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Induction of early mitotic events in vivo and in vitro by maturation promoting factor

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Induction of Early Mitotic Events in vivo and in vitro

by Maturation Promoting Factor

by

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Induction of Early Mitotic Events in vivo and in vitro by Maturation Promoting Factor

Ryn Miake-Lye

Abstract

The major events of prophase - chromatin condensation and nuclear envelope breakdown - have been induced by partially purified maturation-promoting factor (MPF), both in vivo and in vitro. MPF is an M-phase-specific activity that has previously been shown to induce meiosis in oocytes.

In vivo, the embryonic cell cycle can be arrested at the end of S-phase by cycloheximide treatment. By inhibiting cytokinesis in the early embryo until approximately 100 nuclei are present and then injecting cycloheximide, all 100 nuclei can be arrested in a common cytoplasm. Within 5 min of injecting MPF into such embryos, the nuclear envelope disperses, as determined histologically or by immunofluorescent staining of the nuclear lamina. The breakdown of the nuclear envelope occurs at levels of MPF comparable to those required for oocyte maturation. Amplification of MPF activity, however, does not occur in the arrested embryo as it does in the oocyte.

MPF also induces chromatin condensation and nuclear envelope breakdown in somatic interphase nuclei incubated in a cell-free extract of arrested embryos. These events occur rapidly and synchronously in response to the addition of MPF and are reversed when MPF activity disappears. Forty min after MPF addition, nuclear envelopes are dispersed and individual chromosomes are visible. Using this cell-free

system, the temporal relationships among 1) nuclear envelope breakdown, 2) depolymerization of the underlying nuclear lamina, and 3) increased phosphorylation of lamins, the structural proteins of the nuclear lamina, have been examined. Lamins A and C are hyperphosphorylated between 10 and 15 min after MPF addition, followed by a gradual depolymerization of the nuclear lamina. Immunofluorescent staining of nuclei for lamins A and C decreases until it is undetectable 40 min after addition of MPF, when nuclear envelope breakdown occurs. It is suggested that hyperphosphorylation of the lamins trigger the depolymerization of the nuclear lamina and, in turn, nuclear envelope breakdown. These results show that MPF can advance interphase nuclei into the first events of mitosis both in vivo and in vitro.

Additional results presented concern preliminary studies on the in vivo regulation of microtubule assembly in Xenopus oocytes and eggs.

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CHAPTER 1

Introduction

"The cell has no other mode of origin than by division of a pre-existing cell. In the multicellular organism all the tissue-cells have arisen by continued division from the original germ-cell, and this in its turn arose by the division of a cell pre-existing in the parent-body. By cell-division, accordingly, the hereditary substance is split off from the parent-body; and by cell-division, again, this substance is handed on by the fertilized egg-cell or oosperm to every part of the body arising from it. Cell-division is, therefore, one of the central facts of development and inheritance."

E.B. Wilson

The Cell in Development and Inheritance

There are two central events that define a cell's progress through the cell division cycle: the replication of the genome (during S-phase), and the equal partitioning of the genome between newly-formed daughter cells during M-phase. Often there are intervals of time between S- and M- phase, and these have been termed G1 (following M-phase) and G2 (following S-phase). Many studies of the cell cycle have focused on how various parameters, such as enzyme activities, gene expression and cellular structures, change during the cell cycle (for reviews, see Hochhauser et al., 1981; Prescott, 1976). However, these studies do not address the fundamental problem of how progression through the cell cycle is regulated.

Regulation of the cell cycle has been studied in a variety of model systems, each having certain advantages for studying a given type of

regulation. That is to say, depending on the experimental system used, different types of regulation become apparent. Most cells double in mass during the cell cycle, in order to maintain a relatively constant cell size. In these cases, progression through the cell cycle is coupled to conditions required for growth; the most obvious type of cell cycle regulation in these cells is growth regulation.

Studies on cultured somatic cells indicate that there is a "restriction point" during G1 at which cells decide to progress through the cell cycle or remain quiescent, depending on their growth conditions (Pardee et al, 1978; Prescott, 1976). After passage through the restriction point, progress through the remainder of the cell cycle seems to be relatively unaffected by growth conditions. The biochemical nature of the restriction point is unknown, although accumulation of a labile protein may be a necessary step for passage through the restriction point (Croy and Pardee, 1983).

The yeast Saccharomyces offers the additional advantage of genetic analysis (Pringle and Hartwell, 1981; Hartwell, 1978). This cell cycle also has a control point during G1 sensitive to growth conditions (termed "start"), that is conceptually analogous to the restriction point. The availability of genetic approaches has allowed the isolation of several temperature-sensitive mutants (cdc mutants), each of which is arrested at a specific point in the cell cycle. The unique value of the mutants has been to determine which cell cycle events are dependent on each other, and which can proceed independently. In this way, specific cell cycle events have been ordered, and pathways of dependent events have been constructed. The major limitation of this approach has been the lack of a specific selection for mutations in molecules that

regulate the normal cell cycle. For example, it is still unknown what triggers the initiation of DNA synthesis, although mutations in DNA ligase and other replication functions have been isolated. Thus many of the mutations isolated are in substrates upon which the regulatory molecules ultimately act.

While the systems described above have provided a great deal of information about the sequential steps in the cell cycle, and the points during the cell cycle that are sensitive to external growth conditions, they have provided little information about the nature of the endogenous signal that initiates the fundamental phases of the cell cycle: S-phase and M-phase. The molecular nature of these signals is still not known. However, from results of the following experimental systems, it is clear that the state of the cytoplasm determines the cell cycle state of the cell.

Nuclei in one phase of the cell cycle have been exposed to cytoplasm in another phase, either by fusion of cultured cells (Rao and Johnson, 1970; Matsui et al, 1972) or multinucleate syncytia such as Physarum (Kaufmann and Wille, 1975), or by microinjection of nuclei into large cells, such as amphibian eggs (Gurdon, 1968). In these experiments, the nuclei become entrained to the cytoplasmic cell cycle state. Such experiments demonstrate that there are diffusible cytoplasmic factors that can induce both S-phase and M-phase. More recently, it has been shown that other components of the cell, such as DNA (Harland and Laskey, 1980; Forbes et al, 1983) and centrioles (Karsenti et al, 1984) are similarly entrained, demonstrating the importance of the cytoplasm in determining the overall state of the cell cycle.

To study the mechanism of cytoplasmic regulation of the cell cycle, we have chosen the simple cell cycle in early Xenopus embryos as a model system. This system offers several advantages that are shared by other embryonic cell cycles (review: Agrell, 1964; Drosophila: Foe and Alberts, 1983; Stafstrom and Staehelin, 1984; sea urchin: Mazia, 1974): the cell cycle is naturally synchronized, proceeds rapidly in the absence of growth, and oscillates between M- and S-phase only, without G1 and G2 (Graham and Morgan, 1966; Gerhart, 1980; Newport and Kirschner, 1982).

Furthermore, in Xenopus embryos, there are manifestations of the cell cycle that continue in the absence of the nucleus, and are (to date) experimentally inseparable from the fundamental cell-cycle oscillator. First, there are surface contraction waves of the cortex that start at the time of each metaphase-anaphase transition (Hara et al, 1980; Sakai and Kubota, 1981). The surface contraction waves may be more closely linked to the cell-cycle oscillator than many of the substrates that the oscillator acts upon, because the surface contraction waves continue in the absence of some of these substrates, eg. microtubules and the nucleus. The early embryonic cell cycle oscillator is arrested by treatment with cycloheximide (Miake-Lye et al, 1983), or by cytostatic factor, an activity in unfertilized egg cytoplasm that arrests the cell cycle at metaphase (Masui and Markert, 1971; Meyerhof and Masui, 1979). These are also the only conditions known to arrest surface contraction waves (Miake-Lye, 1983; Newport and Kirschner, 1984). Surface contraction waves have been observed in newt (Sawai, 1979) and sea urchin embryos (Yoneda et al, 1978), and are an indication of the cytoplasmic nature of the cell-cycle oscillator.

The biochemical nature of the cell-cycle oscillator is unknown. However, the activity of maturation-promoting factor (MPF) is closely coupled to the cell-cycle oscillator (Gerhart et al, 1984). MPF was originally isolated for its ability to induce meiosis (maturation) in amphibian oocytes (Smith and Ecker, 1971; Masui and Markert, 1971), and its activity cycles with the same period as the embryonic cell cycle in both Xenopus (Wasserman and Smith, 1978; Gerhart et al, 1984) and starfish (Kishimoto et al, 1982), with peaks occurring at M-phase. Like the surface contraction waves, the cycling of MPF activity occurs in the absence of either the nucleus or microtubules, and is arrested by cycloheximide and cytostatic factor (Gerhart et al, 1984). MPF, a partially purified protein with a native molecular weight of approximately 100,000 (Wu and Gerhart, 1980), is a highly conserved activity specific to M-phase; it has been isolated from a wide variety of cells in both meiotic and mitotic M-phase (Kishimoto et al, 1982, 1984), including cultured mammalian cells (Sunkara et al, 1979; Nelkin et al, 1980) and yeast (Weintraub et al, 1982). However, the question still remained as to what the immediate effects of MPF were. Since MPF becomes active during M-phase, we decided to test the possibility that it is involved in the transition from interphase into mitosis.

Several morphological changes that occur in the transition between interphase and mitosis take place during prophase, the first stage of mitosis (Wilson, 1925; Mazia, 1961). First, throughout prophase, there is a gradual condensation of chromatin into chromosomes (Gurley et al, 1978; McKeon et al, 1984). Condensation begins at the periphery of the nucleus (Comings and Okada, 1971) and continues until metaphase (Bajer, 1959). Second, the nucleolus, the site of ribosomal assembly, becomes

dispersed (Brinkley, 1965; Anastassova-Kristeva, 1977). The timing of the dispersal correlates with a decrease in ribosomal RNA synthesis. Third, the centrosomes, each containing a centriole pair, separate and migrate toward the poles of the mitotic spindle. Centriole separation is highly variable in its timing relative to nuclear envelope breakdown, even among cells of the same cultured cell line (Aubin et al, 1980). As the centrosomes separate, asters of microtubules form between them, and the growing microtubules appear to push in the nuclear envelope (Wilson et al, 1925; Stafstrom and Staehelin, 1984). It is in this region that the nuclear envelope begins to break down (Stafstrom and Staehelin, 1984; Moll and Paweletz, 1980). The breakdown of the nuclear envelope, consisting of the vesiculation of the nuclear membranes and the dissolution of the underlying nuclear lamina, marks the end of prophase.

The biochemical mechanisms that underlie these morphological changes are unknown. However, there are a number of observations concerning 1) proteins associated with the structures described above, and 2) properties of partially purified MPF, which indicate that there are differences in patterns of phosphorylation between interphase and mitotic cells.

There is indirect and circumstantial evidence that MPF may be a phosphoprotein and a kinase. MPF is activated by ATP, phosphatase inhibitors stabilize its activity, and it co-purifies with a kinase activity (Wu and Gerhart, 1980) which does not significantly phosphorylate histones, casein or the major enzymes of glycogen metabolism (Maller, 1983). It can induce maturation in the absence of protein synthesis (Wasserman and Masui, 1975; Gerhart et al, 1984), indicating that it acts via post-translational modification; and, in

Xenopus oocytes, MPF activity has the unusual ability to be autoamplified in the absence of protein synthesis (Gerhart et al, 1984; Wasserman and Masui, 1975). One possible explanation for autoamplification would be the self-phosphorylation of a store of inactive MPF by a small amount of active MPF. It has been demonstrated that there is a burst of phosphorylation associated with injection of MPF into oocytes (Maller, Wu and Gerhart, 1977), and that there are mitosis-specific self-phosphorylating kinases present in crude preparations of MPF (Halleck et al, 1984).

There is also evidence that a number of proteins are phosphorylated specifically during mitosis (reviewed in Laskey, 1983). Davis et al (1983) have isolated two monoclonal antibodies that stain only mitotic cells by indirect immunofluorescence; each appears to recognize a large group of mitosis-specific phosphoproteins. Furthermore, proteins associated with structures that undergo morphological changes at the onset of mitosis (i.e., chromatin, nucleoli, centrosomes and the nuclear envelope) are phosphorylated during mitosis. Histone H1 is hyperphosphorylated (Bradbury et al, 1974) and H3 and HMG14 are phosphorylated during mitosis (Gurley et al, 1978; Paulson and Taylor, 1982). An identical pattern of histone phosphorylation occurs in prematurely condensed chromosomes (Ajiro et al, 1983). It should be noted that, while H1 hyperphosphorylation may be necessary for chromosome condensation, it is not sufficient, since chromatin blocked from condensing in sea urchins still have mitotic levels of H1 phosphorylation (Krystal and Poccia, 1981). In Physarum, nucleolar proteins are phosphorylated shortly before mitosis (Shibayama et al, 1983). A subset of the mitosis-specific phosphoproteins recognized by

the monoclonal antibodies mentioned above are located in the centrosome (Vandre et al, 1984). Finally, lamins A, B, and C, the major proteins of the nuclear lamins are hyperphosphorylated during mitosis (Gerace and Blobel, 1980), possibly by a kinase associated with the nuclear envelope that phosphorylates the lamins in vitro (Lam and Kasper, 1979; Agutter et al, 1979).

On the basis of the observations described above, a possible model for the role of MPF activity in regulating the cell cycle is that it could initiate a cascade of mitosis-specific phosphorylation, similar to the activation of glycogen phosphorylase (Cohen, 1982). In principle, this is a testable hypothesis; but previously it has been impossible to study the biochemical mechanism of the onset of mitosis. A major limitation has been the synchrony of the cells used. Although it has been possible to compare cells in interphase to cells already in mitosis, it has not been feasible to obtain highly synchronized populations of premitotic cells, in order to observe the time course of biochemical changes in prophase.

The aim of my studies has been to begin to ask how MPF acts to regulate the cell cycle, particularly during the transition from interphase to mitosis. In doing so, we hope to eventually extend our understanding of the cell cycle from the level of observing morphological changes to the level of determining the biochemical mechanisms of those changes.

CHAPTER 2

Maturation Promoting Factor Induces Nuclear Envelope Breakdown in Cycloheximide-arrested Embryos in Xenopus laevis

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ABSTRACT

We have studied the effect of maturation promoting factor (MPF) on embryonic nuclei during the early cleavage stage of Xenopus development. When protein synthesis is inhibited by cycloheximide during this stage, the embryonic cell cycle arrests in an artificially produced G2-like state, after completion of one additional round of DNA synthesis. Approximately one hundred nuclei can be arrested in a common cytoplasm if cytokinesis is first inhibited by cytochalasin B. Within five minutes after injection of MPF into such embryos, the nuclear envelope surrounding each nucleus disperses, as determined histologically or by immunofluorescent staining of the nuclear lamina with anti-lamin antiserum. The breakdown of the nuclear envelope occurs at levels of MPF comparable to or slightly lower than those required for oocyte maturation. Unlike the oocyte, however, amplification of MPF activity cannot be detected in the arrested egg. These results suggest that MPF can act to advance interphase nuclei into the first events of mitosis and show that the nuclear lamina responds rapidly to MPF.

INTRODUCTION

For cell growth and orderly progression through the cell cycle, many disparate events in the cell must be closely controlled, suggesting the existence of endogenous molecules responsible for the regulation of growth and the cell cycle. The study of oocyte maturation and the early cleavage stage in Xenopus laevis development offers several advantages in distinguishing homeostatic mechanisms for regulating cell growth from mechanisms involved in regulating the progression through the cell cycle. During the first twelve cleavages following fertilization, the embryo does not grow, so the usual requirement of having to double the mass of a cell during each division cycle is obviated. Furthermore, the embryo contains stores of major structural elements, such as histones (39), tubulin (29) and deoxyribonucleotides (22). The cell cycle during this stage is rapid, having a period of approximately thirty minutes, and the cells divide synchronously. There are effectively no G1 and no G2 phases (14), so the cycle consists of only two phases: M (mitosis) and S (DNA synthesis). There is no transcription during this period (28). In short, the primary function of this stage of development seems to be the rapid and orderly replication of DNA and the subdivision of the cytoplasm to prepare for the onset of more complicated developmental patterns, a strategy which is followed in other types of embryos as well (13,41).

The early cell cycle in Xenopus can be best described as being driven by a cytoplasmic oscillator, which entrains nuclear events (19). The oscillator is manifested by contractions of the cortex of the embryo with the same period as the cell division cycle. These contractions continue in the absence of either the centriole or the nucleus (16).

Recent experiments sugggest that even the replication of injected prokaryotic DNA comes under the control of this early cell cycle (17,42). Thus, this rudimentary cell cycle provides an opportunity to study a cell cycle in which only the most basic events controlling the progression through mitosis and DNA synthesis are operative. It also offers the opportunity to study the molecular nature of the oscillatory components.

Although the egg may contain many regulatory factors, maturation promoting factor (MPF) has already been shown to initiate meiotic events in the oocyte, and is also present in mitotic cells. In this paper, we have chosen to study the role of this factor in the mitotic cell cycle. Maturation promoting factor was originally detected as an activity present in the cytoplasm of mature oocytes or unfertilized eggs (26,32). When a small amount of mature cytoplasm is transferred into an immature oocyte, which is naturally arrested in G2 before the first meiotic division, MPF sets off events leading to the completion of meiosis and the maturation of the oocyte. The presence of this activity is easily scored by the breakdown of the germinal vesicle (i.e., oocyte nucleus) in the immature oocytes. MPF has since been shown to be a protein which has been partially purified thirty-fold by Wu and Gerhart (40). It can be found in a wide variety of higher eukaryotic cells, including the mature oocytes of starfish, sea cucumber and frog (20), as well as mitotic HeLa (34) and CHO cells (27). In addition, Wasserman and Smith (37) showed that MPF activity fluctuates with the same period as the cell cycle in Xenopus embryos, having a mitotic rather than meiotic cell cycle. In the embryos, the peak of MPF activity occurs immediately prior to mitosis.

Although other cytoplasmic proteins (including calmodulin (38) and the regulatory subunit of cAMP-dependent protein kinase (23)) have been shown to induce oocyte maturation, MPF can be clearly distinguished from all of these by two criteria. First, MPF injected into a recipient immature oocyte is amplified, such that the recipient will contain enough MPF activity to act as a donor to a second immature oocyte. These transfers have been continued serially up to ten times (30). Second, MPF can induce maturation in the absence of protein synthesis (36,43), whereas these other proteins require new protein synthesis before maturation is induced (23,38). This latter point implies that MPF is acting later in the pathway to effect maturation in immature oocytes.

The way in which MPF induces the meiotic cell cycle is unknown. MPF sets off a complex series of events which cause changes in virtually every part of the cell: the oocyte nucleus breaks down, transcription is shut off, chromosomes undergo meiosis, the cortex of the oocyte is reorganized and the ability of microtubules to assemble changes radically (18) (for reviews, see references 11, 25, 31). It has therefore not been possible to distinguish which events respond immediately to MPF. Since it has proven difficult to dissect the specific role of MPF out of the ongoing cell cycle (either meiotic or mitotic), we have chosen instead to arrest embryonic cells at one point in the mitotic cell cycle and ask what limited set of events MPF initiates in this context.

In this study, we have used cycloheximide to arrest the embryonic cell cycle at a point after the end of DNA synthesis (S phase) but prior to nuclear envelope breakdown and mitosis (M phase). Although the

normal embryo passes rapidly from S phase to M phase, with no detectable G2, the cycloheximide-arrested embryos are blocked at the transition from S to M in an artifical G2-like state. In the blocked state, the level of endogenous MPF is immeasurably low. However, when we microinject these embryos with MPF partially purified from unfertilized Xenopus eggs, there is a dramatic change in the nuclei of these embryos. The nuclear envelope breaks down, and the nuclear lamina disperses. During oocyte maturation, there is a delay of at least ninety minutes between the time of MPF injection and the breakdown of the oocyte nucleus, but the arrested embryonic nuclei respond to MPF injection within five minutes. Typically, the transition from the G2 phase of the cell cycle to mitosis is marked by nuclear envelope breakdown, accompanied by the disassembly of the nuclear envelope lamina. We have therefore demonstrated that: 1) MPF purified from meiotic cells is competent to induce a part of the mitotic cell cycle, and 2) nuclear envelope breakdown is a rapid response to partially purified MPF in vivo.

MATERIALS AND METHODS

Maturation Promoting Factor

MPF was generously provided by Michael Wu and John Gerhart, UC Berkeley. It was purified thirty-fold after the arginine-agarose column, according to the protocol described in reference 40. MPF was extremely stable when stored in small aliquots at -80°C. When necessary, it was diluted into 80 mM β-glycerophosphate, 15 mM $MgCl_2$, 20 mM EGTA, 1 mM ATP, and 1 mM dithiothreitol, pH 7.4 (extraction buffer).

Cycloheximide-arrested embryos

Eggs were synchronously fertilized in vitro, as described by Newport and Kirschner (28). Approximately 45 minutes after fertilization, they were transferred into MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES (pH 7.8) and 0.1 mM EDTA) containing 5% Ficoll, type 400 (Sigma, St. Louis, MO) to facilitate later injections and 5 µg/ml cytochalasin B. Cytochalasin B completely inhibits cytokinesis (15), and allows nuclei to accumulate in a common cytoplasm. Shortly after the time of first cleavage in control eggs (typically 90 minutes after fertilization), cleavage-blocked eggs were judged as fertilized on the basis of a characteristic white stripe across the animal hemisphere, due to the appearance of new membrane at the surface of the egg instead of in the cleavage furrow. Four or five hours after fertilization, the cleavage-blocked embryos were injected with 50 nl of 200 µg/ml cycloheximide in 20 mM potassium phosphate (pH 7.0) and transferred into 10 mls of MMR containing 5% Ficoll and 10 µg/ml cycloheximide (Sigma). (Cytochalasin B was no longer necessary to inhibit cytokinesis at this point; after two to three hours post-fertilization, cytochalasin B-treated embryos will not cleave, even if cytochalasin B is rinsed out of the medium.) Embryos were completely arrested in a G2-like state (as judged by the appearance of their nuclei) 55 to 60 minutes after injection of cycloheximide.

The absence of cleavage and surface contraction waves in embryos treated with cycloheximide (200 µg/ml) or puromycin (600 µg/ml) was observed using time-lapse video recording (28). The recording was played back at a speed such that one hour of real time corresponded to 20 seconds of recording.

DNA Synthesis

Fertilized eggs were pulse-labelled by injecting 50 nl of α-^{32}P-dCTP (10 mCi/ml, 400 Ci/mmol, Amersham Corp., Arlington Heights, IL) into each of three eggs in MMR plus 5% Ficoll. The eggs were incubated for ten minutes. At the end of the labelling period, eggs were rinsed briefly in MMR without Ficoll, and transferred into 100 µl of 10 mM Tris (pH 7.0), 10 mM EDTA and 1% SDS. They were lysed by passing them through a 200 µl Pipetman tip several times. 10 µg Proteinase K (EM Biochemicals, Darmstadt, Germany) was added, and the homogenate incubated at 37°C for 1 hour. After two phenol extractions and ethanol precipitation, the pellet of nucleic acid was resuspended in 10 µl of 20 mM Tris (pH 8.1), 20 mM sodium acetate and 2 mM EDTA (TAE). An equal volume of TAE containing 50% glycerol was added, and samples were loaded onto a 1% agarose-TAE gel (14 cm x 15 cm) and electrophoresed overnight at 35V. The gel was dried onto a paper backing and autoradiographed for 2 days at -70°C, using a Dupont Cronex intensifying screen and Kodak X-omat AR x-ray film.

Visualization of Nuclei

For histology of paraffin sections, cycloheximide-arrested embryos were fixed, either before or after injection of MPF, in a few mls of Tellysnicky's modification of Smith's fixative (0.5 g potassium dichromate, 2.5 ml glacial acetic acid and 10 mls formaldehyde solution, diluted to 100 ml with water) between 2 and 16 hours. The embryos were rinsed in several changes of tap water, after which the embryos were dehydrated and embedded in Paraplast Plus (Lancer, St. Louis, MO). 10 µm sections were cut and floated onto slides with a dilute solution of Mayer's albumin fixative (Harleco, Gibbstown, NJ). After drying, the

slides were stained with Mayer's acid hematoxylin (hematoxylin powder from Chroma-Gesellschaft, Stuttgart, Germany), which stains the chromatin dark purple, and counterstained with 1% Chlorazol Black E (Chroma-Gesellschaft), which stains membranes (including the nuclear membranes) black.

Squashes were prepared as follows: a single embryo was placed on a microscope slide, excess medium was pulled off, and 5 µl of extraction buffer containing 250 mM sucrose and 10 µg/ml Hoechst dye 33258 (bisbenzimide)(Calbiochem-Behring Corp., La Jolla, CA) was added to the embryo. The embryo was then gently lysed by slowly lowering a coverslip onto the slide. The coverslip was sealed using nail polish to prevent dehydration. These squashes were scanned using fluorescence microscopy with a Zeiss photomicroscope to locate nuclei; the state of the nuclear envelope was observed using Nomarski optics.

Indirect Immunofluorescence

Cycloheximide-arrested embryos (either before or after injection with MPF) were lysed into extraction buffer containing 0.1% Triton X-100, 250 mM sucrose and 2% formaldehyde. They were allowed to fix for about 15 seconds, and then mixed by repeated passages through a 200 µl Pipetman tip to remove yolk platelets adhering to the nuclei. The lysate was allowed to settle for 10 minutes onto acid-cleaned coverslips coated with cytochrome c. The coverslips were washed once with 1% bovine serum albumin (BSA, Sigma) and 0.1% Triton X-100 in PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM NaH_2PO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$). The primary antiserum was human serum from a patient with linear scleroderma (44). This serum contains antibodies to lamins, the major proteins of the nuclear envelope lamina (10,21). Sections were

incubated in a 1:1000 dilution of this serum for 15 minutes. After washing off the excess primary antiserum, rhodamine conjugated goat anti-human immunoglobulin antibodies (Cappel Laboratories, Cochranville, PA) were applied as a secondary antibody for 15 minutes. The secondary was washed off with multiple rinses of 1% BSA and 0.1% Triton X-100 in PBS. The penultimate rinse contained 10 µg/ml Hoechst dye in PBS. Coverslips were mounted in 90% glycerol containing 2% propyl gallate (Sigma) to decrease fading of the fluorescent signal (12). Although this method did not allow quantitative recovery of nuclei (many nuclei were washed off the coverslips), more than enough nuclei were retained to allow a clear comparison to be made before and after MPF treatment.

