Other combinations of fractions were not tested.

Table 3. Activated pre-MPF fraction mixing

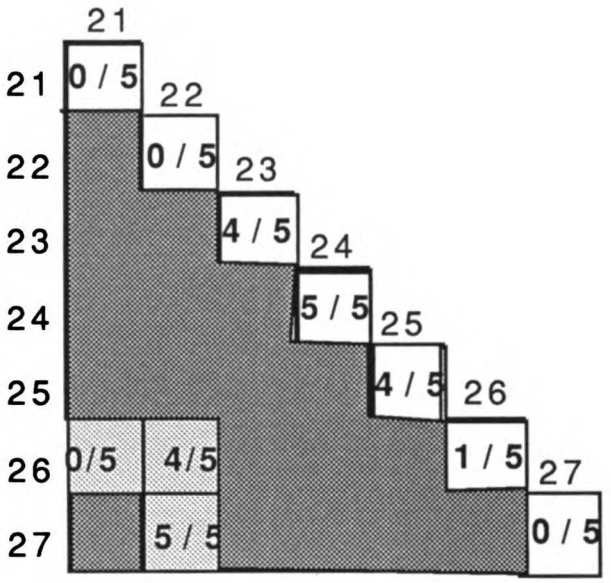

Table I-3. Activated pre-MPF fraction mixing. 320 µl pre-MPF was activated by incubation with ATP, creatine phosphate, and creatine phosphokinase, as described (see chapter 2), resulting in a final MPF concentration of 260 units/µl. This sample was then fractionated by HPLC on a TSK-400 column, 50 nl of each fraction was immediately injected into 5 oocytes, and the number of oocytes undergoing maturation was scored. This data is shown here in the white squares, and graphically in figure 16b, chapter 2. After approximately 4 hours at 0° C, 50 nl of 1:1 (by volume) mixtures of some of the fractions (21 +26, 22 + 26, 22+ 27) were assayed for MPF activity, and the results are presented here, in the stippled squares. Other combinations were not tested.