Cultured Xenopus epithelial cells (A6, from American Type Culture Collection, Rockville, MD) grown on coverslips were extracted and fixed in 1% Triton X-100 and 2% formaldehyde in PBS at room temperature for 10 minutes. They were then processed as for cycloheximide-arrested embryos.

Immunoblotting of Cell Extracts

Confluent A6 cells were trypsinized off plates and washed in PBS without calcium or magnesium chloride. The washed cell pellet was resuspended in the same buffer at 4°C. 20% (v/v) Triton X-100 was added to a final concentration of 0.1%. Cells were extracted for 5 minutes on ice, then re-pelleted (1000 g, 5 minutes). The pellet was resuspended in PBS containing both $CaCl_2$ (0.7 mM) and $MgCl_2$ (0.5 mM) at room temperature, and micrococcal nuclease was added to a final concentration of 100 units/ml. The cells were digested for 15 minutes at room temperature, pelleted in an Eppendorf centrifuge, resuspended in 5% SDS,

20% glycerol, 20 mM Tris (pH 6.8), 2 mM EDTA and 5% β-mercaptoethanol, and heated in a boiling water bath for 3 minutes.

The proteins in this preparation were resolved by one-dimensional electrophoresis in an 8.5% SDS-polyacrylamide gel, and either stained with Coomassie blue (Bio-Rad, Richmond, CA) or transferred to nitrocellulose (Schleicher and Schuell, Inc., Keene, NH)(33). The nitrocellulose transfer was washed and treated with antibody essentially as described by Burnette (2). The primary antiserum used was the human serum described above; IgG purified from goat anti-human immunoglobulin antiserum (Cappel Laboratories) was iodinated by the chloramine T reaction (8) and used instead of iodinated protein A.

MPF Assays

To obtain immature oocytes, a small piece of ovary was surgically removed from a female Xenopus; Stage 6 oocytes (5) were hand-dissected from the surrounding follicle. Unfertilized eggs were dejellied in 2% cysteine (pH 7.8) and rinsed into MMR. Eggs were activated by pricking unfertilized eggs in one-quarter strength MMR with a microinjection needle. Extracts were made from activated eggs 15 minutes after pricking.

To test the cytoplasm of either immature oocytes, unfertilized eggs or activated eggs for MPF activity, extracts were prepared in the following manner: excess medium was removed from five cells, 5 µl of cold extraction buffer was added, and the cells were lysed by repeated passages through a 200 µl Pipetman tip. The cell lysate was transferred into a flared glass capillary with one end flamed shut, and centrifuged for 10 minutes in a Beckman Microfuge B. The capillary was scored and broken just below the interface between the lipid and aqueous phases,

and the aqueous extract was taken up directly from the capillary into a microinjection needle (43).

RESULTS

The effect of cycloheximide on the early cell cycle

The cell cycle during the early cleavage stage of Xenopus development seems to be regulated by a cytoplasmic clock or oscillator. Perhaps the most direct known manifestation of this clock is a series of surface contraction waves (16). These contractions of the cortex occur at metaphase of each cell cycle (42), and can be visualized by time-lapse cinematography (16). The surface contraction waves have been shown to continue in eggs blocked from cleaving by treatment with colchicine or vinblastine as well as in eggs in which the nucleus and the centriole are absent, suggesting that the cell cycle oscillator can be uncoupled from nuclear division and mitosis. We have found that if protein synthesis is inhibited in fertilized eggs by the injection of 30 nl of either cycloheximide (200 µg/ml) or puromycin (600 µg/ml) at 40 minutes after fertilization, neither cleavage nor surface contraction waves occur (data not shown). Control eggs injected with water continue to show at least six surface contraction waves at intervals of thirty minutes. This indicates that cycloheximide might block the cell cycle; but, it is unclear from these results whether cycloheximide-treated embryos are blocked randomly in the cell cycle or at a specific point.

To test whether cycloheximide produces a specific block in the cell cycle, we have examined the timing of DNA synthesis (S phase) in cycloheximide-treated embryos. After fertilization, control eggs are pulse-labeled by injection of α-^{32}P-dCTP for ten-minute intervals.

During the brief S phase, ^{32}P-dCTP is incorporated into DNA and can be visualized by autoradiography. On a 1% agarose gel, ^{32}P-labelled chromosomal DNA enters the gel and runs as a single wide band near the upper limit of resolution. (Using a Bgl II digest of bacteriophage T4 DNA as molecular weight standards, the bands shown in figure 1 comigrate with a 55 kb fragment, above a 17 kb fragment.) The labelled bands are completely DNase-sensitive, and resistant to digestion by RNase. Figure 1a shows the normal times of occurence of the first three rounds of DNA synthesis in Xenopus embryos. If these times are normalized to the average time of first cleavage (90 minutes after fertilization, at 21°C), then the average periods of DNA synthesis are between 24 and 35 minutes after fertilization, between 71 and 85 minutes, and between 105 and 120 minutes. There are two rounds of DNA synthesis before first cleavage because the embryonic nuclei re-form and enter S phase before the onset of cytokinesis at the cell surface (11).

Injection of cycloheximide prior to the first S phase still allows the initiation and termination of the first round of DNA synthesis at the normal time (Figure 1b). The first S phase occurs even though protein synthesis, as assayed by ^{35}S-methionine incorporation into TCA-precipitable material, is inhibited greater than 95% (data not shown) before initiation of DNA synthesis (within 5 minutes of injection). However, subsequent rounds of DNA synthesis are completely inhibited. No cleavage is observed in these cells, consistent with the observations of Wasserman and Smith (37), and as mentioned previously, no surface contraction waves occur. Thus, if cycloheximide is causing a specific temporal arrest, it is after DNA synthesis but before mitosis, when the surface contraction wave occurs.

If cycloheximide is injected at 38 to 48 minutes post-fertilization (i.e., after the first S phase, but before the second), the second round of DNA synthesis begins and ends at the normal time, but the third is prevented (Figure 1c). (In this case, the cells form a partial first cleavage furrow, somewhat later than control eggs; this partial furrow recedes before second cleavage in controls.) Therefore, these embryos also appear to be arrested at a point after the end of chromosomal DNA synthesis (S phase) in the cell cycle, since they complete exactly one round of DNA synthesis after injection of cycloheximide, but do not start subsequent rounds.

It should be noted that the failure to reinitiate DNA synthesis after cycloheximide treatment is not due to a decreased ability of the embryo to replicate DNA, since Harland and Laskey (17) have shown that SV40 DNA injected into cycloheximide-treated eggs can replicate once even at times after chromosomal DNA would normally have finished replication (i.e., up to five hours after cycloheximide injection). In addition, cycloheximide treated embryos are likely to be metabolically competent, since we find that the rATP pool of arrested embryos, as measured by high performance liquid chromatography, does not decrease over a period of at least four hours after injection of cycloheximide (data not shown).

Having determined that cycloheximide blocks embryos after S phase, we wanted to know whether the embryos are arrested in a G2-like state or whether they proceed into mitosis. To accumulate many nuclei in each embryo for histological examination, eggs are incubated in medium containing 5 µg/ml cytochalasin B, which prevents cleavage (15), but allows nuclear division to continue for several hours. Other aspects of

the cell cycle have been shown to continue normally. For example, DNA replication continues at the normal rate in such cleavage-blocked embryos (28). If we continue the incubation for four to five hours after fertilization, approximately 100 nuclei accumulate in a common cytoplasm. The eggs are then injected with cycloheximide, and subsequently incubated in medium containing cycloheximide. Eggs are then fixed and processed for conventional paraffin-section histology. As can be seen in figure 2a, the nuclear envelopes of the nuclei in arrested embryos are intact, and the chromatin has not condensed into chromosomes. Therefore, these embryos do not appear to have proceeded into mitosis.

Although paraffin sections preserve nuclear morphology well, many of the following experiments required a more rapid assay for determining the state of the nuclei. In order to visualize the nuclei easily, single eggs are very gently lysed in the presence of 10 µg/ml Hoechst dye (to fluorescently label the DNA), and viewed by Nomarski and fluorescence microscopy. In some cases the nuclei are also fixed in 1% formaldehyde; this has no effect upon their morphology. Figure 3a shows the characteristic appearance of nuclei in the arrested embryos. Using Nomarski optics, the presence of the nuclear envelope can be seen as a ridge at the periphery of the nuclei; the Hoechst staining pattern of the DNA (figure 3b) confirms that the chromatin has not condensed into chromosomes. The small round particles are yolk platelets, which are weakly autofluorescent.

To confirm that the nuclear envelope is intact in nuclei in cycloheximide-arrested embryos, lysates of arrested embryos are fixed in formaldehyde, and allowed to settle onto coverslips. The coverslips are

then prepared for indirect immunofluorescent staining of the nuclear envelope lamina (the layer of peripheral membrane protein closely underlying the inner nuclear membrane) with antibodies to lamins, the major structural proteins of the nuclear lamina (10,21). The antiserum used is a human serum from a patient with linear scleroderma which has been shown to react very specifically with lamins A and C, two of the three closely related major proteins in the nuclear lamina (44). This antiserum reacts with lamins in a variety of species, including a cultured Xenopus epithelial cell line, A6 (Figure 4a). The staining shows the same morphology as observed by other investigators (10,21). It shows reactivity on nitrocellulose transfers of SDS-polyacrylamide gels with lamins A and C (Figure 4b). (Some staining in the lamin B region is most likely due to a proteolytic fragment of lamin A, based on evidence from two-dimensional gel electrophoresis (44)).

Nuclei in cycloheximide-arrested embryos (identified by Hoechst staining, Figure 5b), which have been stained using this antiserum have a distinct and continuous lamina surrounding the chromatin, seen as strong perinuclear staining in figure 5a. A continuous lamina is the configuration normally seen in interphase cells (10,21), and similar to what we observe in interphase Xenopus cultured cells (Figure 4a). On the basis of the clearly defined nuclear envelope visible both in whole mounts by Nomarski optics and in paraffin sections, and the continuous lamina observed with antibodies against lamins A and C, we conclude that the nuclei are arrested in an interphase state. Since the nuclei have completed DNA synthesis without proceeding into mitosis, we conclude that they are in an artificially extended G2 state, which does not normally occur during the early cleavage stage.

The effect of MPF on nuclei in cycloheximide-arrested embryos

When MPF partially purified from Xenopus eggs is injected into the cycloheximide-arrested embryos (having approximately 100 nuclei in a common cytoplasm), a radical change in nuclear morphology is observed. Within five minutes after injection, the nuclear envelope breaks down, and chromatin in these embryos is no longer contained within a discrete nucleus. In figure 2b, the light microscopic image of paraffin-sectioned material shows a yolk-excluding region which contains darkly staining chromatin. However, the chromatin is no longer dispersed within a clearly defined nuclear envelope (as in figure 2a). In figure 3c, the Nomarski image of a squash again shows a coherent yolk-excluding region with no sign of a nuclear envelope. The fibrous structures shown in the Nomarski image are unambiguously identified as chromatin by the Hoechst dye fluorescence image of the same region (Figure 3d). No changes in the nuclei are observed if the embryos are injected with extraction buffer only.

The breakdown of the nuclear envelope is accompanied by a dissolution of the nuclear lamina. Prior to injection of MPF, all Hoechst-staining chromatin is surrounded by immunofluorescent staining of the nuclear lamina (Figures 5a and 5b). After injection of MPF, there is almost no detectable staining in the area surrounding the DNA, and the very weak residual staining is not organized into a continuous lamina (Figures 5c and 5d). This suggests that the nuclear envelope has broken down, since in cultured cells, anti-lamin antibodies stain the entire cell diffusely during mitosis when the nuclear envelope is disassembled (10,21). (This diffuse staining would not be

distinguishable from background after dilution throughout the entire egg cytoplasm.)

We also observe the disappearance of nuclear lamina staining in frozen sections of cycloheximide-arrested embryos after treatment with MPF, demonstrating that the loss of lamin staining is not an artifact of the lysis procedure described above (data not shown). Just as in figure 5a, intact nuclei in sections show continuous staining at their periphery. After MPF treatment, no staining is observed in the area surrounding the Hoechst-stained chromatin.

Since the MPF injected has not been purified to homogeneity, it could be argued that the factor(s) responsible for the breakdown of the nuclei is not MPF itself, but some other protein which co-purifies with MPF. To address this issue, we exploited the fact that MPF activity is clearly present and absent in different phases of the cell cycle. Immature oocytes have no MPF, mature oocytes (i.e., unfertilized eggs) have MPF, and eggs lose this activity within fifteen minutes after activation (43). Extracts of each of these three cell types were made under identical conditions. When they were injected into oocytes, only the extract from unfertilized eggs induced maturation. As shown in table 1, when the extracts from immature oocytes and activated eggs were injected into arrested embryos, only a small percentage of the nuclei did not have the characteristic spherical morphology; however, this is also the fraction of nuclei which are damaged in a squash of uninjected arrested embryos, or embryos injected with extraction buffer only. In contrast, 83% of the nuclei underwent nuclear envelope breakdown in embryos injected with extract from unfertilized eggs. Therefore, by

this functional definition, it is MPF which is responsible for the breakdown of the nuclear envelope.

The effect of MPF on the cycloheximide-arrested nuclei is extremely rapid. When the partially purified MPF is diluted as much as 1:100 and injected into arrested embryos, the percentage of nuclei broken down is the same at 8' post-injection (56%) as at 68' post-injection (54%). Similar results are found for MPF at a 1:200 dilution: at 5' after injection, 20% of the nuclei had broken down, compared to 19% at 45'.

These results illustrate another interesting and significant fact: at lower concentrations of MPF, there is a graded response of the nuclei to the levels of MPF injected. In other words, the percentage of nuclei broken down is proportional to the concentration of MPF. This is in marked contrast to the effects of MPF on oocyte maturation, where there is a sharp threshold concentration at which MPF becomes effective. Figure 6 shows the percentage of nuclei broken down plotted against the concentration of MPF injected. For purposes of comparison, the response of oocyte nuclei is also plotted against the concentration of MPF. Using a Hill plot as a measure of the cooperativity of this reaction (7), the order of the response is estimated to be 1.2 for the cycloheximide-arrested nuclei compared to 7 for oocyte nuclei (40). (A first-order response is not cooperative; the greater the order of the response, the greater the cooperativity.) At low levels of MPF (eg. 1:100), a certain percentage of the nuclei in cycloheximide-arrested embryos break down, whereas the oocyte nuclei do not. Since the response is graded over a wide range of concentrations, the breakdown of nuclei in arrested embryos may be a useful assay for MPF.

The ability of MPF to induce nuclear envelope breakdown is obviously independent of translation, since the effect occurs in the presence of cycloheximide; it is also independent of transcription, since there is no transcription normally during this period of development, even in the presence of cytochalasin B (28). In addition, we have shown that nuclear envelope breakdown after MPF injection proceeds in the presence of either 100 μM 8-Br-cAMP, 0.5 mM colchicine, 20 mM sodium fluoride (a phosphatase inhibitor) or 1 μg/ml R24571 (a potent calmodulin inhibitor)(Janssen Pharmaceuticals, Beerse, Belgium), when any of these are coinjected with MPF.

Lack of amplification of MPF in cycloheximide-arrested embryos

Prior to addition of exogenous MPF, the endogenous level of MPF in the cycloheximide-arrested embryo has been found to be below the limit of detectability. This is consistent with previous results of Wasserman and Smith (37), who found no MPF activity in cycloheximide-treated fertilized eggs. However, once MPF is injected into the arrested embryos, it is not amplified to detectable levels. This is determined in the following experiment: cycloheximide-arrested embryos are injected with 50 nl of a 1:10 dilution of partially purified MPF. An extract is made of the recipient embryos using conditions which stabilize MPF. This extract, in turn, is injected into immature oocytes to test for the presence of amplified MPF. To be able to detect if MPF activity is transiently amplified and subsequently lost, extracts were made at various times after injection of MPF. No maturation is observed in oocytes injected with extracts made 5, 10, 20 or 30 minutes after injection of MPF. A similar experiment using unfertilized eggs as the source of the injected extract produces maturation in 6/6 oocytes, even

when the extract is diluted threefold before injection. It has also been shown that the levels of MPF during the early cell cycle are as high as those in the maturing oocyte and unfertilized egg (43). Therefore, the level to which the injected MPF is amplified in cycloheximide-arrested embryos is at least threefold less than normal.

Not only is the MPF not amplified in these arrested embryos, but there is indirect evidence to indicate that MPF may be inhibited or degraded at times long after injection. The nuclei in arrested embryos undergo nuclear envelope breakdown within a few minutes in response to MPF. However, if the MPF-injected embryos are left for much longer periods of time (eg., two hours), the nuclei appear as masses of small Hoechst-staining vesicles. This morphology is typical of nuclei which are in the process of reforming. If we postulate that the presence of MPF is necessary to keep the nuclear envelope broken down, then this observation could indicate that MPF activity may disappear after long periods of time. We cannot measure the MPF levels in these embryos directly, since the assay involves a 20-fold dilution of the cytoplasm to be tested in the recipient oocyte, which would put unamplified levels of MPF in the embryo below the level of detectability. However, there are indirect ways to determine the fate of the injected MPF, and we are investigating further the loss of MPF activity at times long after its injection.

DISCUSSION

Periodic surface contraction waves, which are a manifestation of a cytoplasmic cell cycle oscillator in early Xenopus embryos, are blocked by inhibition of protein synthesis. This block appears to occur at a

specific point in the cell cycle. Although protein synthesis is inhibited greater than 95% within five minutes of injection of cycloheximide, the embryos go on to initiate and complete one more round of DNA synthesis, arresting after S phase. Upon cytological examination, the nuclear envelopes in the arrested embryos are found to be intact, and the chromatin relatively dispersed. Therefore, these cycloheximide-arrested embryos have not yet entered into mitosis; they appear to be blocked in a G2-like state.

The state of this arrest is consistent with observations made using sea urchin eggs. Shimada has shown that sea urchin eggs always initiate and complete one round of DNA synthesis subsequent to inhibition of protein synthesis (personal communication). Also, Wagenaar and Mazia (35) have shown that if sea urchin eggs are treated with emetine from the time of fertilization, the one-cell embryo is arrested with its nuclear envelope intact.

It may initially seem surprising that such a general block as inhibition of protein synthesis does not simply kill these embryos, instead of arresting their cell cycle at a specific point. However, in early Xenopus development, protein synthesis is not necessary for growth (since there is no growth during this stage of development) and many of the "housekeeping molecules" (such as histones (39), DNA polymerases (1), tubulin (29), small nuclear RNAs (45), and actin (3)) exist in large intracellular stores accumulated during oogenesis. Since new protein synthesis is not required for many of the basic functions, and also since there is no transcription during this stage, it is conceivable that translation may be playing a more specialized role in these early embryos. For example, many events which occur during this

time (including cell-cycle specific events) may be regulated by the active synthesis (and degradation) of certain proteins. Thus, it could be rationalized that inhibition of protein synthesis has a less global effect on these embryos than in cells which require continuous protein synthesis for growth.

When cleavage-arrested embryos blocked with cycloheximide are injected with MPF, there is a rapid and striking change in the appearance of their nuclei. As visualized by Nomarski optics, the characteristic ridge of the nuclear envelope disappears, and in paraffin sections there is no staining of the nuclear envelope. Furthermore, indirect immunofluorescence with antibodies to lamins A and C shows that the nuclear lamina is dispersed, as is typical of mitotic cells. This is the first demonstration that MPF purified from unfertilized eggs can cause an effect in somatic cells. MPF seems to be able to initiate the next event in the cell cycle which these nuclei would normally undergo (i.e., the first events in mitosis). Additionally, these studies provide a way to experimentally induce nuclear envelope breakdown.

MPF was originally discovered as an activity in the cytoplasm of unfertilized eggs which could initiate a complex series of cortical, cytoplasmic and nuclear events, resulting in meiosis and maturation of immature oocytes. This activity is assayed by the ability of the cytoplasm to induce germinal vesicle breakdown in oocytes. It has been shown by Wasserman and Smith (37) that the cytoplasm from fertilized eggs and early embryos also possesses the ability to induce germinal vesicle breakdown in oocytes. This activity oscillates during the early cleavage period and reaches peaks at times corresponding to mitosis in the embryos. We have now shown that the MPF purified from unfertilized

egg cytoplasm can induce the breakdown of embryonic nuclear envelopes during the early cleavage cycle.

The MPF used in these experiments has been purified about thirty fold. Although it is not purified to homogeneity, we have provided evidence that it is MPF, and not some protein which co-purifies with MPF, which is responsible for nuclear envelope breakdown. MPF can be distinguished from many other cellular proteins in that its activity fluctuates with respect to the cell cycle. Since the ability to break down nuclear envelopes correlates temporally exactly with MPF activity, we can say by this functional definition that it is MPF which causes the breakdown of these nuclear envelopes.

The effect of MPF on nuclear envelopes can be used as a sensitive assay for MPF, since the concentration of MPF is proportional to the fraction of nuclei which break down. This relationship does not have a sharp threshold, which is puzzling in view of the fact that, at least morphologically, the nuclei upon which MPF acts appear to be identical. At least two possibilities exist to explain why this curve does not show a sharp threshold as is the case with the oocyte assay. MPF could be acting stoichiometrically upon the nuclei. In other words, below a certain ratio of MPF molecules to nuclei, there would be no nuclear envelope breakdown. Thus at low dilutions, there would only be sufficient MPF molecules to break down a fraction of the nuclei. Alternatively, there may be heterogeneity among these nuclei, such that some are more sensitive to MPF than others. In this hypothesis, if the behavior of each individual nucleus could be followed, it would show a sharp threshold as in the oocyte assay. However, for all the nuclei in one embryo, these thresholds would occur at a range of MPF

concentrations and when the individual thresholds are summed, a curve without a sharp threshold would result for the overall behavior of the embryo's nuclei.

The fact that the levels of MPF needed to induce nuclear envelope breakdown in cycloheximide-arrested embryos are comparable to or lower than those required for oocyte maturation (figure 6), raises interesting questions about the role of MPF amplification. Injection of MPF in the oocyte can result in amplification of 150 to 300 fold, observable at the time of germinal vesicle breakdown. However, in cycloheximide-arrested embryos, MPF is not amplified to such levels, either at the time of nuclear envelope breakdown, or at later times (up to thirty minutes after injection of MPF). Thus, it seems that only low levels of MPF are needed to induce breakdown of the nuclear envelope.

How does MPF cause nuclear envelope breakdown? It is clear that the effect of MPF is due to post-translational changes, since it occurs rapidly in the presence of cycloheximide. The molecular basis of this effect is unknown. However, there is indirect evidence linking MPF activity to protein phosphorylation. Many of the extraction conditions which stabilize MPF activity also stabilize phosphoproteins and/or inhibit phosphatases. For example, both γ-thio-ATP (a good kinase substrate which can be hydrolyzed very poorly by phosphatases (6)) and β-glycerophosphate stabilize MPF and also competitively inhibit phosphatases. Furthermore, Maller, Wu and Gerhart (24) have shown that there is a 2.5-fold increase in total protein phosphorylation immediately following injection of MPF into immature oocytes. This burst of phosphorylation shortly precedes germinal vesicle breakdown,

and is (to date) experimentally inseparable from germinal vesicle breakdown.

Not only is MPF implicated in phosphorylation, but there is also a link between the nuclear envelope lamina and phosphorylation. Gerace and Blobel (9) have shown that lamins A, B and C, the major proteins of the nuclear lamina underlying the nuclear envelope, are more highly phosphorylated in mitotic cells than in interphase cells. They propose that this hyperphosphorylation may be involved in the depolymerization of the nuclear lamina, and have put forth a model in which the nuclear lamina directly mediates the breakdown and reformation of the nuclear envelope (10).

In view of the evidence cited above, one possibility for the mechanism of nuclear envelope breakdown by MPF is a phosphorylation cascade, similar in concept to the activation of glycogen phosphorylase (see reference 4 for a review). In such a scheme, MPF formation and breakdown may be part of the cytoplasmic cell cycle oscillator, or the oscillator may regulate the synthesis of an activator/kinase, which shifts MPF into an active phosphorylated state. Activated MPF could then initiate a chain of phosphorylation events resulting ultimately in the increased phosphorylation of lamins and the breakdown of the nuclear envelope. This would account not only for the hyperphosphorylation of the lamins in mitotic cells and the apparent phosphorylated nature of MPF, but also for the ability of MPF activity to be amplified in the oocyte in the absence of protein synthesis. This model can be tested by observing biochemical changes in the lamins in response to the addition of MPF to cycloheximide-arrested embryos. The rapid response *in vivo* of nuclei to partially purified preparations of MPF led me to explore the

possibility that the same response could be reproduced in an *in vitro* system. Such experiments will eventually lead to an understanding at the molecular level of how a cytoplasmic oscillator can effect specific biochemical events in the cell cycle.

TABLE 1

MPF is Responsible for Nuclear Envelope Breakdown

Recipients	FRACTION OF NUCLEI OR GERMINAL VESICLES BROKEN DOWN Source of donor extract		
	immature oocyte	mature egg	activated egg
immature oocytes	0/6	6/6	0/6
arrested embryos*	8/153=5%	99/119=83%	4/60=7%

*when arrested embryos were either injected with dilution buffer or not injected at all, 7% of the nuclei were damaged in the squash.

FIGURE LEGENDS

Figure 1: Timing of DNA synthesis in normal and cycloheximide-treated fertilized eggs.

Each lane on the gel is the nucleic acid extracted from a group of three fertilized eggs which have been pulse-labeled with α-^{32}P-dCTP. Labelling was started at the times indicated (shown as minutes after fertilization) by injection of 50nl of α-^{32}P-dCTP. After ten minutes, eggs were lysed in buffer containing 1% SDS to terminate the labelling. Nucleic acid isolated from these cells was run on a 1% agarose gel and autoradiographed for two days. The top quarter of each gel is shown in the figure, since chromosomal DNA migrates near the upper limit of resolution on a 1% agarose gel (greater than 55 kb), and is the only band labelled on the gel.

(a) normal fertilized eggs, (b) cycloheximide (50 nl of 200 µg/ml) injected 12-20 minutes post-fertilization, before first round of DNA synthesis, (c) cycloheximide injected 38-48 minutes post-fertilization, before second round of DNA synthesis.

Figure 2: Detail of paraffin section of arrested cell showing nucleus before and after injection of MPF.

Cells were blocked with cycloheximide as described in Materials and Methods. One hour after injection of cycloheximide, cells were either either fixed (a), or injected with 50 nl of partially purified MPF, incubated for an additional thirty minutes, and then fixed (b). After fixation, both samples were embedded in paraffin, sectioned, and stained with hematoxylin and chlorazol black E. Bar, 10 µ.

Figure 3: Morphology of nuclei in a squash of a cycloheximide-arrested embryo before and after injection of MPF.

Cells were blocked with cycloheximide as described in Materials and Methods. Either before (a and b) or after (c and d) injection of MPF, ten embryos were very gently lysed into 100 µl of buffer containing 1% formaldehyde, allowed to fix for 20 seconds, then mixed thoroughly to detach adhering yolk platelets. This method considerably diluted the amount of surrounding yolk, allowing a clearer image of the nucleus to be seen. However, it also tended to damage a higher percentage of the nuclei. Thus, for purposes of quantitation, simple squashes without agitation were used. Nuclei were then visualized by Nomarski optics (a and c), which showed the presence of the nuclear envelope as a ridge at the periphery of the nucleus (a), which disappeared after MPF treatment (c). b and d show fluorescent Hoechst staining of the DNA in these same nuclei. Bar, 10 µ.

Figure 4: Cross-reactivity of human autoimmune anti-lamin serum with Xenopus nuclear lamina .

(a) Indirect immunofluorescence staining (see Materials and Methods) of Xenopus epithelial cell line (A6) with human serum from a patient with linear scleroderma, containing antibodies against the major nuclear lamina proteins. Note the diffuse staining in the mitotic cell marked M. Bar, 10 µ. (b and c) SDS gel electrophoresis of A6 cells after extraction with Triton X-100 and digestion with micrococcal nuclease. (b) Nitrocellulose transfer of gel reacted with human anti-lamin serum and ^{125}I goat anti-human immunoglobulin secondary. (c)

Identical gel stained with Coomassie blue. Molecular weight markers are shown on right in kilodaltons.

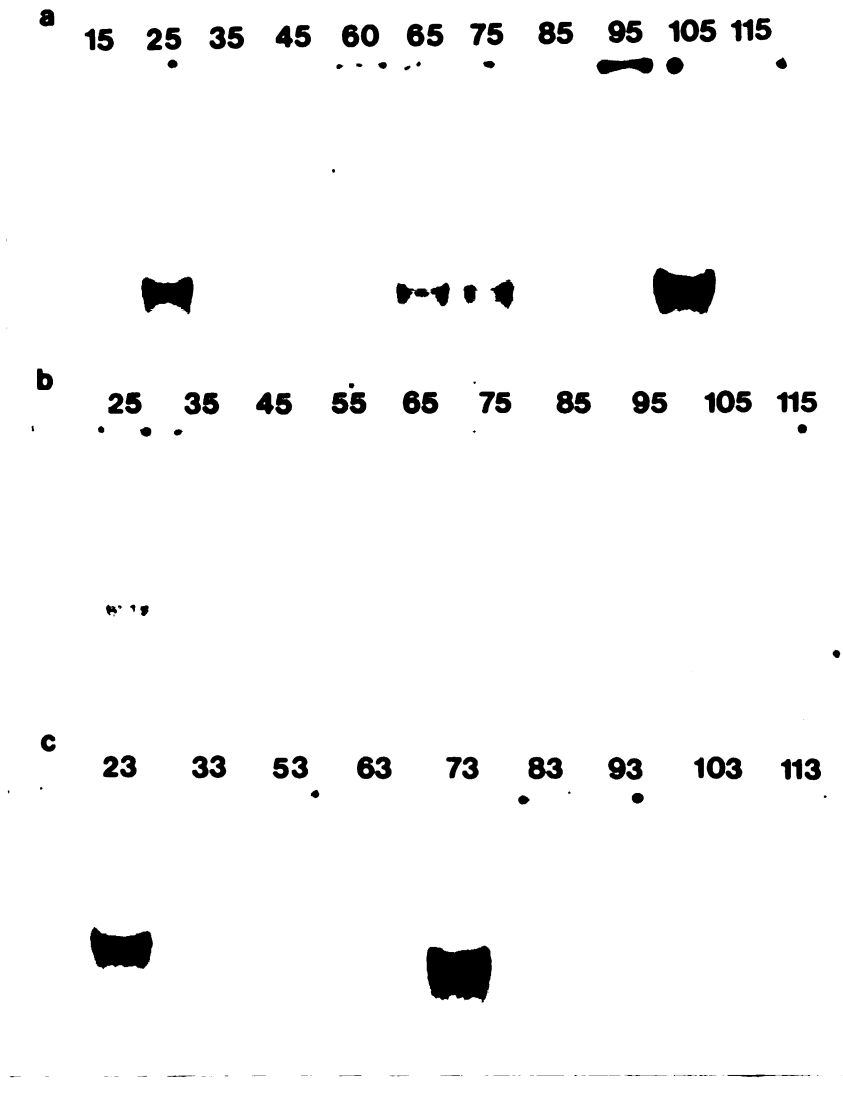
Figure 5: Indirect immunofluorescence staining of the nuclear lamina in lysates of arrested embryos.

Arrested embryos were gently lysed into 2% formaldehyde either before (a and b) or after (c and d) injection of MPF. The lysates were allowed to settle onto coverslips coated with cytochrome c, processed for indirect immunofluorescent staining of the nuclear lamina and stained with Hoechst bisbenzamid (see Materials and Methods). Nuclei in the sections were located by fluorescence of the Hoechst-stained DNA (b and d). Before MPF, a continuous lamina is visible (a); whereas afterwards (c), very little staining of the lamina can be seen. e and f show indirect immunofluorescence and Hoechst-staining, respectively, of a nucleus before MPF treatment using normal human serum. Bar, 10 μ.

Figure 6: Dependence of nuclear envelope breakdown on the concentration of MPF injected.

Partially purified MPF was injected at various dilutions into arrested embryos (closed circles) or immature oocytes (open circles). Undiluted MPF equals 1.0 concentration unit. In the case of arrested embryos, the fraction of nuclei broken down in a squash of an embryo was scored by Hoechst dye staining within thirty minutes after injection of MPF. Immature oocytes were scored for germinal vesicle breakdown three hours after injection of MPF. At each concentration, six oocytes were injected with 30 nl of the appropriate dilution of MPF.

Figure 1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b
25 35 45 55 65 75 85 95 105 115
c
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Figure 2

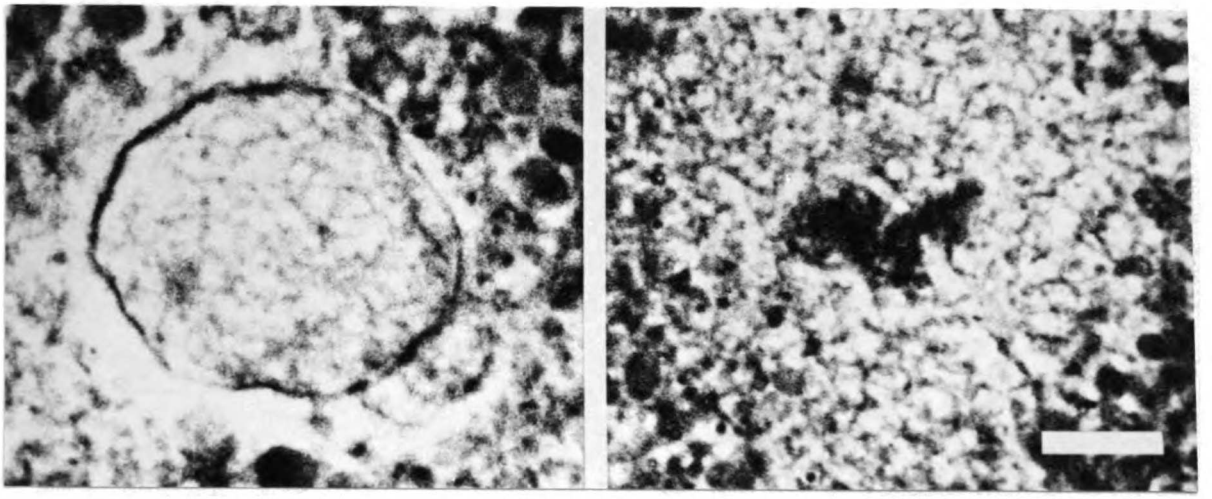
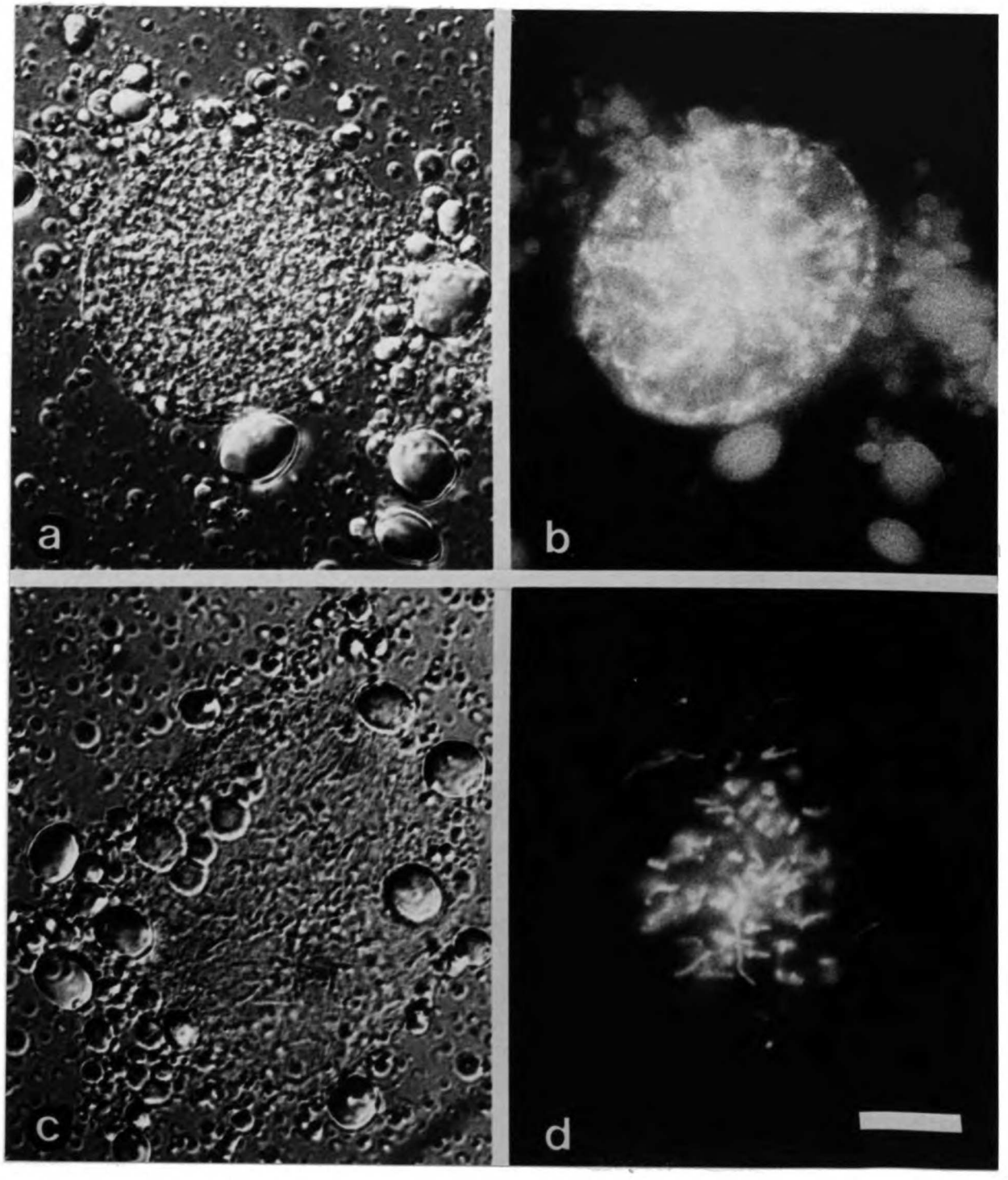


Figure 3



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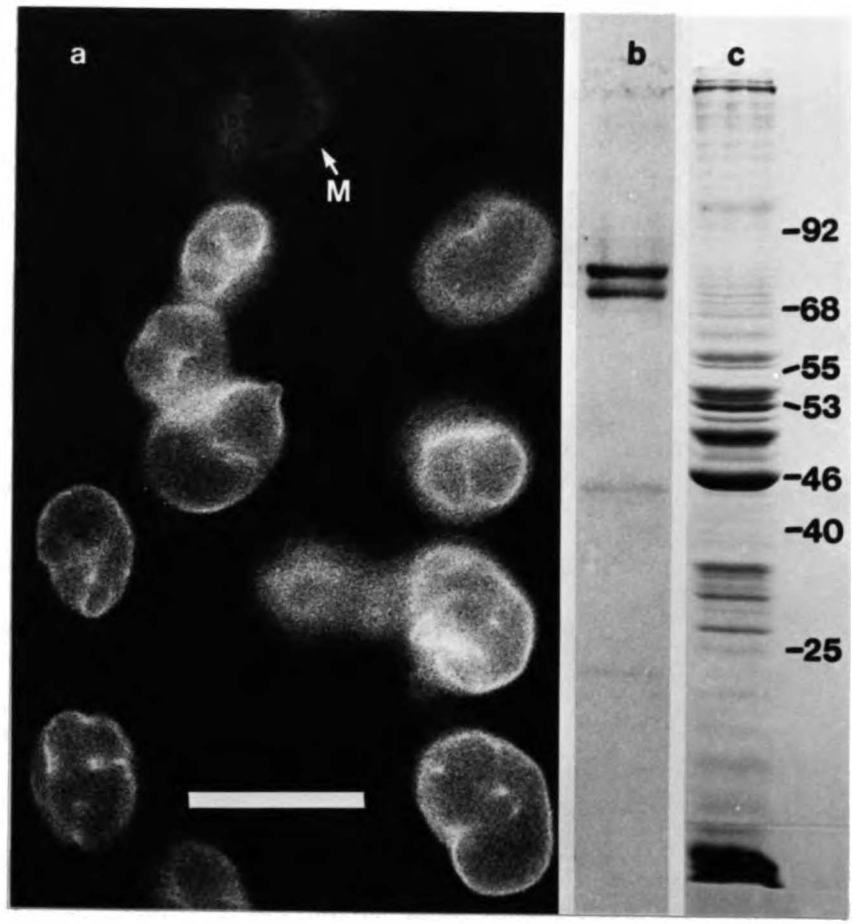


Figure 5

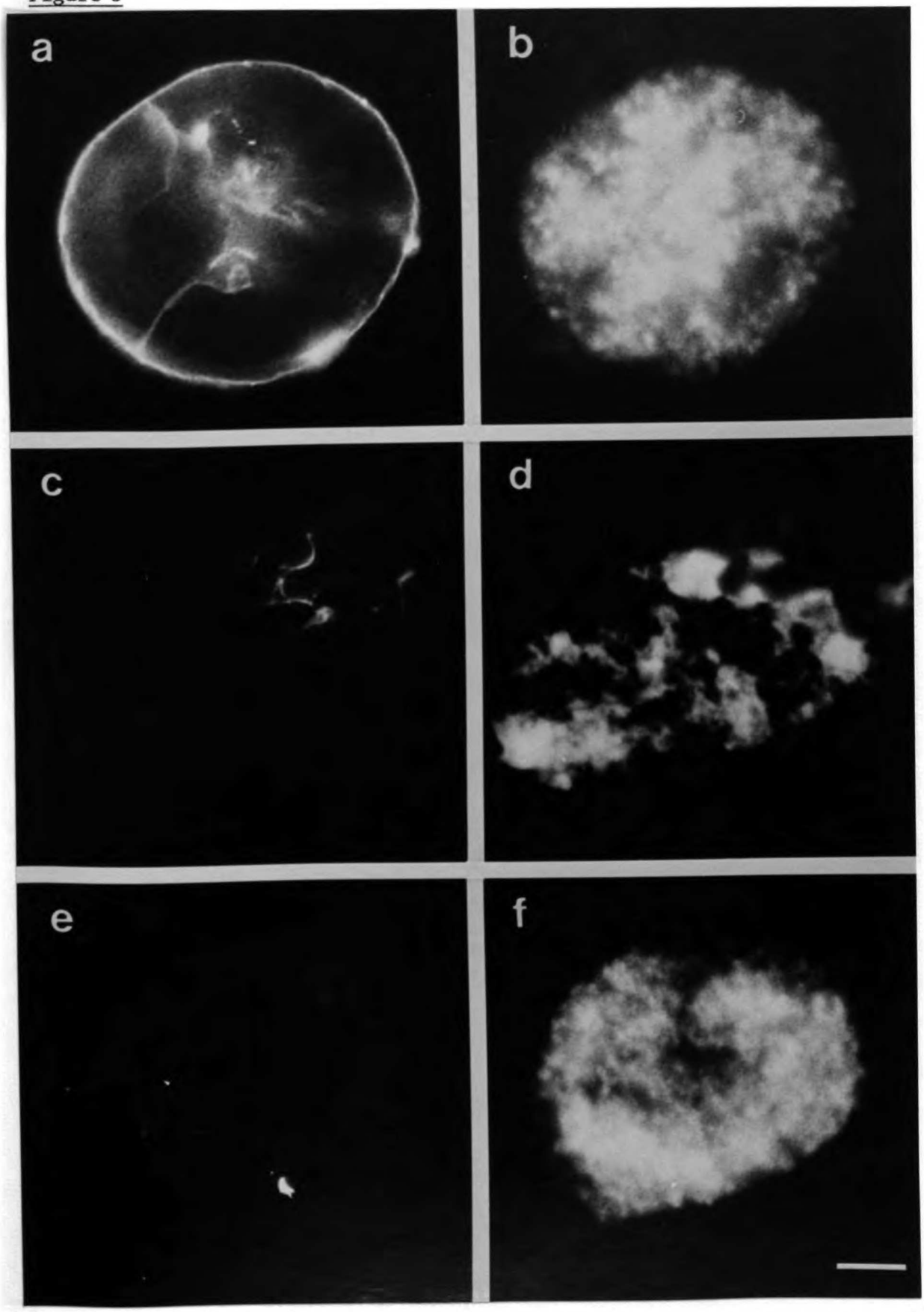
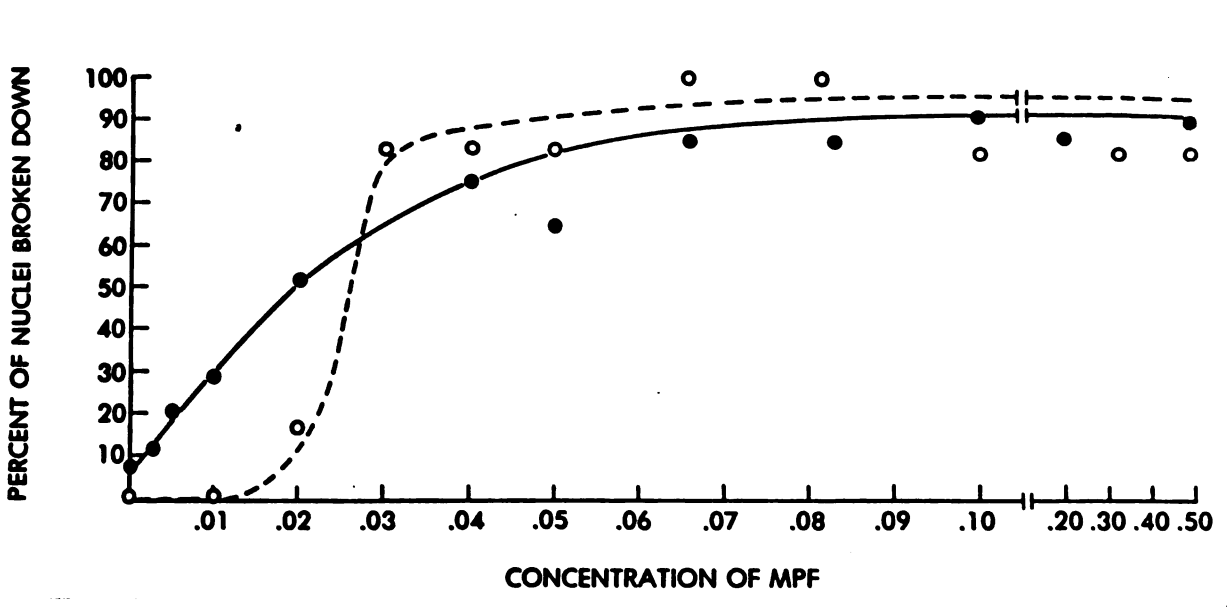


Figure 6


PERCENT OF NUCLEI BROKEN DOWN
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CONCENTRATION OF MPF

CHAPTER 3

Induction of Early Mitotic Events in a Cell-Free System

SUMMARY

The major events of prophase - chromatin condensation and nuclear envelope breakdown - have been induced by partially purified maturation promoting factor (MPF) in somatic interphase nuclei that are incubated in a cell-free extract of Xenopus embryos. MPF has previously been shown to induce meiosis in Xenopus oocytes, and nuclear envelope breakdown and chromosome condensation in Xenopus embryos arrested at the end of S phase. These events occur rapidly and synchronously in response to the addition of MPF and are reversed when MPF activity disappears. Forty min after MPF addition, nuclear envelopes are dispersed and individual chromosomes are visible. Using this cell-free system, we have examined the temporal relationships among nuclear envelope breakdown, depolymerization of the underlying nuclear lamina, and increased phosphorylation of lamins, the structural proteins of the nuclear lamina. We show that lamins A and C are hyperphosphorylated between 10 and 15 min after MPF addition, followed by a gradual depolymerization of the nuclear lamina. Immunofluorescent staining of nuclei for lamins A and C decreases until it is undetectable 40 min after addition of MPF, when nuclear envelope breakdown occurs. These results show that MPF can induce mitotic events in vitro, and suggest that hyperphosphorylation of the lamins could act to trigger the depolymerization of the nuclear lamina, and in turn, nuclear envelope breakdown.

INTRODUCTION

The transition from interphase to mitosis is highly complex, involving chromatin condensation, nuclear envelope breakdown, reorganization of cytoskeletal arrays, and mitotic spindle formation (Karsenti et al, 1984). From nuclear transplantation and cell fusion studies, there is evidence that this transition is under cytoplasmic control. Gurdon (1968) demonstrated that nuclei transplanted into Xenopus oocytes become entrained to the cell cycle state of the cytoplasm. Furthermore, when an interphase nucleus is exposed to cytoplasm from a mitotic cell by cell fusion, it undergoes premature chromatin condensation and nuclear envelope breakdown (Rao and Johnson, 1970; Matsui et al, 1972). Although it appears that this transition is regulated by cytoplasmic factors, the biochemical mechanisms that underlie the interphase-to-mitosis transition have been impossible to study in the absence of an in vitro system in which entry into mitosis could be synchronously induced.

On the basis of its activity in vivo, maturation promoting factor (MPF) is an excellent candidate for an inducer of the interphase-to-mitosis transition. MPF, a partially purified protein (Wu and Gerhart, 1980), was originally isolated for its ability to induce meiosis in frog oocytes (Masui and Markert, 1971; Smith and Ecker, 1971). MPF activity has been found in a wide variety of cells in M-phase (Kishimoto et al, 1982, 1984), including yeast (Weintraub et al, 1982) and mammalian cultured cells (Sunkara et al, 1979; Nelkin et al, 1980). A role for MPF in the mitotic cell cycle was suggested by the fact that its activity fluctuates with the same period as the cell cycle, with peaks during mitosis (Wasserman and Smith, 1978; Gerhart et al, 1984).

More directly, MPF has been shown to induce nuclear envelope breakdown and chromatin condensation within 5 min of injection into early Xenopus embryos that have been arrested at the end of S phase by cycloheximide treatment (Miake-Lye et al, 1983; Halleck et al, 1984a). In fact, Newport and Kirschner (1984) have recently shown that addition and breakdown of MPF activity is sufficient to drive the cell cycle of Xenopus eggs. Thus, the problem of cell cycle regulation in this simplified system can be reduced to questions of 1) what is the pathway by which MPF acts to induce the interphase-to-mitosis transition, 2) how is MPF itself regulated, and 3) which events are dependent on MPF, and which are not?

In this work, we begin to explore the pathway of mitotic events that occur in response to MPF. We describe a cell-free system in which parts of the mitotic process can be induced by MPF, using interphase somatic nuclei as a substrate. This system gives us the first opportunity to determine the temporal relationships between biochemical changes that have been correlated with mitosis and the morphological changes of mitosis. To date such studies have been impossible because of the difficulty of obtaining highly synchronized premitotic cells and because of the relatively short duration of mitosis. Although a given biochemical event can be correlated with mitosis, in vivo systems lack the temporal resolution to ask whether the biochemical event could be a cause or a result of a particular morphological change.

In particular, the major structural proteins of the nuclear lamina, lamins A, B, and C, have been shown by Gerace and Blobel (1980, 1982) to be hyperphosphorylated during mitosis, compared with lamins in interphase cells. They propose that this biochemical change may be

directly responsible for a key event of mitosis, which has been defined morphologically as the breakdown of the nuclear envelope. However, it is unknown when the increase in phosphorylation occurs relative to nuclear envelope breakdown.

Using our cell-free system, we are able to directly examine the temporal relationship between nuclear envelope breakdown and hyperphosphorylation of the lamins. We show that the increase in lamin phosphorylation occurs 25 - 30 min before nuclear envelope breakdown. Concomitant with the increase in lamin phosphorylation, we observe a structural weakening of the nuclear envelope, followed by the gradual depolymerization of the nuclear lamina.

EXPERIMENTAL PROCEDURES

Preparation of nuclei

Chinese hamster ovary (CHO) cells were grown in monolayer in minimum essential medium alpha without nucleosides, supplemented with 10% fetal calf serum. Cultures were radioactively labeled 10 - 16 hr in methionine-free medium containing 100 μCi/ml ^{35}S-methionine and 10% normal medium.

7 week old female Sprague-Dawley rats were used for thymocytes. The thymus was minced into phosphate buffered saline, and filtered through a 50 μ Nitex screen to obtain a single-cell suspension.

After sedimenting either type of cell, the pellet was resuspended in a small volume of Buffer A (80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EDTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 15 mM PIPES, pH 7.4) and added to 10 volumes of cold Buffer A containing 0.2% Triton X-100. After a 3 min extraction on ice, the nuclei were pelleted in a

swinging-bucket rotor at 100 g for 5 min. The pellet of nuclei was resuspended in 1 volume of Buffer A without detergent.

Preparation of extract

Xenopus eggs were fertilized and dejellied as described in Newport and Kirschner (1982). About 30' after fertilization, eggs were transferred into MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1 mM EDTA, 5 mM HEPES, pH 7.8) containing 5% (w/v) ficoll and 100 µg/ml cycloheximide. After 1 hr incubation at room temperature, the eggs were rinsed twice in an equal volume of MPF extraction buffer (Wu and Gerhart, 1980) containing 250 mM sucrose and 10 µg/ml cytochalasin B. The interstitial volume between the eggs was minimized by centrifuging the eggs at 35 g for 1 min, and immediately removing the excess buffer. The eggs were lysed using a P200 Pipetman and centrifuged at 12,000 g for 5' at 4°C. The cytoplasmic layer (between the lipid supernatant and the yolk pellet) was removed. Creatine phosphokinase was added to 5 µg/ml, and creatine phosphate to 10 mM.

Typically, approx. 10^5 nuclei were incubated in 30 µl of extract for 2 hr at room temperature. Nuclear envelope breakdown and chromatin condensation were induced by the addition of 1 µl of MPF. The MPF used in these experiments was purified (approximately 30-fold), through the polyethylene glycol precipitation step, according to Wu and Gerhart (1980).

Immunoprecipitation analysis

At the appropriate time after MPF addition, 100 µl reactions were stopped by adding 0.9 mls of 0.5% SDS, 50 mM Tris (pH 7.4), 100 mM NaCl and 2 mM EDTA (Gerace and Blobel, 1980), and boiling for 2 min. Triton X-100 was added to 2% and a mixture of protease inhibitors (2.5 mg/ml

leupeptin, 2.5 mg/ml aprotinin, 100 mM benzamidine, 50 mM PMSF, 1.0 mg/ml pepstatin) was diluted 100-fold into each sample. Samples were cleared of large insoluble aggregates by centrifugation for 2 min at 12,000 g.

Immunoprecipitation was carried out by adding 4 µl human autoimmune serum against lamins A and C (McKeon et al, 1983) to each sample and incubating 30 min at room temperature. Twenty µl of an immunoadsorbent, Pansorbin, was added and incubated for 5 min. The remainder of the immunoprecipitation procedure was carried out according to Gerace and Blobel (1980).

Electrophoresis

2-D NEPHGE gels were prepared and run according to O'Farrell et al (1977), using LKB ampholines. Samples for 2-D gels were prepared as described in Gerace and Blobel (1980). After electrophoresis, the gels were silver stained (Merrill et al, 1981), treated with en^{3}Hance and autoradiographed.

Immunofluorescence

At the appropriate time, 30 µl cell-free reactions were fixed by dilution into 1 ml of 10 mM ethylene glycol bis (succinimidyl succinate) (EGS) in Buffer A without sucrose, spermine, or spermidine. The fixative was prepared immediately before use by diluting 100 mM EGS in DMSO into Buffer A. After fixation for 10 - 15 min at 37°C, each sample was layered onto a cushion of Buffer A containing 33% glycerol in a tube with a polylysine-coated coverslip at the bottom. Nuclei and chromosomes were sedimented onto the coverslip in a swinging bucket rotor at 10,200 g for 10 min.

The procedure for immunofluorescent staining of the coverslips was the same as in Miake-Lye et al (1983), except that the wash solution and the antibody dilutent used was Buffer A without sucrose (but with spermine and spermidine), instead of phosphate buffered saline. To estimate the intensity of lamin staining, photographic negatives of nuclei were digitized using an image processor. The optical density of a small area (49 pixels) at the edge of the nucleus was averaged, and the optical density of the background was subtracted for each measurement.

Electron microscopy

100 µl cell-free reactions were diluted into 1% glutaraldehyde in Buffer A without sucrose or polyamines, and fixed 10 min on ice. Nuclei were pelleted through a cushion of 33% glycerol in a swinging-bucket rotor at 15,900 (10K rpm) for 10'. The pelleted material was post-fixed in osium textroxide, stained with uranyl acetate, dehydrated and embedded in Araldite. Thin sections were cut and viewed at 7000 - 9000x.

RESULTS

Establishment of an MPF-dependent cell-free system

To test the ability of MPF to act in vitro, we extended our previous findings that in vivo, Xenopus embryonic nuclei arrested at the end of S phase (by cycloheximide treatment) undergo nuclear envelope breakdown and chromatin condensation rapidly in response to MPF (Miake-Lye et al, 1983; Halleck et al, 1984a). In preliminary experiments using thymocyte nuclei injected into arrested Xenopus embryos, we determined that the nuclei needed to be incubated in the cytoplasm for a minimum of two hours to become entrained to the stae of the cytoplasm. Nuclei incubated for shorter times failed to condense

their chromatin in response to subsequent injection of MPF. To determine the initial conditions for the in vitro system, we preincubated nuclei from either thymocytes (Fig. 1 a,b) or CHO cells in a cell-free extract of such arrested embryos, before adding MPF.

After a two hour incubation (Fig. 1 c,d), the nuclei had increased in volume approx. 20-fold, and were all in an interphase configuration. Heterochromatin in these nuclei was completely decondensed, and nucleoli were dispersed. This was particularly apparent in the CHO nuclei (compare figures 4a and 4b). Nuclear swelling, chromatin decondensation and nucleolar dispersal have been observed when somatic nuclei are microinjected into Xenopus oocytes (Gurdon, 1976).

After this incubation, nuclear envelope breakdown and chromatin condensation were induced by the addition of maturation promoting factor (MPF). The response of thymocyte nuclei to MPF is rapid, uniform and synchronous. By contrast, only 75 - 90% of CHO nuclei respond to MPF. However, both thymocyte and responding CHO nuclei show the same kinetics in responding to MPF. In both cases, chromatin condensation starts to become apparent 25 - 30 min after addition of MPF. Forty min after the addition of MPF, nuclear envelopes are dispersed and individual chromosomes are visible (Fig. 1 e,f). While the timing of nuclear envelope breakdown is very consistent and reproducible, the extent of chromosome condensation is somewhat more variable. Furthermore, chromatin condensation is often not complete until after nuclear envelope breakdown.

The induction of mitotic events is completely dependent upon addition of MPF. In the absence of MPF, the nuclei remain arrested in an interphase state. The final concentration of MPF needed to completely

induce nuclear envelope breakdown and chromatin condensation in vitro is approximately 15-fold higher than is required in vivo (Miake-Lye et al, 1983). As the amount of MPF was decreased below this level, fewer nuclear responded to MPF; it was difficult to quantitate this trend more precisely, since the exact levels of MPF required for a complete response varied as much as 3-fold, depending on the cell-free extract used.

The existence of the mitotic state is transient. Sixty to 70 min after MPF addition, chromosomes spontaneously decondense and are re-packaged into nuclei (Fig. 1 g,h). Nuclear envelope breakdown occurs 40 min after MPF addition; thus the mitotic state lasts only 20 - 30 min. It is probable that the return to the interphase state is due to the inactivation or degradation of the added MPF, since adding MPF 3 times at 15 min intervals can prolong the lifetime of the mitotic state, to greater than 1 hr. The instability of MPF activity is consistent with what we know about the state of cycloheximide-arrested embryos. In this state, there is no endogenous MPF activity, and there is an MPF-inactivating factor that is present at times in the cell cycle when MPF activity is not present (Adlakha et al, 1983; Gerhart et al, 1984).

We found that the extraction buffer used in the purification of MPF was optimal for preparing the cell free extracts, probably because it maximized the stability of MPF added to induce mitotic events. The extract was free of yolk platelets and embryonic nuclei although other organelles (such as mitochrondria) remained in the supernatant (see Fig. 4). Nuclei incubated in extracts that had been frozen did not respond to MPF, although nuclear swelling and decondensation of heterochromatin did occur. Nuclei were not stable in extracts prepared by centrifugation at

100,000 g for 30' also lost MPF responsivity and became abnormally condensed, even when the pellet and supernatant were re-mixed.

The induction of nuclear envelope breakdown and chromatin condensation requires ATP and a high concentration of cytoplasm in the cell-free extract. In the extract used here, cytoplasm was diluted only one-third to one-quarter by buffer (as determined by isotope dilution). If the buffer exceeded one-half the volume of the extract, no induction was observed. No effect of MPF was seen in the absence of an ATP regenerating system, although 5 mM ATP in the buffer could substitute less effectively for the regenerating system. The induction was also abolished by omitting magnesium in the buffer, increasing the concentration of β-glycerophosphate to 120 mM or lowering the temperature of the reaction to 4°C. However, β-glycerophosphate was not required, since its omission from the buffer permitted normal induction.

Relationship of lamin phosphorylation to nuclear envelope breakdown

Using the cell-free system, we have examined the timing of lamin phosphorylation relative to nuclear envelope breakdown. It was necessary to first determine whether lamin phosphorylation actually occurred in the cell-free reaction. To test whether lamins are phosphorylated in response to MPF, ^{35}S-methionine-labeled CHO nuclei were incubated in unlabelled cell-free extract, so that the CHO nuclear proteins could be seen independently from the extract proteins (which are present in roughly 1000-fold excess over the CHO proteins). Forty min after adding MPF to induce a mitotic state, lamins A and C were isolated by immunoprecipitation from parallel reactions, with and without MPF. The immunoprecipitates were resolved on 2-D NEPHGE gels (O'Farrell, 1977). Figure 2 shows autoradiograms of the gels from this experiment. It can

be seen that lamins A and C undergo an acidic shift in their isoelectric point in response to MPF. Such a shift is indicative of phosphorylation; we showed that the shift was indeed due to phosphorylation, since the shift could be nearly completely reversed by treatment with alkaline phosphatase (Fig. 3e).

We could now determine the timing of the lamin hyperphosphorylation relative to nuclear envelope breakdown by following the time course of lamin phosphorylation after addition of MPF using immunoprecipitation and 2D gel analysis. Again, nuclei were prepared from CHO cells that had been metabolically labeled with ^{35}S-methionine and incubated in unlabelled extract. At various times after MPF addition, the reaction was quenched by boiling in SDS. Figure 3 a-d shows autoradiograms of lamins A and C immunoprecipitated at various times after MPF addition, and resolved on 2-D NEPHGE gels. It is clear that the entire increase in phosphorylation takes place within 15 min of MPF addition. This is long before nuclear envelope breakdown at 40 min after MPF addition. Time points taken 2, 5, 10 and 15 min after MPF addition show that no increase in phosphorylation has taken place 10 min after MPF addition; thus virtually the entire increase in phosphorylation occurs between 10 and 15 min after MPF addition.

Because lamin hyperphosphorylation occurs long before (25-30 min) nuclear envelope breakdown is detectable by light microscopy, we verified that no morphological change in the nuclear envelope had occurred concomitant with lamin phosphorylation using electron microscopy. Nuclei incubated in cell-free extract were fixed in 1% glutaraldehyde at various times after addition of MPF. Figure 4 shows micrographs of thin sections cut from 2 samples: one before the addition

of MPF (Fig. 4b), and 20 min after the addition of MPF, when the lamins are fully phosphorylated. In both cases, the nuclei have a definite boundary. At the time the lamins are fully phosphorylated, no obvious morphological change in the nuclear envelope is observable by electron microscopy (fig. 4).

From this study, we conclude that hyperphosphorylation of the lamins does not occur as an immediate response to MPF, but does occur well before nuclear envelope breakdown. This order of events is consistent with lamin phosphorylation being necessary (but not sufficient) for nuclear envelope breakdown.

Relationship of depolymerization of the nuclear lamina to nuclear envelope breakdown

We have shown that lamin phosphorylation precedes nuclear envelope breakdown by 25 - 30 min (Fig. 3), and is completed at a time when there is not yet any obvious morphological change in the nuclear envelope (fig. 4). This is somewhat puzzling, in view of the fact that the phosphorylated state of the lamins correlates very well with the brief period during the cell cycle when the lamins are soluble proteins, and are not assembled into an insoluble nuclear lamina (Gerace and Blobel, 1980, 1982).

In order to determine whether lamin phosphorylation is linked to a change specifically in the nuclear lamina (as opposed to the overall breakdown of the nuclear envelope), indirect immunofluorescence was used to visualize the nuclear lamina at various times after adding MPF. At the appropriate time, cell-free reactions were fixed in solution, using ethylene glycol bis(succinimidyl succinate) (EGS), a bifunctional protein cross-linker. The nuclei and chromosomes were then sedimented

onto polylysine-coated coverslips. Lamins A and C were visualized by indirect immunofluorescence, using a human antiserum against lamins A and C (McKeon et al, 1983). Spermine and spermidine were present during the immunofluorescence procedure to stabilize chromatin structure. Using these conditions, we see two changes in the nuclear lamina that occur in response to MPF.

The first change occurs abruptly, coincident with lamin phosphorylation. At this time (between 10 and 15 min after addition of MPF), the MPF-treated nuclei appear more flattened than control nuclei sedimented under identical conditions. This is manifested by a loss of characteristic peripheral staining of the nuclei by anti-lamin serum, by an inability to "focus through" the nuclei and by an increase in the diameter of the nuclei on the coverslips (compare fig. 5a and fig. 5c). We believe the flattening of the nuclei to be indicative of a structural weakening of the nuclear envelope. Apparent weakening of the nuclear envelope before its dissolution in mitosis has been observed in vivo: the nuclear envelope, which is quite resistant to mechanical disruption during interphase (Wilson, 1925), is deformed and involuted by growing asters of microtubules during prophase (Wilson, 1925; Stafstrom and Staehelin, 1984; Moll and Paweletz, 1980).

The abrupt weakening of the nuclear envelope was followed by a gradual loss of immunofluorescent staining of the nuclear lamina (fig. 5 c, e) until the time of nuclear envelope breakdown, when no staining could be seen (fig. 5g). A rough estimate of the relative intensity of lamin staining was made by densitometry of photographic negatives of nuclei stained for lamins A and C by immunofluorescence. Densitometry verified that there was a gradual loss of lamin staining from 10 min

after MPF addition until the time of nuclear envelope breakdown (fig. 6). The gradual uniform loss of staining is suggestive of a slow depolymerization of the nuclear lamina, culminating in nuclear envelope breakdown.

The temporal relationships among lamin hyperphosphorylation, nuclear lamina depolymerization and nuclear envelope breakdown are summarized in Fig. 6. Following a 10 min lag period, the lamins are maximally phosphorylated within a 5 min interval. Coincident with the increase in lamin phosphorylation, there is an apparent weakening of the nuclear envelope, followed by the gradual depolymerization of the nuclear lamina, as assayed by the decreasing intensity of immunofluorescence of lamins A and C. During this period of lamina depolymerization, chromosome condensation begins. Finally 40 min after the addition of MPF, nuclear envelope breakdown occurs and individual condensed chromosomes can be visualized.

MPF-dependent events prior to lamin phosphorylation

The results above demonstrate that lamins A and C are not hyperphosphorylated immediately in response to MPF, but that there is a 10 - 15 min lag phase after the addition of MPF before any increase in phosphorylation occurs. To ask if there are any events induced by MPF that take place during the lag phase, we analyzed 2D gels of ^{35}S-methionine labeled CHO nuclei, at 10 min after MPF addition. More than 100 CHO proteins could be resolved, and nearly all of these proteins remained unchanged in their mobility. However, 3 species of MW 86,000, 73,000 and 65,000 had undergone an acidic charge shift within 10 min of MPF addition (fig. 7). In all three cases, the charge shift could be reversed by alkaline phosphatase (data not shown). The

phosphorylation of these 3 proteins indicates that there are early biochemical events that occur rapidly in response to MPF, before any apparent morphological changes.

DISCUSSION

Dramatic changes occur in cell structure when a cell enters mitosis. It has been virtually impossible to examine the mechanisms regulating these changes, since generally it has only been possible to study either the interphase state or the mitotic state, but not the transition between the two states. Numerous biochemical events have been correlated with morphological changes in each of these states, but assigning causal relationships to the events has been difficult, in the absence of a system where one can initiate and follow the actual transition from interphase to mitosis.

In this study, we have demonstrated the ability of partially purified purified MPF to induce nuclear envelope breakdown and chromatin condensation in a cell-free reaction. The somatic interphase nuclei in the reaction respond to MPF rapidly and synchronously, making this an easily manipulable system for understanding not only how MPF acts to induce mitotic events but also how each mitotic event is itself regulated. After incubation of either CHO or thymocyte nuclei in a cytoplasmic extract of Xenopus embryos that have been arrested at the end of S phase, MPF was added to induce chromatin condensation and nuclear envelope breakdown. Although the extent of chromatin condensation was somewhat variable, nuclear envelope breakdown occurred quite reproducibly at 40 min after the addition of MPF.

Compared to the onset of mitosis in vivo, the temporal order of morphological events is retained in the cell-free system. Prophase, the first stage of mitosis, has generally been defined as beginning when chromatin starts to condense, and as ending with nuclear envelope breakdown (e.g., Wolfe, 1972; Mazia, 1961). In the cell-free system, chromatin condensation is usually apparent 25 - 30 min after addition of MPF, followed by nuclear envelope breakdown 10 - 15 min later. The interval between these two events in vitro is roughly the same duration as prophase for cultured animal cells (10 - 35 min, depending on cell type) (Mazia, 1961), although slower than in Xenopus embryos. Furthermore, similar kinetics for loss of lamin staining and nuclear envelope breakdown have been observed in cell fusion experiments (Jost and Johnson, 1983).

Although we do not see spindle formation as a response to MPF, this may simply be due to the fact that the mitotic spindle is a highly labile and complicated structure, and our extraction conditions were not optimized for spindle formation or preservation. Alternatively, spindle formation and nuclear envelope breakdown may be regulated independently. Independent regulation of nuclear envelope breakdown and spindle formation could account for the observation that the timing of nuclear envelope breakdown is highly variable with respect to the migration of centriole duplexes to form the poles of the mitotic spindle (Aubin et al, 1980). However, it may be possible to induce spindle formation using cytostatic factor (Masui and Markert, 1971; Meyerhof and Masui, 1979), which arrests the cell cycle in a mitotic state and stabilizes MPF activity. Using CSF to stabilize MPF activity, Newport and Kirschner (1984) observed spindle formation in response to MPF in vivo.

Instability of MPF activity may also account for the variability in the extent of chromatin condensation. However, the fact that the chromatin continues to condense after nuclear envelope breakdown has been observed previously in vivo (Bajer, 1959; Johnson and Roberts, 1964; Murray et al, 1965).

An obvious advantage of this inducible cell-free system is the ability to establish the temporal relationships between morphological changes observed at the onset of mitosis and biochemical events associated with those changes. To this end, we have established the temporal relationship between nuclear envelope breakdown, and the hyperphosphorylation and depolymerization of the lamins, the major structural proteins of the nuclear lamina.

Gerace and Blobel (1980) had previously shown that the nuclear lamina was reversibly depolymerized during mitosis. Furthermore, they found that lamins isolated from mitotic cells were hyperphosphorylated relative to those in interphase cells. They proposed that phosphorylation of the lamins could be a possible mechanism for the depolymerization of the nuclear lamina. The depolymerization, in turn, could directly mediate nuclear envelope breakdown. This model predicts that lamin phosphorylation should either precede or coincide with depolymerization of the lamina, and nuclear envelope breakdown. We have demonstrated that lamin phosphorylation occurs 10 - 15 min after MPF addition, coincident with the start of the depolymerization of the lamina. Depolymerization continues until the lamina is no longer detectable by immunofluorescence, at which point nuclear envelope breakdown takes place, 40 min after MPF addition. The order of events is consistent with the model described above.

How does the addition of MPF lead to phosphorylation of the lamins? In view of the circumstantial and indirect evidence that MPF may be an autocatalytic kinase (Maller, Wu and Gerhart, 1977; Gerhart et al, 1984; Halleck et al, 1984b), one intriguing possibility is that MPF initiates a phosphorylation cascade. The isolation of monoclonal antibodies that appear to recognize a class of mitosis-specific phosphoproteins (Davis et al, 1983) provides indirect support for such a possibility. But in the absence of direct evidence, the initiation of a phosphorylation cascade by MPF remains a tantalizing conjecture.

We have shown that the lamins are not phosphorylated immediately in response to MPF; there is a 10 - 15 min lag period after MPF addition before any increase in lamin phosphorylation is seen. So, MPF itself is probably not the lamins kinase. If this is the case then there must be intermediate steps between MPF addition and lamin phosphorylation. We have shown there are in fact early MPF-dependent phosphorylations that occur during the lag period. Further studies should reveal whether these phosphorylations are steps in the same pathway leading to lamin phosphorylation, or whether they are part of an independent pathway.

In summary, we have shown that MPF induces chromatin condensation and nuclear envelope breakdown in a cell-free system. Using this cell-free system, three distinct stages in the onset of nuclear envelope breakdown can now be defined. During the early stage before lamin hyperphosphorylation, there are no detectable morphological changes, but a few proteins are phosphorylated in response to MPF. The increased phosphorylation of the lamins, 10 - 15 min after the addition of MPF and the coincident weakening of the nuclear envelope mark the beginning of the middle stage. During this stage, the nuclear lamina gradually

depolymerizes and chromatin begins to condense. Finally, there is the late stage, when nuclear envelope breakdown and depolymerization of the nuclear envelope breakdown occurs, along with the continued condensation of chromatin.

A plausible model to account for these events is that MPF initiates a series of events, at least one of which leads to lamin phosphorylation. Phosphorylation could provide the lamins with the capacity to depolymerize; but if the depolymerized form of the lamins is only slightly more energetically favorable, the lamins could leak out of the lamina slowly. Since our antibody does not react with lamin B, which is thought to be membrane-associated (Gerace and Blobel, 1980, 1982) we do not know if it depolymerizes like lamins A and C, or if it remains associated with the nuclear membranes. In either case, when the nuclear envelope is depleted of lamins A and C, the nuclear membranes could then vesiculate, resulting in nuclear envelope breakdown. Ultimately, we could test the necessity of lamin phosphorylation if we could specifically inhibit the lamins kinase in this system.

Although we have focused on the nuclear envelope, with this cell-free system it should be possible to look in detail at other mitotic events, such as chromatin condensation and spindle formation, and study the coordination of the entire mitotic process. Ultimately this system could also provide more direct substrates and biochemical assays for MPF, although at present the in vivo systems are more sensitive. The ability to induce chromatin condensation and nuclear envelope breakdown under the control of endogenous regulatory molecules makes this system uniquely attractive for structural and biochemical studies of these cellular processes.

FIGURE LEGENDS

Figure 1. Response of Thymocyte Nuclei to MPF in vitro.

Fluorescence (a,c,e,g) and phase (b,d,f,h) microscopy of thymocyte nuclei at various times after incubation in extract and addition of MPF. Nuclei were fixed in 2% formaldehyde in Buffer A containing 10 µg/ml Hoechst 33258 (bisbenzimide), a fluorescent DNA-binding dye. Scale bar = 10 µ.

(a,b) before incubation in extract

(c,d) after 2 hr incubation in extract, immediately before addition of MPF

(e,f) 40 min after addition of MPF

(g,h) 75 min after addition of MPF

Figure 2. MPF-dependent acidic charge shift of lamins A and C

Autoradiograms of lamins A and C immunoprecipitated from cell-free reactions (a) in the absence of MPF, and (b) in the presence of MPF. Both reactions were stopped 40 min after the addition of MPF to (b). 2-D NEPHGE gels were aligned with respect to actin (a) and vimentin (v) contaminants in the immunoprecipitate. The acidic side of the gel is to the right. A typical cell-free reaction before immunoprecipitation can be seen in Figure 7.

Figure 3. Time course and reversibility of lamin hyperphosphorylation

Autoradiogram of immunoprecipitation of lamins A and C from cell-free reactions (a) 0, (b) 15, (c) 30 and (d) 45 min after adding MPF. (e) 45 min after addition of MPF followed by digestion with 8 units of alkaline phosphatase for 30 min at room temperature. Since these

immunoprecipitates were not contaminated by sufficient actin and vimentin (as in figure 2), the 2-D NEPHGE gels were aligned with respect to nonradioactive silver-stained spots visible on the original gels (oval outlines). The acidic side of the gel is to the right.

Figure 4. Ultrastructure of CHO nuclei before nuclear envelope breakdown

Electron micrographs of CHO nuclei fixed in 1% glutaraldehyde, embedded in Araldite and thin-sectioned. Nuclei (a) before exposure to cell-free extract, (b) after 2 hr incubation in cell-free extract, (c) 20 min after addition of MPF, when lamins A and C are fully phosphorylated. Scale bar = 1.0 μ

Figure 5. Time course of depolymerization of nuclear lamina

Indirect immunofluorescence of lamins A and C (a,c,e,g,) and fluorescent staining of DNA (b,d,g,h,) of CHO nuclei fixed in 10 mM ethylene glycol bis(succinimidyl succinate) and sedimented onto polylysine-coated coverslips. (a,b) 10 min, (c,d) 15 min, (e,f) 30 min and (g,h) 40 min after addition of MPF, Scale bar = 10 μ

Figure 6. Temporal relationships among lamin hyperphosphorylation, depolymerization of the nuclear lamina and nuclear envelope breakdown

Fluorescent staining of DNA, relative charge shift (indicating increase in lamin phosphorylation) and intensity of indirect immunofluorescence of lamins A and C (indicating degree of depolymerization of nuclear lamina) of CHO nuclei are shown as a function of time after addition of MPF. See text for description. DNA was fluorescently stained with

Hoechst 33258 (bisbenzimide). The scale of relative charge shift was set by letting the position of lamins A and C before MPF be equal to zero and designating the position of fully hyperphosphorylated lamins to be 1.0. The actual distance from 0 to 1.0 was approximately 10 mm on the autoradiographs. Intensity of perinuclear lamin staining was measured using a digitized image of photographic negatives of immunofluorescent staining of lamins A and C. The optical density of a 49-pixel area on the edge of the nucleus was averaged, and compared to the optical density of the background. 10 nuclei were measured for each time point; the standard deviation at each point was between 12.0 and 14.7 units.

Figure 7. Early MPF-dependent phosphorylation of CHO proteins
Nuclei were isolated from ^{35}S-methionine-labeled CHO cells, and incubated in unlabeled cell-free extract. MPF was added to one of two parallel reactions. 10 min after addition of MPF both reactions were quenched by boiling in SDS. Proteins were resolved on 2-D NEPHGE gels. Ovals show the position of the three proteins that were phosphorylated within 10 min of MPF addition. Squares show the position of the same proteins in the absence of MPF.

Figure 1

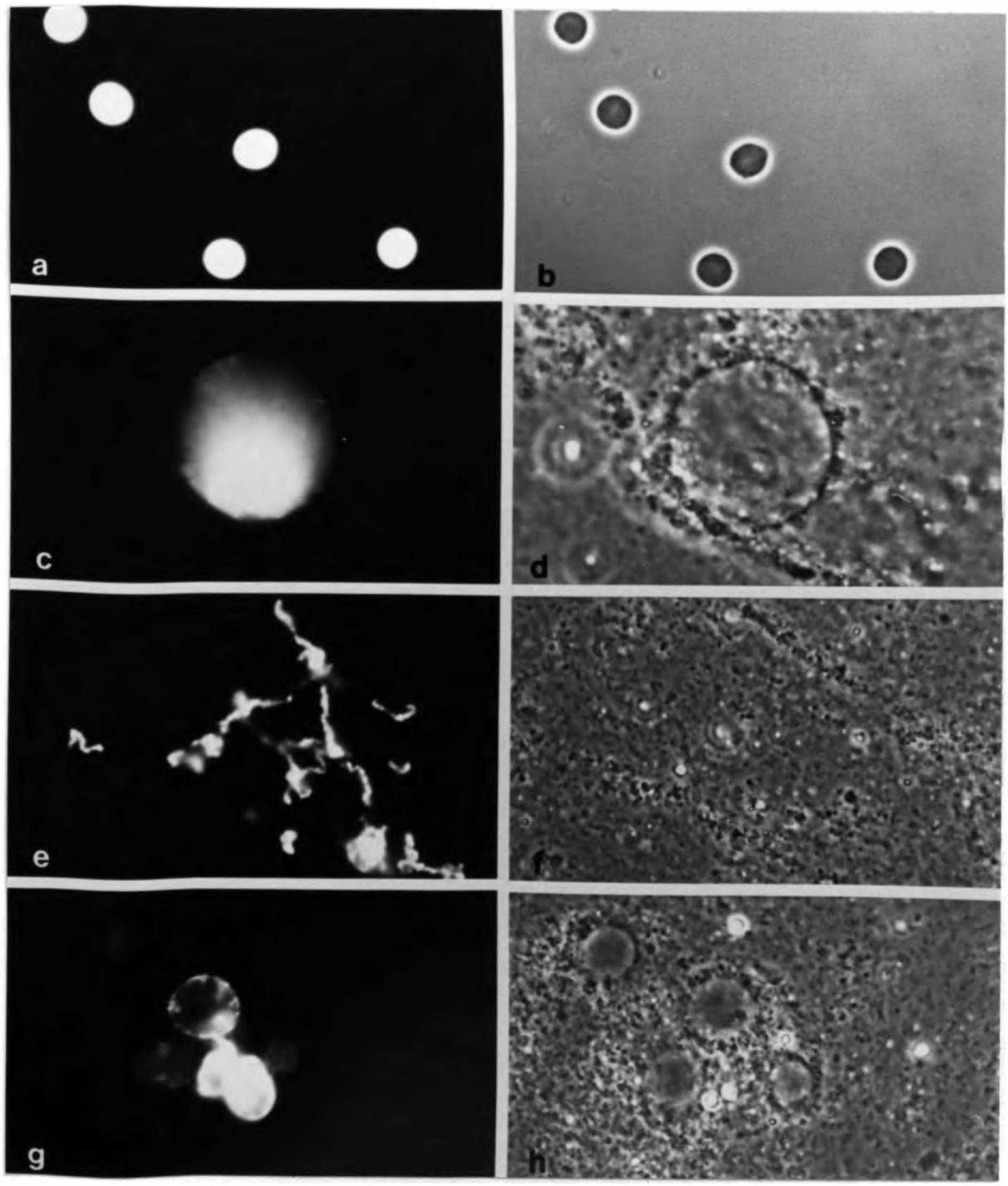
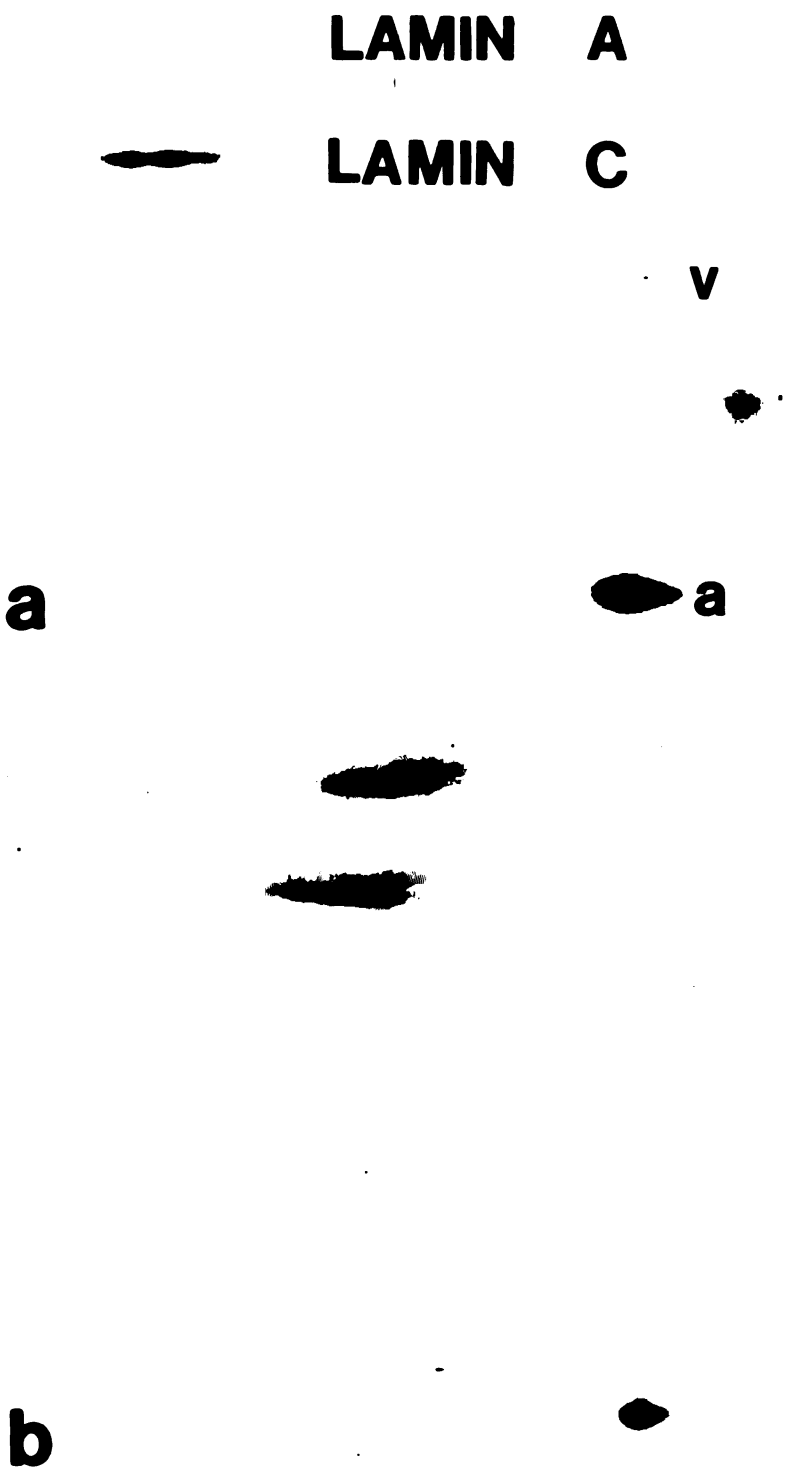


Figure 2



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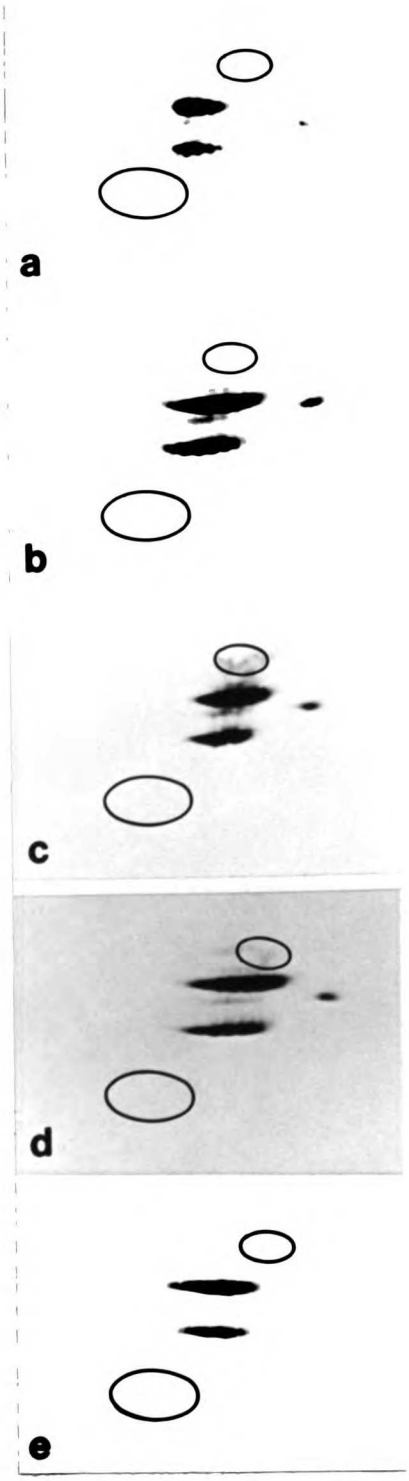


Figure 4

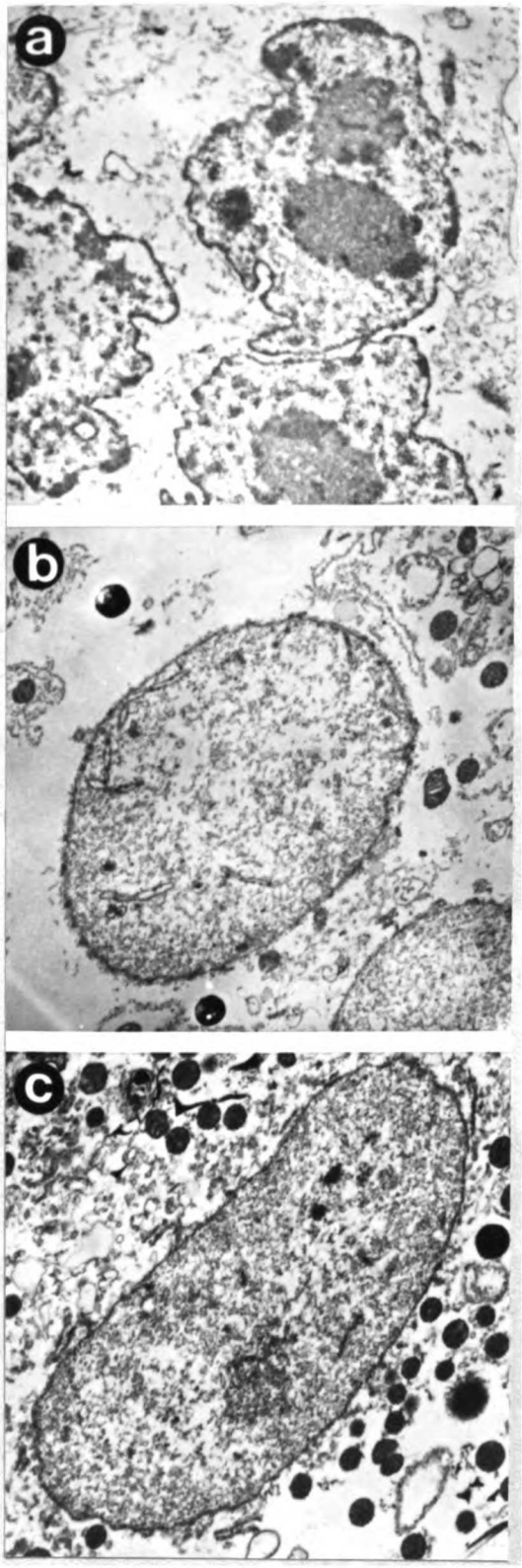


Figure 5

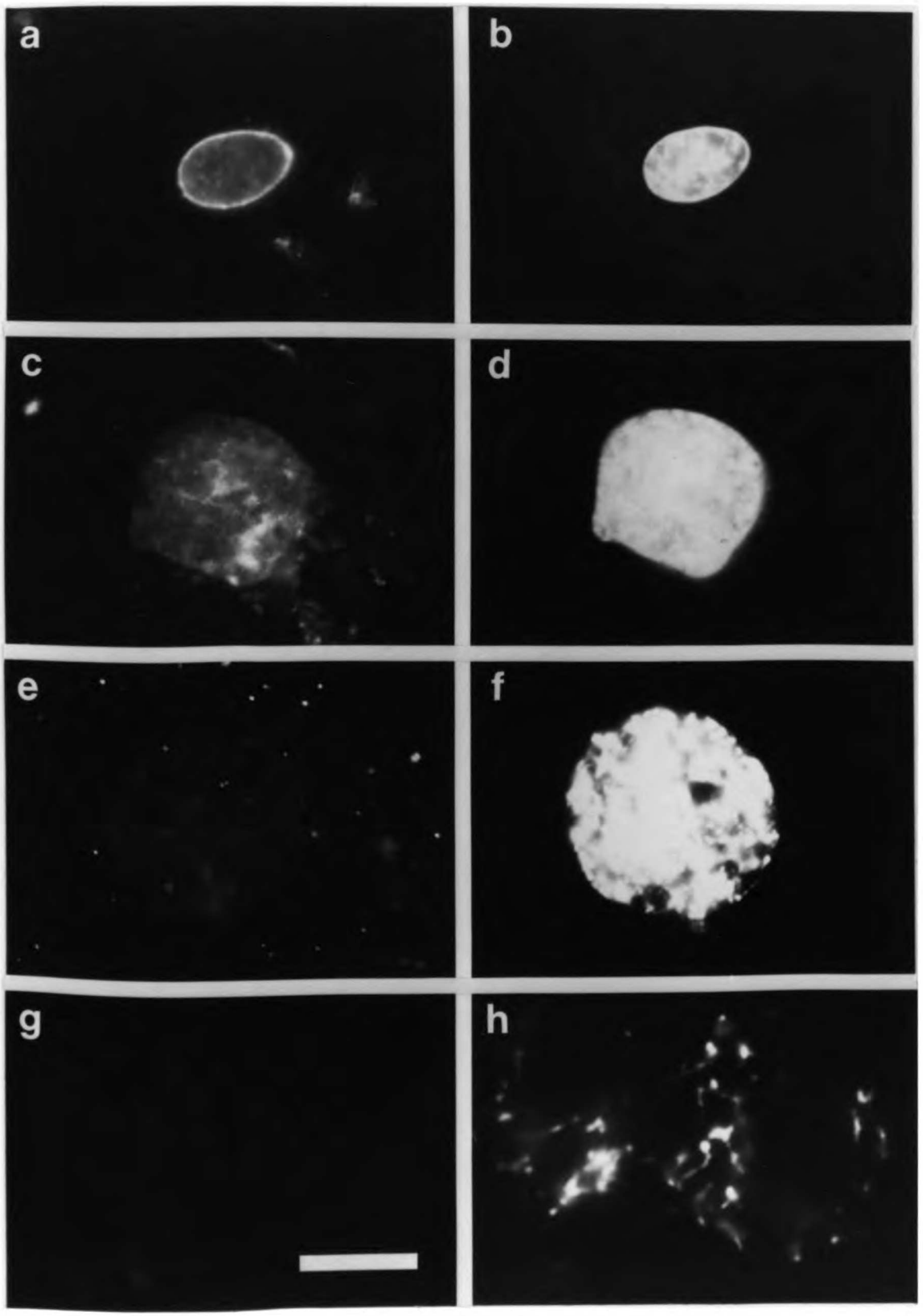


Figure 6

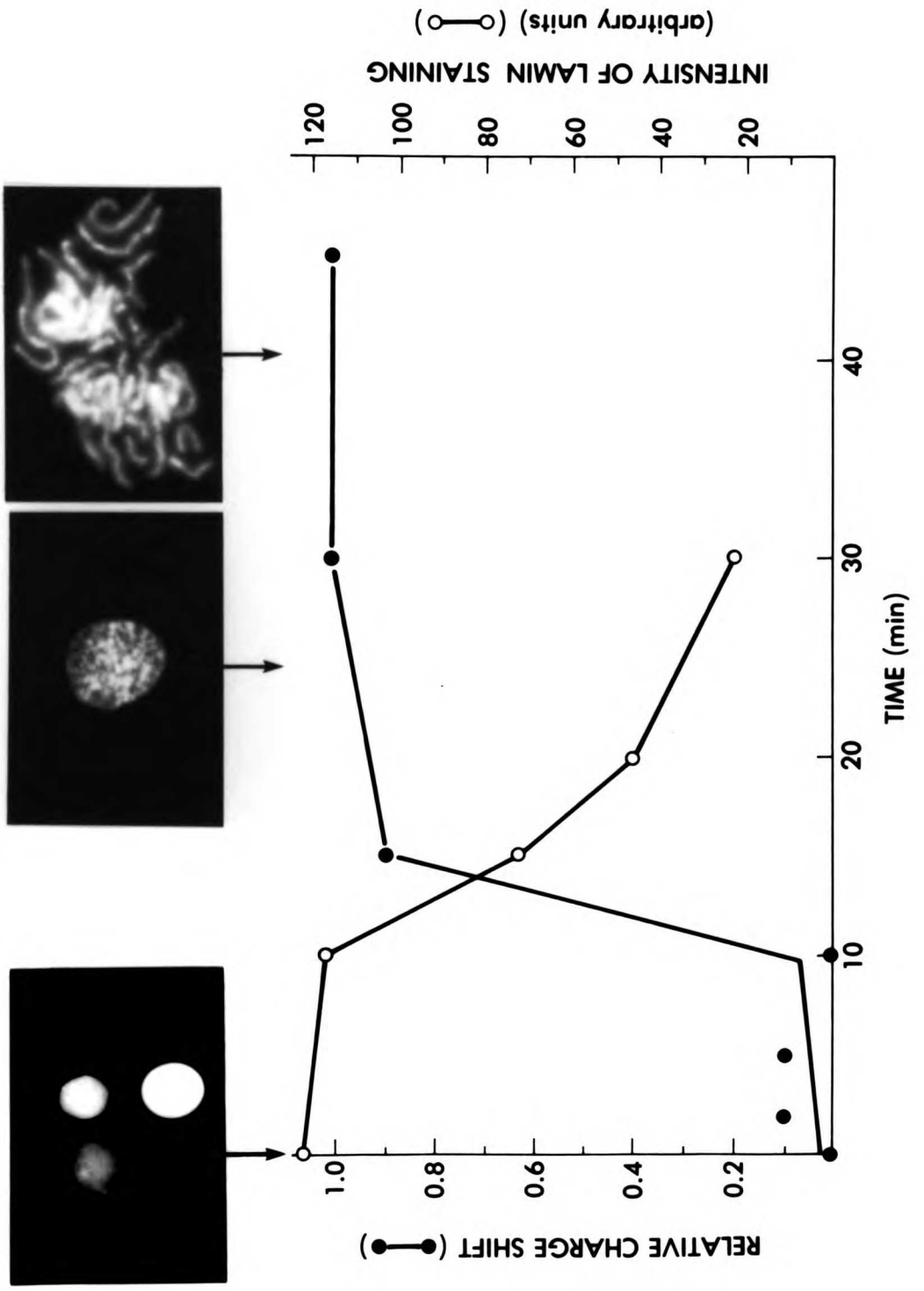
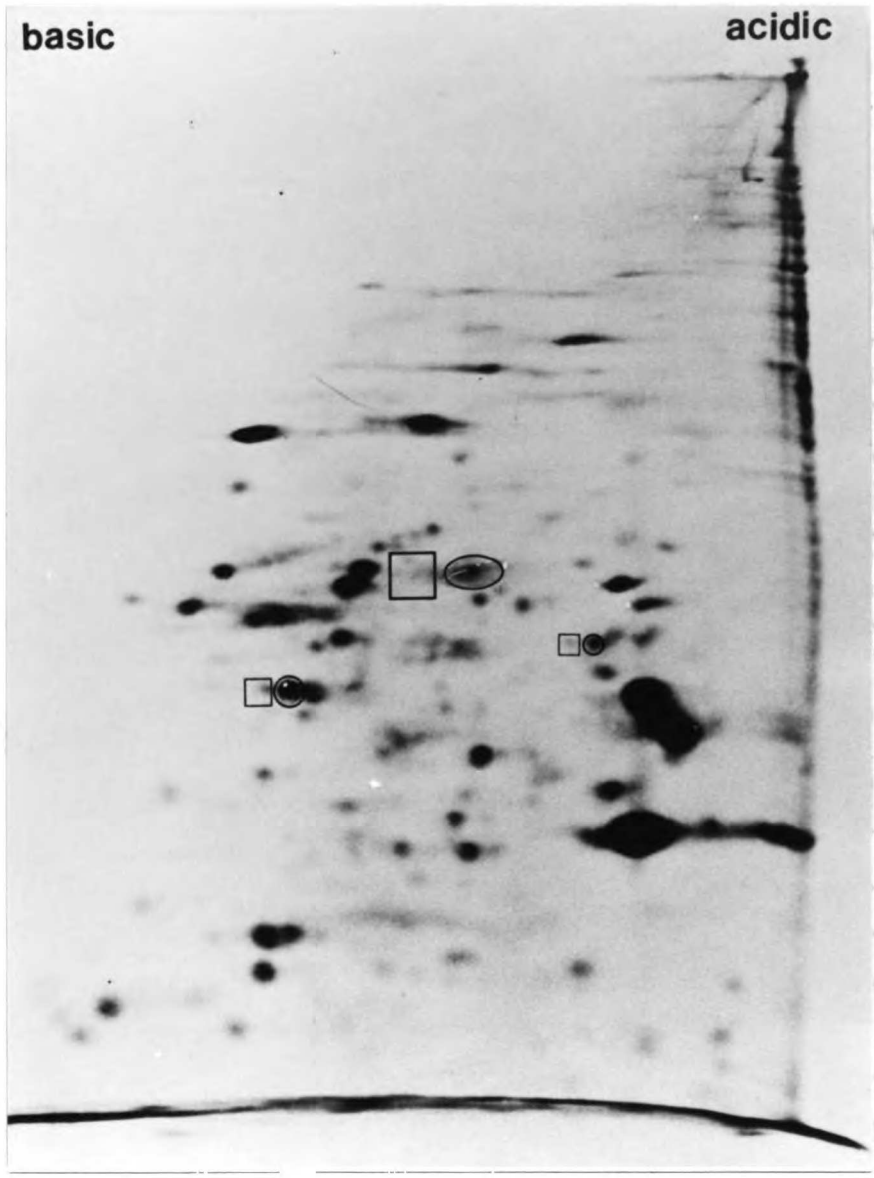

RELATIVE CHARGE SHIFT (●—●)
1.0
0.8
0.6
0.4
0.2
INTENSITY OF LAMIN STAINING
(arbitrary units) (○—○)
120
100
80
60
40
20
10
20
30
40
TIME (min)

Figure 7



CHAPTER 4

Preliminary Studies on Regulation of Microtubule Assembly in Xenopus Eggs and Oocytes

ABSTRACT

The Xenopus oocyte cannot be induced to form asters of microtubules under any circumstances, including injection of centrioles or taxol, or incubation in D_2O. But when it matures into an unfertilized egg, it acquires the ability to assemble asters in response to taxol or D_2O. We have studied various aspects of this experimental system in an attempt to begin to understand the mechanism of this change in microtubule assembly in vivo. We have shown that microtubules injected into oocytes are less stable than in eggs, although we could detect no inhibitory activity of free microtubule assembly in oocyte extracts. The change in ability to assemble asters in vivo is independent of new protein synthesis. Furthermore, tubulin from oocytes and from eggs are equally competent to polymerize in vitro.

INTRODUCTION

The cellular microtubule array is a highly dynamic structure. The spatial distribution of microtubules is dramatically reorganized via localized assembly and disassembly during such fundamental processes as cell division, neurite outgrowth and cell movement. While there are well-characterized factors that promote microtubule assembly in vitro (Olmsted and Borisy, 1975; Weingarten et al, 1975; Murphy and Borisy, 1975), much less is known about the regulation of microtubule organization and assembly in vivo.

A comparison of the oocyte and the unfertilized egg of Xenopus offers several advantages as a model system for studying the regulation of microtubule organization and assembly in vivo. The oocyte and the unfertilized egg are both naturally arrested at points in the cell cycle that have very different properties with respect to microtubule organization and assembly. These differences have been assayed by comparing the oocyte's or the egg's ability to form asters, radially symmetric arrays of microtubules (Heidemann and Kirschner, 1975, 1978). The fully grown oocyte is arrested at the end of premeiotic prophase (diplotene). In this state, asters of microtubules cannot be induced to form under any circumstances, including incubation of the oocyte in D_2O or injection of centrioles, basal bodies or taxol, a drug which promotes microtubule assembly (Heidemann and Kirschner; Heidemann and Gallas, 1980). After a fully grown oocyte has matured into an unfertilized egg, asters still will not form if centrioles or basal bodies are injected (Karsenti et al, 1984) but they can be induced to form (even in the absence of injected centrioles) under conditions that drive microtubules to assemble, such as D_2O (Karsenti et al, 1984) or taxol (Heidemann and

Gallas, 1980). Thus, a comparison of the oocyte and the egg with respect to their responses to D_2O and taxol provides an experimental system with plus/minus states of microtubule assembly. The oocyte (i.e., minus state) cannot be induced to form asters, while the unfertilized egg can. This situation is more amenable to study than many other types of microtubule changes in vivo that often involve changes in spatial arrangement that are difficult to assay biochemically. In principle, the striking changes seen here can provide the basis for isolating proteins that are involved in the regulation of microtubule assembly in vivo.

It should be noted that there is a third state of microtubule assembly in which aster formation occurs spontaneously when centrioles are injected (Karsenti et al, 1984). This state happens when an unfertilized egg is activated or fertilized. However, the studies described here are restricted to a comparison of the first two states: the oocyte (in which aster formation does not occur), and the unfertilized egg (in which aster formation can be induced to occur).

As an extension of the studies carried out by Heidemann and Kirschner (1975, 1978) that characterized this experimental system, we wanted to know more about the nature of the difference between the oocytes and the egg. To this end, we asked the following questions:

1) are injected microtubules equally stable in oocytes and eggs?
2) is there an inhibitor in extracts of oocytes that prevents assembly of free microtubules?
3) is there a difference in the ability of tubulin from oocytes and from eggs to copolymerize with brain microtubule protein?

4) is new protein synthesis necessary to acquire the ability to form asters?

Our results show that injected microtubules are more rapidly disassembled in oocytes than in eggs. However, the tubulin from both cell types is equally competent to polymerize. Also, the ability to form asters can be acquired in the absence of new protein synthesis, indicating a possible role of post-translational modifications in regulating microtubule assembly in vivo.

MATERIALS AND METHODS

Injection of microtubules

Chicken brain microtubule protein was purified according to Weingarten et al (1974). Microtubules to be injected were radioactively labeled by adding microtubule protein (1.5 ng) to 200 µCi α^{32}P-GTP and incubating 30 min at 37°C in the presence of acetate kinase and acetyl phosphate as a GTP regenerating system (MacNeal et al, 1977). The polymerized microtubules were separated from free counts and aggregates of protein by sedimentation through a 50% sucrose cushion (Margolis and Wilson, 1978). The pellet was resuspended in 75 µl of polymerization buffer (PB) (100 mM MES, 0.5 mM $MgCl_2$, 2 mM EGTA, 0,1 mM EDTA, 1 mM β-mercapoethanol, pH 6.5) containing the GTP regenerating system. The resuspended microtubules were kept at 37° until use. 50 nl were microinjected into oocytes or eggs. Oocytes were incubated in MMR (Karsenti et al, 1984); eggs were dejellied in 2% cysteine, pH 7.8 and incubated in non-activating medium (100 mM KCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 mM MES, pH 7.0). At the appropriate time, 3 injected cells were lysed into 4 ml of a microtubule-stabilizing buffer, and remaining

microtubules were retained on a GF/F filter, according to Wilson et al (1982) 0.4 ml of the cell lysate was counted to determine the total counts injected. Filters and cell lysates were counted in Aquasol.

Turbidimetry

Oocyte extracts were prepared by rinsing Stage 5 and 6 Xenopus oocytes extensively in PB and homogenizing them in 1 volume of PB. The extract was centrifuged 10K rpm for 10' to sediment yolk platelets, then at 40K rpm for 40 min in a 50Ti rotor. The clear cytoplasmic layer was removed, and glycerol was added to 4 M.

Assembly of microtubule protein, or mixtures of oocyte extract and microtubule protein, was initiated by warming the reaction to 37°C in a temperature-controlled cuvette. Turbidity was monitored at 380 nm.

Copolymerization

Oocytes were labeled overnight at 19°C in MMR containing ^{35}S-methionine at a concentration of 2 mCi/ml. Eggs were labeled by injecting them each with 0.5 µCi ^{35}S-methionine, followed by incubation at 19°C for 4 hrs. Extracts were prepared by rinsing the cells extensively in PB, lysing the cells into an equal volume of PB with a P200 pipetman, and centrifuging for 10' at 15K rpm. Identical extracts were prepared from unlabeled oocytes and eggs. The oocyte copolymerization mixture consisted of 1.2 ml labeled oocyte extract, 0.35 ml unlabeled egg extract and 1.0 ml brain microtubule protein. The egg copolymerization mixture contained the same volumes of extract, except that the egg extract was labeled and the oocyte extract unlabeled.

Copolymerization was then carried out, according to Spiegelman et al (1977). Protein was determined by the Lowry procedure (1951).

SDS-polyacrylamide gels were run according to Laemmli (1970) and fluorographed according to Bonner and Laskey (1974).

Induction of Aster Formation

Protein synthesis was inhibited by incubating oocytes in 50 µg/ml cycloheximide in MMR for 1 hr. Oocytes to be matured were injected with 50 nl of partially purified MPF. The MPF was the generous gift of Michael Wu and John Gerhart. After oocytes had undergone germinal vesicle breakdown (as judged by white spot formation), they were incubated (along with control oocytes) in 75% D_2O in MMR for 1 hr. At the end of the incubation, the oocytes were fixed, embedded in paraffin and sectioned, as described by Karsenti et al (1984).

RESULTS

In a simplified model, the difference between the oocyte and the egg in their abilities to form asters could be ascribed to a difference in either of two factors: the ability of microtubules to elongate, or the activity of microtubule organizing centers (MTOCs) in nucleating microtubule growth. Since MTOCs had not yet been purified at the time these studies were carried out, our efforts to characterize this system focused on factors that might affect the ability of microtubules to elongate, and did not address possible differences in nucleating ability (but see Karsenti et al, 1984).

Microinjected microtubules are more stable in eggs than in oocytes

We wanted to know if we could detect the difference between oocytes and eggs by a more quantitative and rapid assay than histological detection of asters. Since unfertilized eggs will support formation of asters, while oocytes do not, we thought there might also be an

observable difference in the stability of free microtubules injected into these two cell types. Such a difference could form the basis of a more quantitative assay. To test this, we chose to measure the lifetime of brain microtubules assembled in vitro after they had been injected into either oocytes or eggs. We radioactively labeled the microtubules to high specific activity, by assembling them in the presence of α-^{32}P-GTP and a GTP regenerating system (MacNeal et al, 1977). Labeled microtubules were separated from aggregates of microtubule protein by pelleting the microtubules through a cushion of sucrose (Margolis and Wilson, 1978). They were resuspended in a small volume of warm polymerization buffer, and microinjected into either oocytes or eggs. At various times after injection, the cells were lysed into a microtubule stabilizing buffer, and the amount of labeled microtubule protein in polymer form was determined by a filter assay, in which polymer is retained on a glass fiber filter (Wilson et al., 1982). For each measurement, the counts in polymer were normalized to the total counts injected, which was determined by counting an aliquot of the cell lysate before filtration. Taxol-stabilized microtubules and cold-treated microtubule protein were injected as positive and negative controls for the assay, respectively.

Figure 1 shows a plot of the fraction of radioactively labeled microtubule protein remaining in polymer as a function of time after injection. The control time course with taxol-stabilized microtubules shows that, by this assay, approx. 70% of the labeled microtubule protein was in polymer form. For unfertilized eggs the amounts of polymer remaining in injected eggs decreased montonically for the first 7 min then reached a plateau of approx. 10% which remained constant,

even until 45 min after injection. In contrast, microtubules injected into oocytes were much less stable. Only 1 minute after injection, less than 10% of the injected microtubules remained in the polymer form in oocytes, whereas 3 times that amount was still in the polymer form in eggs at the same time. Polymer levels remained low in the oocyte for the duration of the time course. This result suggests that there may be a difference between oocytes and eggs in their ability to disassemble free microtubules.

Since the fraction of microtubule protein in polymer was not very high, even in the case of the unfertilized egg, we wanted to know if the process of microinjection was shearing the microtubules to the point that they were depolymerizing before we could assay them. To determine the extent of shearing, we microinjected a solution of polymerized microtubules into a drop of warm polymerization buffer, which was then drop loaded onto a grid and negatively stained (Witman et al, 1976). The lengths of both injected and uninjected microtubules were determined by measuring the lengths of projected images of electron micrographs. While uninjected microtubules were often greater than 10μ in length, microinjection sheared the microtubules to an average of 3.4 μ (n=156). At an in vitro rate of depolymerization of 113 subunits/sec (Karr et al, 1980), which was observed for microtubules diluted into buffer, microtubules 3.4 μ in length would be completely depolymerized in less than 50 sec. Although the injected microtubules are not completely stable in eggs, they depolymerize more slowly than in oocytes; in oocyte cytoplasm, the microtubules depolymerize as rapidly as if they had been diluted into buffer.

Oocyte extract does not inhibit polymerization of free microtubules

One possible explanation for the inability of the oocyte to form asters could be that the oocyte cytoplasm contains an inhibitor of free microtubule assembly. We tested this possibility by asking if cytoplasmic extracts of oocytes could inhibit assembly of free microtubules, as assayed by turbidimetry. Cytoplasmic extracts were prepared by homogenizing the oocytes in an equal volume of polymerization buffer, followed by centrifugation. Oocyte cytoplasmic extract, brain microtubule protein and GTP were premixed on ice and then warmed to 37° in controlled-temperature cuvettes. Figure 2 shows the increase in turbidity observed when either microtubule protein alone, or an equal amount of oocyte extract and microtubule protein was allowed to assemble. Under these conditions, no inhibition of free microtubule polymerization was seen, even when the ratio of extract:microtubule protein was as high as 5:1. That there was insufficient tubulin in the cytoplasmic extract to contribute to the assembly is shown by the fact that the cytoplasmic extract alone did not assemble microtubules. This result indicates that there is no detectable soluble inhibitor of free microtubule assembly in the cytoplasm of oocytes.

Oocyte tubulin and egg tubulin form copolymers with equal efficiency

Heidemann and Kirschner (1975) had previously shown that the oocyte and the egg contain equal amounts of tubulin as assayed by colchicine-binding. Since no active inhibition of free microtubule assembly was observed in oocyte cytoplasm, we tested the possibility that oocyte tubulin itself was incapable of polymerizing.

To see if oocyte tubulin had undergone some modification which irreversibly affected its ability to assemble, we asked if it could

coassemble into microtubules with brain microtubule protein. The brain microtubule protein would provide sufficient carrier tubulin and all the necessary microtubule-associated proteins (Spiegelman et al, 1977). The oocytes and the eggs used for copolymerization were metabolically prelabeled with ^{35}S-methionine, to distinguish their proteins from the carrier microtubule protein. Either labeled oocyte extract or labeled egg extract was mixed with brain microtubule protein, and the mixture was subjected to three rounds of warm assembly and cold disassembly. Figure 3 shows an autoradiogram of oocyte and egg copolymerizations. The lanes show successive steps of assembly and disassembly. Clearly, tubulin from both oocytes and from eggs forms copolymers with brain microtubule protein.

To quantitate the efficiency of the copolymerization, tubulin bands were excised from polyacrylamide gels, counted and an activity ratio of ^{35}S-methionine radioactivity to total protein was determined (Spiegelman et al, 1977). Figure 4 shows this activity ratio as a function of the number of cycles of purification. In the initial step, the activity ratio is quite high, since all the radioactively labeled proteins are present in the extract. However, this value drops rapidly, as the fraction of oocyte or egg protein that copolymerizes with brain microtubules is purified away from bulk labeled protein. After 4 rounds of copolymerization, virtually the same fraction of tubulin copolymerized from the oocyte and from the egg. 8% of the labeled soluble protein from the oocyte had copolymerized, and 10% from the egg. It can be seen that the copolymerized fraction is mostly tubulin (figs. 3i and 3s). Thus, both oocyte and egg tubulin are equally competent to

polymerize, when provided with carrier tubulin and microtubule-associated proteins.

No new protein synthesis is necessary to acquire the ability to form asters

During the time a fully grown oocyte matures into an unfertilized eggs, it gains the ability to form asters. As an initial approach to determining at what level this change in ability is regulated, we asked if de novo protein synthesis is required to be able to form asters, or, if instead, the ability to form asters is regulated post-translationally.

This question was addressed in the following manner: oocytes were matured in the absence of protein synthesis, and subsequently tested for their ability to assemble asters. Protein synthesis was inhibited by incubation of the oocytes in cycloheximide. Although protein synthesis is required during the early states of progesterone-induced maturation (Wasserman and Masui, 1975), maturation through the first meiotic division can proceed in the absence of protein synthesis if it is induced by the injection of maturation-promoting factor (Wasserman and Masui, 1975; Gerhart et al, 1984). After injection of maturation-promoting factor the oocytes were allowed to mature. When their germinal vesicles (nuclei) had broken down (as judged by the appearance of a white spot in the animal hemisphere of the oocyte), the oocytes were incubated in medium containing 75% D_2O to see if asters could be induced to form. Table 1 shows that oocytes matured by MPF gained the ability to assemble asters whether or not protein synthesis was inhibited. As previously observed, no asters could be detected in immature oocytes. This demonstrates that de novo protein synthesis is

not required in order to gain the ability to form asters. These results indicate that the difference between the oocyte and the egg might be due to post-translational changes.

DISCUSSION

The unfertilized egg of Xenopus gains the ability to form asters of microtubules in the few hours required for it to mature from being a fully grown oocyte. Comparing the oocyte and the egg provides an easily accessible model system for studying the regulation of microtubule assembly in vivo, since the two cell types are naturally arrested in completely different states with respect to aster formation. Whereas the unfertilized egg can be induced to form asters, the oocyte does not form asters under any conditions, providing a plus/minus experimental system for microtubule assembly.

In these studies, we have examined the nature of the difference between the oocyte and the egg. We have shown that injected microtubules are less stable in oocytes than in eggs. However, we could not detect an inhibitor of free microtubule assembly in oocyte cytoplasm. We also demonstrated that the tubulin from oocytes and from eggs is equally competent to polymerize into microtubules in vitro, when provided with carrier tubulin and microtubule-associated proteins. Finally, it was shown that protein synthesis is not required to acquire the ability to form asters.

We have shown that the oocyte and the egg behave differently not only with respect to aster formation, but also with respect to the stability of free microtubules injected into these cells. While microtubules injected into eggs persisted for nearly 10 min, less than

10% of microtubules injected into oocytes were in polymer form 1 min after injection. The depolymerization rate observed in oocytes is similar to the rate observed for microtubules diluted into buffer, whereas microtubules injected into eggs are stabilized and depolymerize more slowly. Because asters in the eggs can be induced to form by incubation in D_2O, it would be interesting to know if injected microtubules would be even more stable if they were injected into an egg in D_2O. This result also offers an alternative assay for aster formation other than paraffin sections and histological staining of oocytes and eggs. It would be particularly useful if the observed difference in microtubule stability in vivo could be preserved in extracts of oocytes and eggs.

Asters cannot be induced to form in oocytes, and microtubules injected into oocytes depolymerize rapidly. Yet, when cytoplasmic extracts of oocytes were mixed with brain microtubule protein, we detected no inhibition of free microtubule assembly, as assayed by turbidimetry. One resolution of this apparent paradox is that the oocyte inhibitory activity may have been lost or inactivated in the process of making the extract. Another possibility is that turbidimetry may not be the most sensitive assay for detecting the inhibitory activity, since it detects assembly of free microtubules as opposed to nucleated microtubules. Given the high tubulin concentration and the abundance of brain microtubule-associated proteins used in the turbidimetric assay, it is possible that the oocyte inhibitory activity could not be detected under conditions that so strongly favor assembly of free microtubules. This possibility has been substantiated by recent experiments in this laboratory. Using purified MTOCs and tubulin

depleted of microtubule-associated proteins, David Gard has recently been able to demonstrate an activity in oocyte cytoplasm that inhibits nucleated growth off MTOCs (personal communication).

We have also shown that tubulin from oocytes and from eggs forms copolymers with brain microtubule protein with equal efficiency. Since the tubulin from both cell types is competent to polymerize when provided with brain microtubule-associated proteins, this result suggests that the difference between the oocyte and the egg is not at the level of tubulin itself, but possibly at the level of microtubule-associated proteins or nucleating activity of MTOCs.

Finally, we have demonstrated that the acquisition of the ability to form asters is independent of new protein synthesis, implying that post-translational changes may be responsible for the difference between the oocyte and the egg with respect to aster formation. This result is not surprising since only a few hours are required for an oocyte to mature into an egg and gain the ability to form asters. Compared to the months required for oogenesis (during which the oocyte accumulates large stores of proteins required for early development), the process of maturation is relatively rapid. In view of this, the post-translational activation of pre-synthesized components could be a more efficient way to acquire the ability to assemble asters, than to synthesize those components de novo.

These studies provide no conclusive answers at the molecular level about the mechanism of regulation of aster formation and microtubule assembly in these two cell types. This is due in part to the difficulty in distinguishing between changes in ability to elongate microtubules versus changes in nucleation activity. At the time these studies were

undertaken, MTOCs had not yet been purified. Thus, it was not possible to reproduce aster formation or nucleated microtubule growth in vitro. This, in fact, has proven to be a fruitful approach: David Gard has begun to purify both an oocyte inhibitory activity and a stimulatory activity from activated eggs (personal communication). This approach should lead to the isolation of the first proteins that can be shown to regulate the assembly of microtubules in vivo.

Table I

Ability to form asters is independent of protein synthesis

	ASTER FORMATION			
oocytes	untreated		cycloheximide	
-MPF	-	(n=3)	-	(n=3)
+MPF	+	(n=6)	+	(n=12)

FIGURE LEGENDS

Figure 1. Stability of microtubules injected into oocytes and eggs. Microtubules were radioactively labeled by assembly in the presence of α ^{32}P-GTP. Approx. 50 nl of microtubules were injected into each egg or oocyte. At the appropriate time cells were lysed into a microtubule-stabilizing buffer and filtered through GF/F filters, which retain polymer. Amount of polymer is expressed as the fraction of total cpm injected. Total cpm injected was approximately 15,000 cpm. 3 injected cells were pooled for each time point. Taxol-stabilized microtubules were treated with 60 μM taxol prior to injection. Cold-treated microtubule protein was depolymerized 30' on ice before injection.

Figure 2. Lack of inhibition of free microtubule assembly by oocyte extract.
Microtubule protein (final concentration = 2.0 mg/ml), oocyte cytoplasmic extract (4.3 mg/ml) or equal amounts of both (by mg of total protein) were mixed with GTP on ice. Polymerization was initiated by transferring the reaction to a temperature-controlled cuvette at 37°C. Turbidity was monitored at 380 nm.

Figure 3. Copolymerization of oocyte and egg tubulin.
Autoradiograms of successive steps of copolymerization of unlabeled brain microtubule protein with ^{35}S-methionine-labeled oocyte proteins (lanes a-i) and egg proteins (lanes k-s). For each step of purification, H and C refer to a sedimentation at 25°C and 4°C, respectively; the

subscript is the number of cycles or polymerization. Lanes a) and k), initial extract; b) and l), C_1S; c) and m) H_2S; d) and n) H_2P; e) and o)H_3P; g) and q) C_3S; h) and r) H_4S; i) and s) H_4P. Lanes j) and u) are molecular weight standards of 90, 68 and 43 kilodaltons. Lane t) is chicken brain microtubule protein (^{14}C-labeled by reductive methylation). Lanes a) - c) and k) - m) each were loaded with 25 µg protein; lanes d) - i) and n) - s) each contain 5 µg protein.

Figure 4. Efficiency of cycles of copolymerization.

^{35}S-methionine labeled microtubule proteins from oocytes and from eggs were purified by copolymerization with brain microtubule protein (see Materials and Methods). An activity ratio of the total cpm to the total protein (x 10^2 for the egg copolymerization; x 10^3 for the oocyte) is plotted against the stage of purification. H refers to centrifugation at 25°C; P refers to the pellet of the centrifugation; the subscript is the number of cycles of polymerization. The final pellet of the oocyte copolymerization (H_4P) contained 1.35 x 10^6 cpm and 420 µg total protein; for the egg copolymerization H_4P had 4.33 x 10^4 cpm and 430 µg protein.

Figure 1

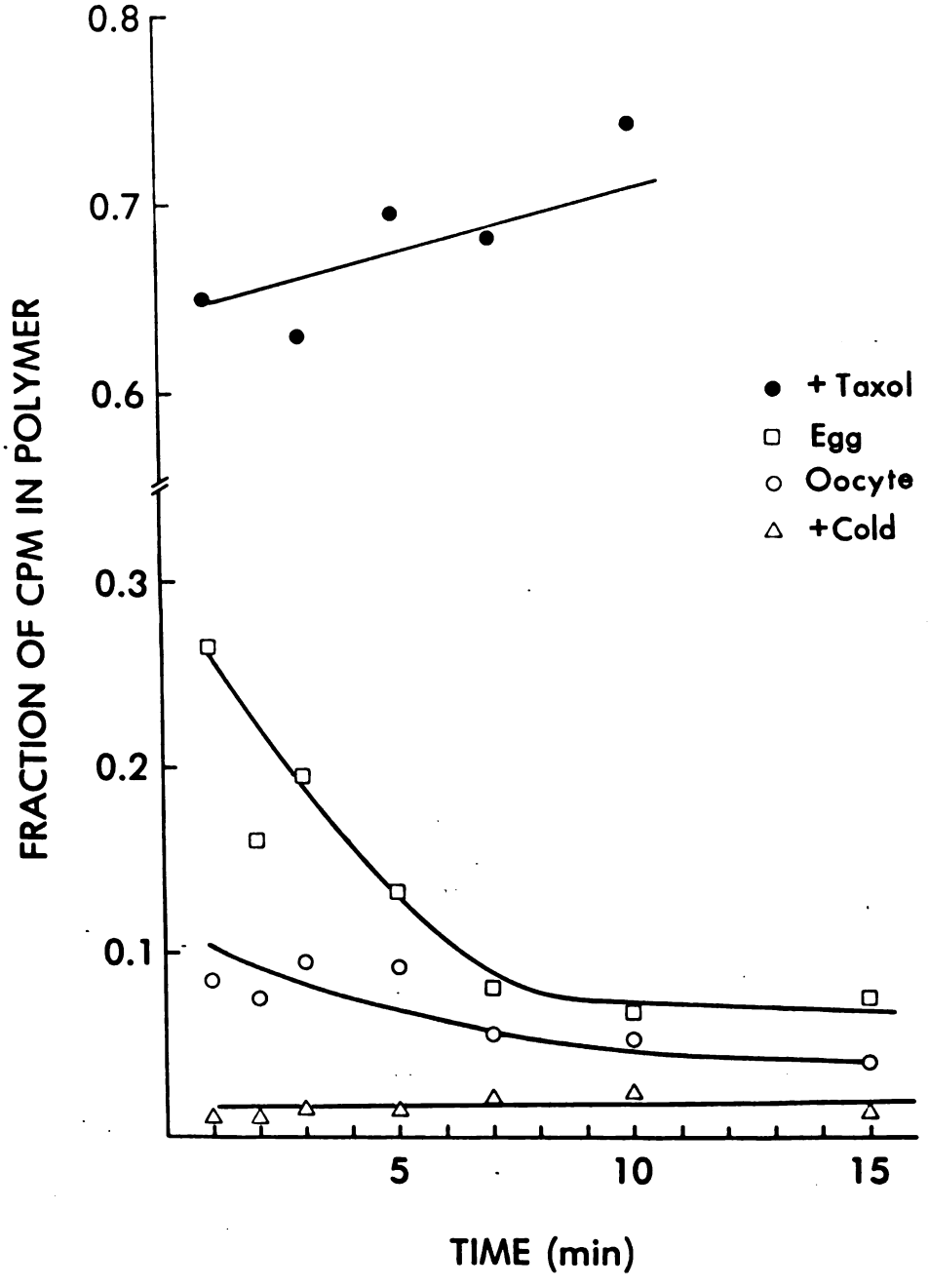
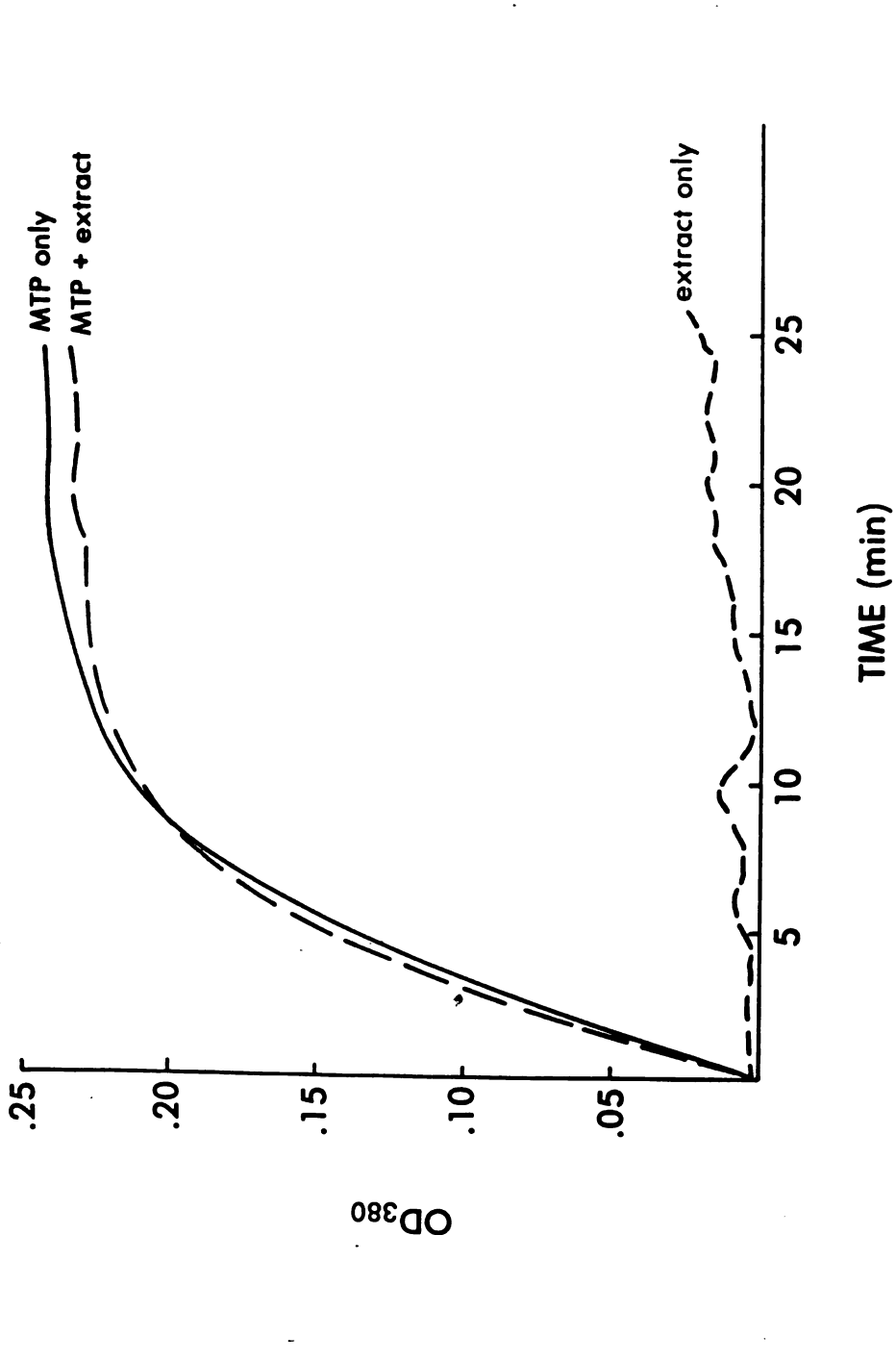

0.8
0.7
0.6
0.3
0.2
0.1
FRACTION OF CPM IN POLYMER
+ Taxol
Egg
Oocyte
+Cold
5
10
15
TIME (min)

Figure 2


MTP only
MTP + extract
extract only
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.20
.15
.10
.05
OD380
5
10
15
20
25
TIME (min)

Figure 3

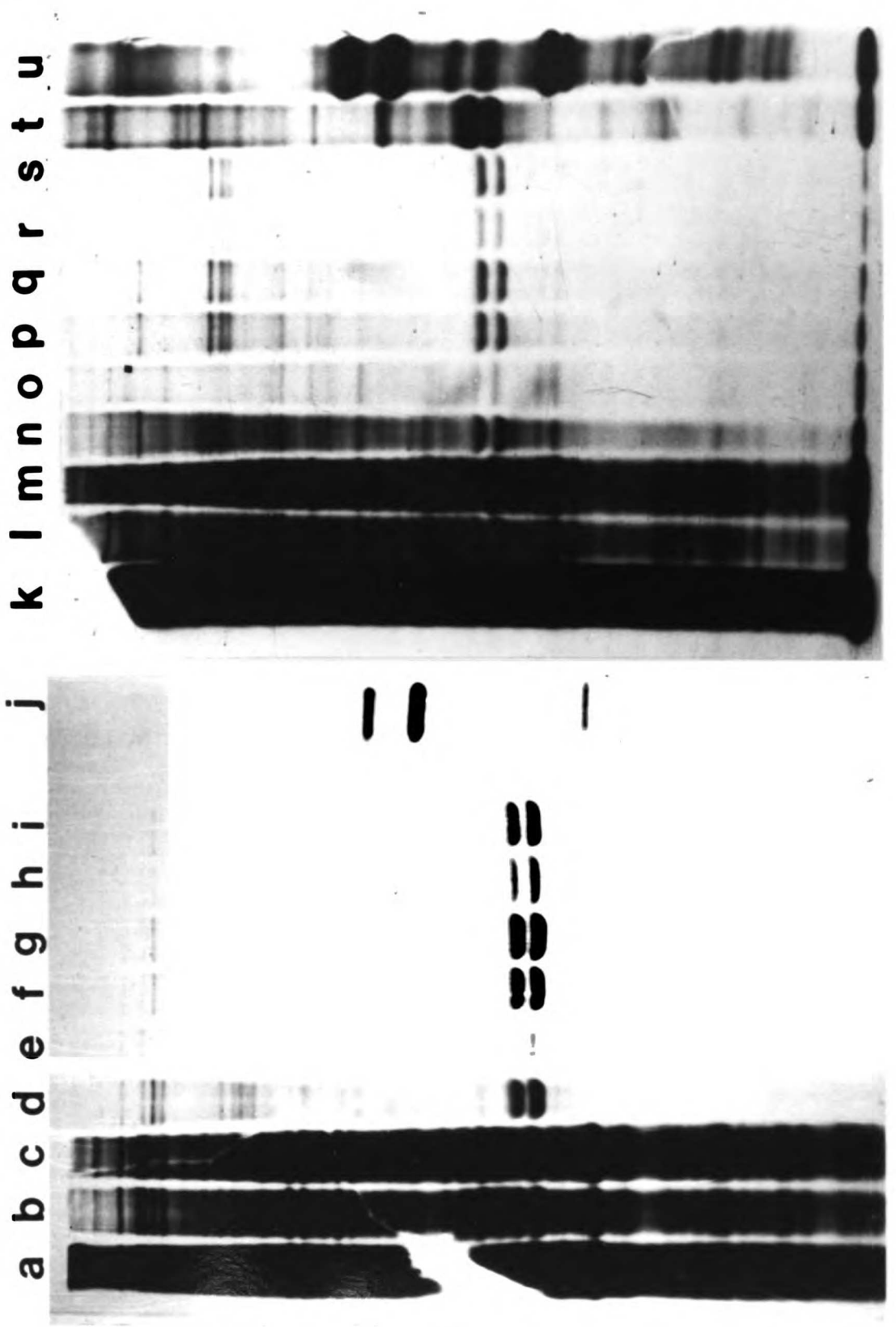
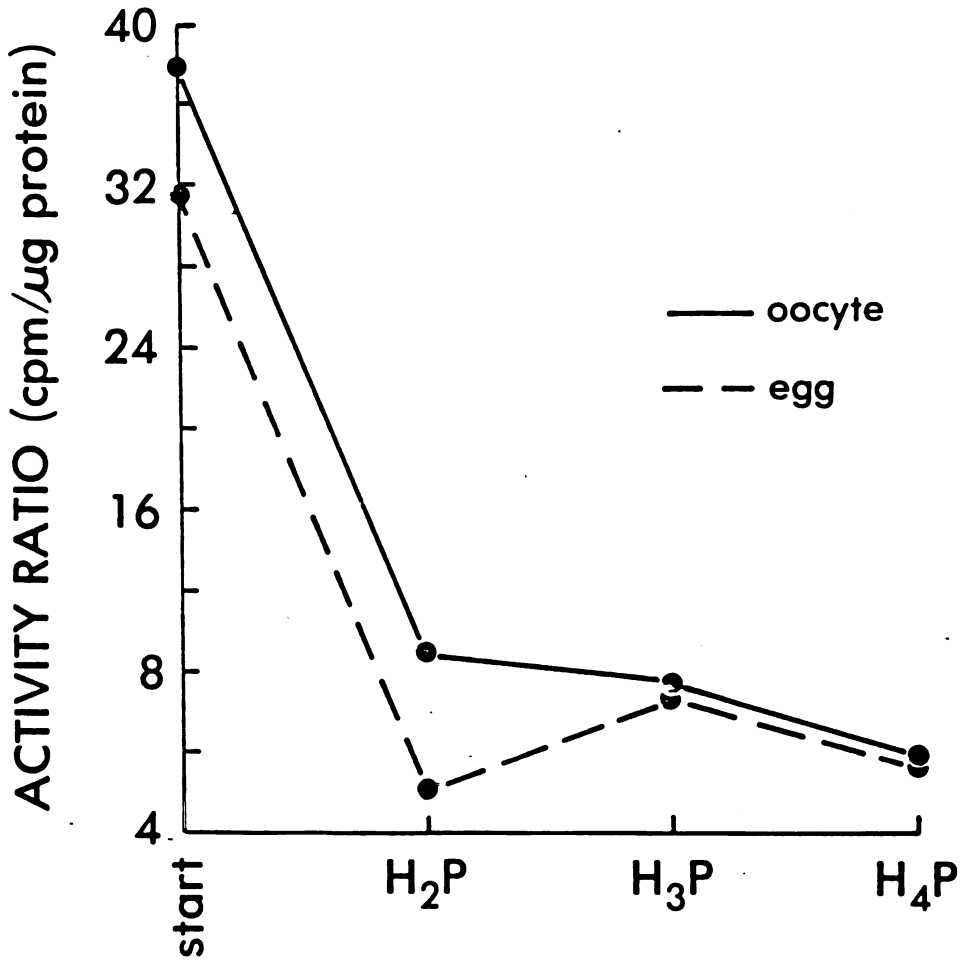


Figure 4


ACTIVITY RATIO (cpm/μg protein)
40
32
24
16
8
4
start
H2P
H3P
H4P
oocyte
egg

CHAPTER 5

Perspectives and Conclusions

Prior to these studies, it was known that diffusible cytoplasmic factors could induce mitotic events, such as chromatin condensation and nuclear envelope breakdown, in interphase nuclei. Interphase nuclei were exposed to mitotic cytoplasm, either by microinjection into Xenopus eggs naturally arrested in M-phase (Gurdon, 1968) or by fusion of interphase and mitotic cultured cells (Rao and Johnson, 1970). Lohka and Masui (1984a) further showed that these events could occur in vitro. After incubating demembranated sperm nuclei for three hours in cytoplasmic extracts of eggs naturally arrested in M-phase, individual chromosomes were seen, and no nuclear envelope had been assembled around the chromatin. While it was clear that factors in mitotic cytoplasm could induce early events of mitosis (i.e., chromosome condensation and nuclear envelope breakdown) in interphase nuclei, the nature of these factors was unknown.

It had also been shown that control of the meiotic cell cycle was determined by a cytoplasmic factor. Masui and Markert (1971) and Smith and Ecker (1971) observed that small amounts of cytoplasm transferred from mature oocytes, arrested in meiotic M-phase, could induce completion of meiosis (maturation) in immature oocytes arrested in meiotic G2 or prophase. This maturation-promoting factor (MPF) was partially purified (Wu and Gerhart, 1980), using breakdown of the oocyte nuclear envelope (germinal vesicle) as an assay. When it was discovered that this activity cycled with the same period as the mitotic cell cycle, with peaks of activity occurring during M-phase, (Wasserman and Smith, 1978; Kishimoto et al, 1982; Gerhart et al, 1984) the possibility was raised that MPF might also regulate the mitotic cell cycle, as well as the meiotic cycle. This possibility was strengthened by the isolation

of MPF activity from cultured mammalian cells in mitotic M-phase (Sunkara et al, 1979; Nelkin et al, 1980). However, it was unknown what the immediate effects of MPF were in either the mitotic or the meiotic cell cycles.

These studies identify MPF as the diffusible cytoplasmic factor that induces prophase in interphase nuclei. We have shown that MPF can act both in vivo and in vitro to rapidly induce nuclear envelope breakdown and chromatin condensation. In order to examine the immediate effects of MPF in vivo, we arrested the embryonic cell cycle in a premitotic state (by cycloheximide treatment). Within 5 min of injection of MPF into such arrested embryos, nuclear envelope breakdown occurs in a dose-dependent manner. In vitro, interphase nuclei from cultured cells were fist entrained to a premitotic state by incubating them in a cell-free extract of arrested embryos. Addition of MPF to the nuclei in the cell-free extract resulted in nuclear envelope breakdown and individual chromosomes 40 min later.

When taken in conjunction with other recent results, these studies help to demonstrate the role of MPF as an endogenous regulator that initiates M-phase in the cell cycle. Gerhart et al (1984) have shown that MPF activity is experimentally inseparable from the cell-cycle oscillator. Furthermore, Newport and Kirschner (1984) have shown that the addition and breakdown of MPF alone is sufficient to drive the simple embryonic cell cycle.

MPF-dependent changes in the nuclear envelope

The nuclear envelope defines the boundary between the nucleus and the cytoplasm, and as such, is a definitive characteristic of eukaryotic cells. Most studies have focused on the interphase structure of the

nuclear envelope (for reviews, see Kay and Johnston, 1973; Franke and Scheer, 1974; Fry, 1976). The nuclear envelope consists of two membrane bilayers: an outer nuclear membrane, which is continuous with the rough endoplasmic reticulum, and an inner nuclear membrane. The biochemical composition and properties of both membranes are very similar to endoplasmic reticulum. Closely apposed to the inner nuclear membrane is the nuclear lamina. The major components of the nuclear lamina membrane are three closely related proteins, lamins A, B and C (Gerace et al, 1978; Krohne et al, 1978). The inner and outer membranes are joined at the sites of nuclear pore complexes, structurally complex organelles (Unwin and Milligan, 1982) that traverse the layers of the nuclear envelope.

The complex structure of the nuclear envelope is reflective of its association with a variety of functions. As the boundary between the nucleus and the cytoplasm, it is necessarily involved in the transport of proteins and RNA between these two compartments (DeRobertis, 1983). Small molecules can diffuse through the nuclear pores, which have a functional diameter of 9 nm (Paine et al, 1975). The detailed mechanism of transport of larger proteins into the nucleus is unknown, although there is evidence for a specific transport mechanism that recognizes signals encoded in the amino acid sequence of the protein (Hall et al, 1984; Dingwall et al, 1982; Kalderon et al, 1984). In addition, the nuclear envelope serves as an anchoring site for chromatin. Heterochromatin is in close association with the nuclear envelope (Bostock and Sumner, 1978). In most meiotic cells (Moens, 1969 and references therein) and in some plant cells (Fussell, 1975), the nuclear-envelope-associated heterochromatin is a specific subset of the

genome, since it has been shown in these cases that both telomeres of each chromosome are attached to the nuclear envelope. Consistent with this function is the finding that the major proteins of the nuclear lamina bind DNA in vitro (Lebkowski and Laemmli, 1982). Chromatin condensation also begins at the nuclear envelope (Comings and Okada, 1971; McKeon et al, 1984). Finally, the nuclear envelope also acts as an anchoring site on its cytoplasmic face, in this case, for the centrosome that nucleates the cytoplasmic microtubule array (Kuriyama and Borisy, 1981; Bornens, 1977).

Models of interphase structure and function of the nuclear envelope must be reconciled the fact that the nuclear envelope is a dynamic structure. Except in some unicellular organisms (Heath, 1980), the nuclear envelope is disassembled at the end of prophase, and reassembled at the end of mitosis, during telophase. Electron-microscopic observations of nuclear envelope breakdown (Stafstrom and Staehelin, 1984a; Zatsepina et al, 1977; Moll and Paweletz, 1980) showed vesiculation of nuclear membranes, beginning near the centrosomes (where asters of microtubules indented the nuclear envelope). There is variation in how dispersed these vesicles become. In some cases, they remain around the mitotic spindle and form a spindle envelope. The subsequent disappearance of nuclear pore complexes at metaphase was presumed to be disassembly of the pore complexes into their component parts.

The behavior during mitosis of the nuclear lamina has been studied by indirect immunofluorescence (Gerace et al, 1978; Jost and Johnson, 1981; Fuchs et al, 1983), since peripheral heterochromatin often obscures the nuclear lamina in electron microscopic studies. In mitotic

cells, staining of the lamins diffuses throughout the cell. Gerace and Blobel (1980, 1982) have extended the indirect immunofluorescence data by characterizing the dynamics of the nuclear lamina biochemically. They showed that the nuclear lamina is reversibly depolymerized into monomeric subunits of lamins A, B and C, and that the depolymerized lamins are not degraded, but are used to form the nuclear lamina of the daughter cells. Finally, they demonstrated that the lamins are hyperphosphorylated in mitotic cells, and proposed the change in phosphorylation as a possible mechanism for the depolymerization of the nuclear lamina.

Using the ability of MPF to induce nuclear envelope breakdown in a cell-free system, we have been able to examine in detail the kinetics of the increase in lamin phosphorylation and its relationship to depolymerization of the nuclear lamina and nuclear envelope breakdown. We found that, coincident with lamin hyperphosphorylation, the nuclear lamina began to slowly depolymerize until it was no longer visible by indirect immunofluorescence. At this time, nuclear envelope breakdown took place.

The ability to induce nuclear envelope breakdown has enabled us to begin to determine steps in the pathway of the disassembly of the nuclear envelope. Recently, it has become possible to analyze the converse process, assembly of the nuclear envelope, both in vivo (Forbes et al, 1983) and in vitro (Lohka and Masui, 1984b). A combination of these approaches will make it possible to understand the biochemical mechanism of the disassembly and assembly of the nuclear envelope.

Future prospects

At this point, we have worked out a set of conditions such that MPF can induce some early mitotic events in vitro, and we have used this system to examine one of these events, nuclear envelope breakdown, in greater detail than was previously possible. Possible approaches for the future have been divided into the following (somewhat arbitrary) categories. First, it is clear that the cell-free system itself can be refined. Independently of further refinement, it can be used 2) to map pathways of MPF-dependent events, and 3) for further studies on the nuclear lamina. Finally, the cell-free system can be used to examine other mitotic events.

Optimization of the cell-free system

The existing cell-free system is a powerful functional assay for MPF, in that the response is rapid, and morphologically striking. However, the components of the cell-free reaction are far from purified. The MPF used is not purified to homogeneity, the nuclei are simply detergent extracted, and the cell-free extract, which is a postnuclear supernatant that has been cleared of yolk platelets, is essentially total cytoplasm. Attempts to further purify MPF, using chromatographic methods or by isolating specific antibodies to MPF are already underway (Talma Scherson and Martha Cyert, personal communication). It is also possible to fractionate both the nuclei used and the cell-free extract to ask what components are necessary for MPF responsivity. As a specific example, the nuclei could be extensively digested with DNAase or salt-washed before incubation in the cell-free extract to determine if DNA or loosely bound nuclear proteins, respectively, are required either for nuclear envelope breakdown, or the increase in lamin phosphorylation. Similarly, the cell-free extract could be fractionated

to determine what is required in the cytoplasm for MPF responsivity. For example, I have previously found that postmicrosomal supernatants would not allow nuclei to swell or to respond to MPF (see Results, Chapter 4). By contrast, Lohka and Masui (1984b) were able to use such high-speed supernatants for nuclear envelope assembly, provided that the "fluffy layer" of the pellet, containing cytoplasmic membrane vesicles, was re-mixed with the supernatant. This approach could be tried for the cell-free extracts used for nuclear envelope breakdown. Furthermore, the supernatants of a series of centrifugations of increasing force could be tested for their ability to confer MPF responsivity on incubated nuclei; in those cases where MPF responsivity was lost, electron microscopic examination of the sedimented material could determine at least the morphological nature of the subcellular fraction required for MPF responsivity.

A second approach to optimization of the cell-free reaction is to simplify the procedure. Currently, the cell-free extracts of arrested embryos are prepared on a daily basis. Variability in the extract would be reduced, and the time required to set up each experiment would be considerably less, if larger batches of cell-free extract could be frozen in aliquots. This might be accomplished by a few simple procedural changes. First, it is easier to activate large numbers (eg. greater than 2000) of unfertilized eggs than it is to fertilize them. John Newport has been able to use extracts of cycloheximide-arrested activated eggs for nuclear envelope assembly, as long as the eggs are allowed to incubate for 90 min after activation before extracting them (personal communication). I had found that extracts frozen in liquid nitrogen allowed nuclear swelling, but there was no response when MPF

was added. It might be possible to regain MPF responsivity either by preparing the extracts in the presence of protease inhibitors, or by preventing ice-crystal formation by increasing the concentration of sucrose in the extraction buffer.

The cell-free system might be further simplified if the requirement for preincubation of the nuclei in the extract were better defined. For example, we do not know whether, during the preincubation, the nuclei acquire components from the cell-free extract that are required for the ability to respond to MPF. On the other hand, it is also possible that the preincubation could be eliminated entirely, if the nuclei used were already in a premitotic state. Recently, it has become possible to reversibly arrest cultured cells in G2 (Charp et al, 1983). Nuclei prepared from such G2-arrested cells could be used in the cell-free system to test the possibility that nuclei from cells of the appropriate phase of the cell cycle might not require preincubation.

Pathways of MPF-dependent events

While it is becoming clear that MPF initiates M-phase, we have only begun to learn what the immediate effects of MPF are. One approach to dissecting the response to MPF would be to define separate steps in the response and order them in time. Although genetic approaches are not possible in this system, it may be possible to define separate steps by their differential sensitivity to chemical inhibitors. Briefly, a variety of inhibitors could be screened by adding them at the same time as MPF, and asking if they inhibit the morphological response. Those which do inhibit could be tested further by adding them at later times after MPF addition. If addition of the inhibitor at a later time fails to inhibit the morphological response, then it would have been

determined that an inhibitor-sensitive step occurred between the time of MPF addition and the time the inhibitor was added, and that no subsequent steps were inhibitor-sensitive. Examples of inhibitors to screen are nonhydrolyzable NTP analogs, ATP-γS (which can be used preferentially by kinases, and not phosphatases (Eckstein, 1975)), sulfhydryl-blocking reagents such as N-ethyl malemide and iodoacetamide, and quercetin (which has been shown to inhibit a variety of ATPases (see introduction of Gschwendt et al, 1983)). There is precedent for using chemical inhibitors to order events in complex systems. For example, there is a step sensitive to CCCP (an uncoupler of oxidative phosphorylation) prior to transport of the VSV G protein to the Golgi apparatus (Fries and Rothman, 1980); a second example is the fact that MPF acts to induce maturation after a cycloheximide-sensitive step (Wasserman and Masui, 1975; Gerhart et al, 1984).

Another approach to mapping pathways of MPF-dependent events is to examine the biochemical steps between MPF addition and the increase in lamin phosphorylation. If very early substrates for MPF-dependent phosphorylation could be determined, this approach has the potential for isolating possible substrates for a biochemical assay for MPF. We have shown that only a few proteins in labeled CHO nuclei undergo MPF-dependent phosphorylation before the increase in lamin phosphorylation (Results, Chapter 4). However, the total concentration of CHO proteins in the cell-free reaction is approximately 1000-fold less than the protein concentration of the cell-free extract itself. In order to isolate a relatively abundant protein that is rapidly phosphorylated in response to MPF, one could ask what proteins in the cell-free extract are phosphorylated shortly after MPF addition. The

isolation of such a protein for use as a kinase substrate would provide a less direct assay for MPF, but a linear, biochemical assay would be more quantitative and much simpler than the existing assay.

Further studies on the nuclear lamina

Using the cell-free system, it should be possible to purify the lamins kinase. As a first step, one could test whether the lamins kinase in this system has the same properties as the kinase endogenous to the nuclear envelope that appears to phosphorylate one of the lamins in vitro (Lam and Kasper, 1979; Agutter et al, 1979). For example, Agutter et al observed that the kinase activity was inhibited both by ATP-γS and quercetin. If either of these inhibits the MPF-dependent increase in lamin phosphorylation, it would also finally be possible to test the necessity of lamin phosphorylation for nuclear lamina depolymerization, and subsequent nuclear envelope breakdown. It should be noted, however, that neither ATP-γS or quercetin is a specific inhibitor of the nuclear envelope kinase. Quercetin, in particular, has been shown to inhibit a wide variety of enzymes (including some kinases) and transport systems, as well as DNA, RNA and protein synthesis (see introduction of Gschwendt et al, 1983). Nevertheless, the response in the cell-free system is quite rapid, and is independent of many of the processes inhibited by quercetin (eg. DNA, RNA and protein synthesis, and lactate or glucose transport).

The cell-free system also offers the opportunity to study the exchange of lamins into and out of the intact nuclear lamina. During earlier attempts to follow lamina depolymerization biochemically, there were indications that labeled CHO lamins in intact nuclei exchange with unlabeled frog lamins. Although the total amount of labeled lamins did

not change during the time the nuclei were incubated in the cell-free extract, we observed a monotonic decrease in the amount of labeled lamins that cosedimented with the nuclei (data not shown). Because the cell-free extract is prepared from Xenopus embryos that have large cytoplasmic stores of components required in development, there is probably a large store of labeled lamins in the cell-free extract. Thus, the simplest explanation for this observation would be that the amount of lamins in the nuclei were not decreasing, but that there was exchange of unlabeled lamins into the nuclei. Acquisition of lamins into intact nuclear lamina has been observed previously. Cells of two different species were fused and the anti-lamin antibody used to visualize the nuclear lamina reacted with only one of the two species (Jost et al, 1979). In that case, the acquisition of lamins was slow (several days) and dependent on de novo biosynthesis of lamins. By using the cell-free system, we have an opportunity to begin to examine the process of lamins exchange in an biochemically manipulable system. Several questions are completely unresolved: is the process of lamins exchange regulated ? (i.e., how is the amount of lamins in the nuclear lamina determined if there is a large excess of cytoplasm?) Are the cytoplasmic lamins soluble or insoluble? If they are soluble, while the nuclear lamins are insoluble, how is the difference maintained? On the other hand, if the cytoplasmic lamins are also in an insoluble form, are they depolymerized and repolymerized during mitosis like the nuclear lamina? One intriguing possibility for the location of cytoplasmic stores of lamins would be the cytoplasmic arrays of nuclear pore complexes known as annulate lamellae (Kessel, 1983). The annulate lamellae are known to disassemble during M-phase, as the nuclear envelope does (Imoh et al, 1983), and may

function as a store of excess nuclear pore complexes (Stafstrom and Staehelin, 1984b). Using the cell-free system it should be possible to approach several of these questions, and ask if the annulate lamellae contain lamins.

Examination of other mitotic events

We have shown that nuclear envelope breakdown and chromatin condensation occur in interphase nuclei exposed to MPF. There are other events associated with prophase or the onset of mitosis, and it would be interesting to ask if these are also MPF-dependent events. Another morphological event of prophase is the splitting of the centrosome and the migration of the two centriole pairs to the poles of the mitotic spindle. Using the anti-centrosome antisera available in this laboratory (Tuffanelli et al, 1983), it should be possible to determine whether the centriole migration is an MPF-dependent event. A biochemical event associated with mitosis is the phosphorylation of a group of proteins, which is recognized by monoclonal antibodies that were isolated for their ability to stain exclusively mitotic cells by indirect immunofluorescence (Davis et al, 1983). If these mitotic phosphorylation could be shown to be MPF-dependent, it could provide another possible biochemical assay for MPF activity.

Chromatin condensation was one of the events shown to be induced by MPF addition. In the same way that we determined the temporal relationship between nuclear envelope breakdown and the increase in lamin phosphorylation, it should be possible to examine the temporal relationship between chromatin condensation and histone phosphorylation. Increased phosphorylation of histone 1 and phosphorylation of histone 3 has been observed in mitotic cells (Paulson and Taylor, 1982; Gurley et

al, 1978), and has been postulated as a possible mechanism for chromatin condensation. This could be tested in the cell-free system, although the analysis would be facilitated by the use of the appropriate anti-histone antibodies, since purification of the CHO histones from the total protein in the cell-free extract is not straightforward. Furthermore, preliminary attempts to address this question were hindered by the variability in the extent of chromosome condensation. Nevertheless, one might expect histone phosphorylation to occur, since mitotic patterns of histone phosphorylation have been observed in prematurely condensed chromosomes (Ajiro et al, 1983).

Finally, it may be possible to examine later events in mitosis in this cell-free system by the use of cytostatic factor (CSF), in addition to MPF. CSF is a cytoplasmic activity from unfertilized eggs that arrests the cell cycle at metaphase (Masui and Markert, 1971; Meyerhof and Masui, 1979). When Newport and Kirschner (1984) injected a combination of MPF and CSF into cycloheximide-arrested embryos, they observed not only nuclear envelope breakdown and chromatin condensation, but also spindle formation. A similar approach in the cell-free system might make in vitro studies on spindle formation possible. By such an approach, it would be possible to extend the studies of Karsenti et al (1984), which distinguished the relative contributions of the nucleus, the centrosome and the cytoplasm to the mitotic spindle. Also, the presumptive disassembly of nuclear pore complexes does not occur until metaphase (Stafstrom and Staehelin, 1984a), so this is another morphological event that could potentially be studied biochemically in the cell-free system.

In conclusion, the ability to induce mitotic events in a cell-free system makes is feasible to examine a number of morphological events of mitosis biochemically. In addition, this system offers the potential of studying the regulation by MPF of the initiation of M-phase and its coordination of mitotic events.

REFERENCES

Chapter 1

1. Ajiro, K., Nishimoto, T. and Takahashi, T. (1983) Histone H1 and H3 phosphorylation during premature chromosome condensation in a temperature-sensitive mutant (tsBN2) of baby hamster kidney cells. J. Biol. Chem. 94: 1579-1586.
2. Agrell, I. (1964) Natural division synchrony and mitotic gradients in metazoan tissues. In Synchrony in Cell Division and Growth. E. Zeuthen, ed. (Interscience Publishers, New York) pp. 39-67.
3. Agutter, P.S., Cockrill, J.B., Lavine, J.E., McCaldin, B. and Sim, R.B. (1979) Properties of mammalian nuclear-envelope nucleoside triphosphatase. Biochem. J. 181: 647-658.
4. Anastassova-Kristeva, M. (1977) The nucleolar cycle in man. J. Cell Sci. 25: 103-110.
5. Aubin, K.E., Osborn, M. and Weber, K. (1980) Variations in the distribution and migration of centriole duplexes in mitotic PtK2 cells studied by immunofluorescence microscopy. J. Cell Sci. 43: 177-194.
6. Bajer, A. (1959) Change of length and volume of mitotic chromosomes in living cells. Hereditas 45: 579-596.
7. Bradbury, E.M., Inglis, R.J. and Matthews, H.R. (1974) Control of cell division by very lysine rich histone (F1) phosphorylation. Nature 247: 257-261.

8. Brinkley, B.R. (1965) The fine structure of the nucleolus in mitotic divisions of Chinese hamster cells in vitro. J. Cell Biol. 27: 411-422.

9. Cohen, P. (1982) The role of protein phosphorylation in neural and hormonal control of cellular activity. Nature 296: 613-620.

10. Comings, D.E. and Okada, T.A. (1971) Condensation of chromosomes onto the nuclear membrane during prophase. Exp. Cell Res. 63: 471-473.

11. Croy, R.B. and Pardee, A.B. (1983) Enhanced synthesis and stabilization of Mr 68,000 protein in transformed BALB/c-3T3 cells: candidate for restriction point control of cell growth. Proc. Natl. Acad. Sci. 80: 4699-4703.

12. Davis, F.M., Tsao, R.Y., Fowler, S.K. and Rao, P.N. (1983) Monoclonal antibodies to mitotic cells. Proc. Natl. Acad. Sci. 80: 2926-2930.

13. Foe, V.E. and Alberts, B.M. (1983) Studies of nuclear and cytoplasmic behavior during the five mitotic cycles that precede gastrulation in Drosphilia embryogenesis. J. Cell Sci. 61: 31-70.

14. Forbes, D.J., Kirschner, M.W. and Newport, J.W. (1983) Spontaneous formation of nucleus-like structures around bacteriophage DNA microinjected into Xenopus eggs. cell 34: 13-23.

15. Gerace, L. and Blobel, G. (1980) The nuclear envelope lamina is reversibly depolymerized during mitosis. Cell 19: 277-287.

16. Gerhart, J.C. (1980) Mechanisms regulating pattern formation in the amphibian egg and early embryo. in Biological Regulation and Development, Vol. 2, R.F. Goldberger, ed. (Plenum Press, New York) pp. 145-154.

17. Gerhart, J., Wu, M. and Kirschner, M. (1984) Cell cycle dynamics of an M-phase specific cytoplasmic factor in Xenopus laevis oocytes and eggs. J. Cell Biol. 98: 1247-1255.

18. Graham, C.F. and Morgan, R.W. (1966) Changes in the cell cycle during early amphibian development. Dev. Biol. 14: 439-460.

19. Gurdon, J.B. (1968) Change in somatic cell nuclei inserted into growing and maturing amphibian oocytes. J. Embryol. Exp. Morph. 20: 401-414.

20. Gurley, L.R., D'Anna, J.A., Barham, S.S., Deaven, L.L. and Tobey, R.A. (1978) Histone phosphorylation and chromatin structure during mitosis in Chinese hamster cells. Eur. J. Biochem. 84: 1-15.

21. Halleck, M.S., Lumley-Sapanski, K., Reed, J.A., Iyer, A.P., Mastro, A.M. and Schlegel, R.A. (1984) Characterization of protein kinases in mitotic and meiotic cell extracts. FEBS Lett. 167: 193-198.

22. Hara, K., Tydeman, P., and Kirschner, M.W. (1980) A cytoplasmic clock with the same period as the division cycle in Xenopus eggs. Proc. Natl. Acad. Sci. 77: 462-466.

23. Harland, R.M. and Laskey, R.A. (1980) Regulated replication of DNA microinjected in eggs of X. laevis. Cell 21: 761-771.

24. Hartwell, L.H. (1978) Cell division from a genetic perspective. J. Cell Biol. 77: 627-637.

25. Hochhauser, S.J., Stein, J.L. and Stein, G.S. (1981) Gene expression and cell cycle regulation. Int. Rev. Cytol. 71: 96-243.

26. Karsenti, E., Newport, J., Hubble, R. and M. Kirschner (1984) The interconversion of metaphase and interphase microtubule arrays, as studied by the injection of centrosomes and nuclei into Xenopus eggs. J. Cell Biol. 98: 1730-1745.

27. Kauffman, S.A. and Wille, J.J. (1975) The mitotic oscillator of Physarum polycephalum. J. Theor. Biol. 55: 47-93

28. Kishimoto, T., Yamakazi, K., Kato, Y., Koide, S.S. and Kanatani, H. (1984) Induction of starfish oocyte maturation by maturation-promoting factor of mouse and surf clam oocytes. J. Exp. Zool. 231: 293-295.

29. Krystal, G.W. and Poccia, D.L. (1981) Phosphorylation of cleavage state histone H1 in mitotic and prematurely condensed chromosomes. Exp. Cell Res. 134: 41-48.

30. Lam, K.S. and Kasper, C.B. (1979) Selective phosphorylation of a nuclear envelope polypeptide by an endogenous protein kinase. Biochem. 18: 307-311.

31. Maller, J., Wu, M. and Gerhart, J.C. (1977) Changes in protein phosphorylation accompanying maturation of Xenopus laevis oocytes. Dev. Biol. 58: 295-312.

32. Laskey, R.A. (1983) Phosphorylation of nuclear proteins. Phil. Trans. R. Soc. Lond. Series B 302: 143-150.

33. Maller, J.L. (1983) Interaction of steriods with the cyclic nucleotide system in amphibian oocytes. Adv. Cyc. Nuc. Res. 15: 295-336.

34. Masui, Y. and Markert, C.L. (1971) Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J. Exp. Zool. 177: 129-146.

35. Matsui, S.-I., Yoshida, H., Weinfeld, H. and Sandberg, A.A. (1972) Induction of prophase in interphase nuclei by fusion with metaphase cells. J. Cell Biol. 54: 120-132.

36. Mazia, D. (1961) Mitosis and the physiology of cell division. in The Cell, Vol 3, J. Brachet and A.E. Mirsky, eds. (Academic Press, New York) pp. 77-412.

37. Mazia, D. (1974) Chromosome cycles turned on in unfertilized sea urchin eggs exposed to NH_4OH. Proc. Natl. Acad. Sci. 71: 690-693.

38. McKeon, F.D., Tuffanelli, D.L., Kobayashi, S. and Kirschner, M.W. (1984) The redistribution of a conserved nuclear envelope protein during the cell cycle suggests a pathway for chromosome condensation. Cell 36: 83-92.

39. Meyerhof, P.G., Masui, Y. (1979) Properties of a cytostatic factor from Xenopus eggs. Dev. Biol. 72: 182-187.

40. Miake-Lye, R., Newport, J.W. and Kirschner, M.W. (1983) Maturation promoting factor induces nuclear envelope breakdown in cycloheximide-arrested embryos of Xenopus laevis. J. Cell Biol. 97: 81-91.

41. Moll, E. and Paweletz, N. (1980) Membranes of mitotic apparatus of mammalian cells. Eur. J. Cell Biol. 21: 280-287.

42. Nelkin, B., Nichols, C. and Vogelstein, B. (1980) Protein factor(s) from mitotic CHO cells induce meiotic maturation in Xenopus laevis oocytes. FEBS Lett. 109: 233-238.

43. Newport, J.W. and Kirschner, M.W. (1982) A major developmental transition in early Xenopus embryos: I. Characterization and timing of cellular changes at the midblastula stage. Cell 30: 675-686.

44. Pardee, A.B., Dubrow, R., Hamlin, J.L. and Kletzien, R.F. (1978) Animal cell cycle. Ann Rev. Biochem. 47: 715-750.

45. Paulson, J.R. and Taylor, S.S. (1982) Phosphorylation of histones 1 and 3 and nonhistone high mobility group 14 by an endogenous kinase in HeLa metaphase chromosomes. J. Biol. Chem. 257: 6064-6072.

46. Prescott, D.M. (1976) Reproduction of eukaryotic cells. (Academic Press; New York).

47. Pringle, J.R. and Hartwell, L.H. (1981) The Saccharomyces cell cycle. in The Molecular Biology of the Yeast Saccharomyces. J.N. Strathern, E.W. Jones, J.R. Broach, eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) pp. 97-142.

48. Rao, P.N. and Johnson, R.T. (1970) Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. Nature 225: 159-164.

49. Sakai, M. and Kubota, H. (1981) Cyclic surface changes in non nucleate egg fragment of Xenopus laevis. Dev. Growth Diff. 23: 41-49.

50. Sawai, T. (1979) Cyclic changes in the cortical layer of non-nucleated fragments of the newt's egg. J. Embryol. Exp. Morph. 51: 183-193.

51. Shibayama, T., Nakaya, K. and Nakamura, Y. (1983) Change in phosphorylation of nucleolar proteins of Physarum polycephalum during the cell cycle in vivo and in vitro. J. Biochem. 94: 1579-1586.

52. Smith, L.E. and Ecker, R.E. (1971) The interaction of steriods with Rana pipiens oocytes in the induction of maturation. Dev. Biol. 25: 232-247.

53. Stafstrom, J.P. and Staehelin, L.A. (1984) Dynamics of the nuclear

envelope and of nuclear pore complexes during mitosis in the Drosophila embryo. Eur. J. Cell Biol. 34: 179-189.

54. Sunkara, P.S., Wright, D.A. and Rao, P.N. (1979) Mitotic factors from mammalian cells: a preliminary characterization. J. Supramol. Struct. 11: 195-198.

55. Vandre, D.D., Davis, F.M., Rao, P.N. and Borisy, G.G. (1984) Phosphoproteins are components of mitotic microtubule organizing centers. Proc. Natl. Acad. Sci. 81: 4439-4443.

56. Wasserman, W.J. and Smith, L.D. (1978) The cyclic behavior of a cytoplasmic factor controlling nuclear membrane breakdown. J. Cell Biol. 78: R15-R22.

57. Waserman, W. and Masui, Y. (1975) Effects of cycloheximide on a cytoplasmic factor initiating meiotic maturation in Xenopus oocytes. Exp. Cell Res. 91: 381-388.

58. Weintraub, H., Buscaglia, M., Ferrez, M., Weiller, S., Boulet, A., Fabre, F. and Baulieu, E.E. (1982) Mise en evidence d'une activite "MPF" chez Saccharomyces cerevisiae. C.R. Acad. Sc. (Paris) Ser. III 295: 787-790.

59. Wilson, E.B. (1925) The cell in development and heredity. (Macmillan Co., New York).

60. Wu, M. and Gerhart, J.C. (1980) Partial purification and characterization of the maturation-promoting factor from eggs of Xenopus laevis. Dev. Biol. 79: 465-477.

61. Yoneda, M., Ikeda, M. and Washitani, S. (1978) Periodic change in the tension at the surface of activated non-nucleate fragments of sea-urchin eggs. Dev. Growth Diff. 20: 329-336.

Chapter 2

1. Benbow, R.M. and C.C. Ford. 1975. Cytoplasmic control of nuclear DNA synthesis during early development of Xenopus laevis: a cell-free assay. Proc. Nat. Acad. Sci. 72:2437-2441.
2. Burnette, W.N. 1981. "Western blotting": electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Analyt. Biochem. 112:195-203.
3. Clark, T.G. and R.W. Merriam. 1977. Diffusible and bound actin in nuclei of Xenopus laevis oocytes. Cell. 12:883-891.
4. Cohen, P. 1982. The role of protein phosphorylation in neural and hormonal control of cellular activity. Nature. 296:613-620.
5. Dumont, J.N. 1972. Oogenesis in Xenopus laevis. J. Morph. 136:153-180.
6. Eckstein, F. 1975. Investigation of enzyme mechanisms with nucleoside phosphorothioates. Angew. Chem. 14:160-166.
7. Fersht, A. 1977. Enzyme structure and function. W.H. Freeman and Co., San Francisco. 217-220.
8. Freeman, T. 1967. Trace labeling with radioiodine. in Handbook of Experimental Immunology. F.A. Davis Co., Philadelphia. 597-607.
9. Gerace, L. and G. Blobel. 1980. The nuclear envelope lamina is reversibly depolymerized during mitosis. Cell. 19:277-287.

10. Gerace, L., A. Blum and G. Blobel. 1978. Immunocytochemical localization of the major polypeptides of the nuclear pore complex-lamina fraction: interphase and mitotic distribution. J.Cell Biol. 79:546-566.

11. Gerhart, J.C. 1980. Mechanisms regulating pattern formation in the amphibian egg and early embryo. in Biological Regulation and Development, Volume 2. Plenum Press, New York. 145-154.

12. Giloh, H. and J. Sedat. 1982. Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. Science. 217:1252-1255.

13. Giudice, G. 1973. Developmental biology of the sea urchin embryo. Academic Press, New York.

14. Graham, C.F. and R.W. Morgan. 1966. Changes in the cell cycle during early amphibian development. Dev.Biol. 14:439-460.

15. Hammer, M.G., J.D. Sheridan and R.D. Estersen. 1971. Cytochalasin B. II. Selective inhibition of cytokinesis in Xenopus laevis eggs. Soc.Exp.Biol.Med. 136:1158-1162.

16. Hara, K., P. Tydeman and M. Kirschner. 1980. A cytoplasmic clock with the same period as the division cycle in Xenopus eggs. Proc.Nat.Acad.Sci. 77:462-466.

17. Harland, R.M. and R.A. Laskey. 1980. Regulated replication of DNA microinjected into eggs of Xenopus laevis. Cell. 21:761-771.

18. Heidemann, S.R. and M.W. Kirschner. 1975. Aster formation in eggs of Xenopus laevis: induction by isolated basal bodies. J.Cell Biol. 67:105-117.

19. Kirschner, M.W., K.A. Butner, J.W. Newport, S.D. Black, S.R. Scharf and J.C. Gerhart. 1981. Spatial and temporal changes in early amphibian development. Netherlands J. of Zool. 31:50-77.

20. Kishimoto, T., R. Kuriyama, H. Kondo and H. Kanatani. 1982. Generality of the action of various maturation-promoting factors. Exp.Cell Res. 137:121-126.

21. Krohne, G., W.W. Franke, S. Ely, A. D'Arcy and E. Jost. 1978. Localization of a nuclear envelope-associated protein by indirect immunofluorescence microscopy using antibodies against a major polypeptide from rat liver fractions enriched in nuclear envelope-associated material. Cytobiologie. 18:22-38.

22. Landstrom, U. and S. Lovtrup. 1977. Deoxynucleoside inhibition of differentiation in cultured embryonic cells. Exp. Cell Res. 108:201-206.

23. Maller, J.M. and E.G. Krebs. 1977. Progesterone-stimulated meiotic cell division in Xenopus oocytes. J. Biol. Chem. 252:1712-1718.

24. Maller, J., M. Wu and J.C. Gerhart. 1977. Changes in protein phosphorylation accompanying maturation of Xenopus laevis oocytes. Dev.Biol. 58:295-312.

25. Masui, Y. and H.J. Clarke. 1979. Oocyte maturation. Int.Rev. Cytology. 57:185-282.

26. Masui, Y. and C.L. Markert. 1971. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J.Exp.Zool. 177:129-146.

27. Nelkin, B., C. Nichols and B. Vogelstein. 1980. Protein factor(s) from mitotic CHO cells induce meiotic maturation in Xenopus laevis oocytes. FEBS Lett. 109:233-238.

28. Newport, J. and M. Kirschner. 1982. A major developmental transition in early Xenopus embryos: I. Characterization and timing of cellular changes at the midblastula stage. Cell. 30:675-686.

29. Pestell, R.Q.W. 1975. Microtubule protein synthesis during oogenesis and early embryogenesis in Xenopus laevis. Biochem J. 145:527-534.

30. Reynhout, J.K. and L. D. Smith. 1974. Studies on the appearance and nature of a maturation-inducing factor in the cytoplasm of amphibian oocytes exposed to progesterone. Dev. Biol. 38:394-400.

31. Smith, L.D. 1975. Molecular events during oocyte maturation. in Biochemistry of Animal Development, Volume 3. Academic Press, New York. 1-46.

32. Smith, L.D. and R.E. Ecker. 1969. Role of the oocyte nucleus in physiological maturation in Rana pipiens. Dev. Biol. 19:281-309.

33. Stick, R. and G. Krohne. 1982. Immunological localization of the major architectural protein associated with the nuclear envelope of the Xenopus laevis oocyte. Exp. Cell Res. 138:319-330.

34. Sunkara, P.S., D.A. Wright and P.N. Rao. 1979. Mitotic factors from mammalian cells: a preliminary characterization. J.Supramol.Struc. 11:198-195.

35. Wagenaar, E.B. and D. Mazia. 1978. The effect of emetine on first cleavage division in the sea urchin, Strongylocentrotus purpuratus. in Cell Reproduction. Academic Press, Inc., New York. 539-545.

36. Wasserman, W.J. and Y. Masui. 1975. Effects of cycloheximide on a cytoplasmic factor initiating meiotic maturation in Xenopus oocytes. Exp. Cell Res. 91:381-388.

37. Wasserman, W.J. and L.D. Smith. 1978. The cyclic behavior of a cytoplasmic factor controlling nuclear membrane breakdown. J.Cell Biol. 78:R15-R22.

38. Wasserman, W.J. and L.D. Smith. 1981. Calmodulin triggers the resumption of meiosis in amphibian oocytes. J. Cell Biol. 89:389-394.

39. Woodland, H.R. and E.D. Adamson. 1977. The synthesis and storage of histones during the oogenesis of Xenopus laevis. Dev. Biol. 57:118-135.

40. Wu, M. and J.C. Gerhart. 1980. Partial purification and characterization of the maturation-promoting factor from eggs of Xenopus laevis. Dev.Biol. 79:465-477.

41. Zalokar, M. 1976. Autoradiographic study of protein and RNA formation during early development in Drosophila eggs. Dev. Biol. 52:31-42.

42. Newport, J.W. and M. Kirschner. 1984. Regulation of the cell cycle during early Xenopus development. Cell 37:731-742.

43. Gerhart, J., M. Wu and M. Kirschner. 1984. Cell cycle dynamics of an M-phase specific cytoplasmic factor in Xenopus laevis oocytes and eggs. J. Cell Biol. 98: 1247-1255.

44. McKeon, F., Tuffanelli, D., Fukuyama, K. and M. Kirschner. 1983. Autoimmune response directed against conserved protein determinants of nuclear envelope in a patient with linear scleroderma. Proc. Natl. Acad. Sci. 14: 4374-4378.

45. Forbes, D.J., Kornberg, T.B. and M. Kirschner. 1983. Small nuclear RNA transcription and assembly in early Xenopus development. J. Cell Biol. 97: 62-72.

Chapter 3

1. Adlakha, R.C., Sahasrabuddhe, C., Wright, D.A., Sahasrabuddhe, H., Bigo, H. and Rao, P.N. (1983) Evidence for the presence of inhibitors of mitotic factors during G1 period in mammalian cells. J. Cell Biol. 97: 1707-1713.

2. Aubin, J.E., Osborn, M. and Weber, K. (1980) Variations in the distribution and migration of centriole duplexes in mitotic PtK2 cells studied by immunofluorescence microscopy. J. Cell Sci. 43: 177-194.

3. Bajer, A. (1959) Change of length and volume of mitotic chromosomes in living cells. Hereditas 45: 579-596.

4. Davis, F.M., Tsao, T.Y., Fowler, S.K. and Rao, P.N. (1983) Monoclonal antibodies to mitotic cells. Proc. Natl. Acad. Sci. 80: 2926-2930.

5. Gerace, L. and Blobel, G. (1980) The nuclear envelope lamina is reversibly depolymerized during mitosis. Cell 19: 277-287.

6. Gerace, L. and Blobel, G. (1982) Nuclear lamina and the structural organization of the nuclear envelope. Cold Spring Harbor Symp. Quant. Biol. 46: 967-978.

7. Gerhart, J., Wu, M. and Kirschner, M. (1984) Cell cycle dynamics of an M-phase-specific cytoplasmic factor in Xenopus laevis oocytes and eggs. J. Cell Biol. 98: 1247-1255.

8. Gurdon, J.B. (1968) Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. J. Embryol. Exp. Morph. 20: 401-414.

9. Gurdon, J.B. (1976) Injected nuclei in frog oocytes: fate, enlargement, and chromatin dispersal. J. Embryol. Exp. Morph. 36: 523-540.

10. Halleck, M.S., Lumley-Sapanski, K., Reed, J.A., Iyer, A.P., Mastro, A.M. and Schlegel, R.A. (1984a) Characterization of protein kinases in mitotic and meiotic cell extracts. FEBS Lett. 167: 193-298.

11. Halleck, M.S., Reed, J.A., Lumley-Sapanski, K. and Schlegel, R.A. (1984b) Injected mitotic extracts induce condensation of interphase chromatin. Exp. Cell Res. 153:561-569.

12. Johnson, R.F. and Roberts, K.B. (1964) The growth and division of human small lymphocytes in tissue culture: an electron microscopic study. J. Anat. 98: 303-311.

13. Jost, E. and Johnson, R.T. (1983) New patterns of nuclear lamina induced by cell fusion. Eur. J. Cell Biol. 30: 295-304.

14. Karsenti, E., Newport, J., Hubble, R. and Kirschner, M. (1984) The interconversion of metaphase and interphase microtubule arrays, as studied by the injection of centrosomes and nuclei into Xenopus eggs. J. Cell Biol. 98:1730-1745.

15. Kishimoto, T., Kuriyama, R., Kondo, H. and Kantani, H. (1982) Generality of the action of various maturation-promoting factors. Exp. Cell Res. 137: 121-126.

16. Kishimoto, T., Yamazaki, K., Kato, Y., Koide, S.S. and Kanatani, H. (1984) Induction of starfish oocyte maturation by maturation-promoting factor of mouse and surf clam oocytes. J. Exp. Zool. 231: 293-295.

17. Maller, J., Wu, M. and Gerhart, J.C. (1977) Changes in protein phosphorylation accompanying maturation of Xenopus laevis oocytes. Dev. Biol. 58: 295-312.

18. Masui, Y. and Markert, C.L. (1971) Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J. Exp. Zool. 177: 129-146.

19. Matsui, S-I, Yoshida, H., Weinfeld, H. and Sandberg, A.A. (1972) Induction of prophase in interphase nuclei by fusion with metaphase cells. J. Cell Biol. 54:120-132.

20. Mazia, D. (1961) Mitosis and the physiology of cell division. In The Cell 3, J. Brachet and A.E. Mirsky, eds. (New York: Academic Press)

21. McKeon, F.D., Tuffanelli, D.L., Fukuyama, K. and Kirschner, M.W. (1983) Autoimmune response directed against conserved determinants of nuclear envelope proteins in a patient with linear scleroderma. Proc. Nat. Acad. Sci. 80:4374-4378.

22. Merril, C.R., Goldman, D., Sedman, S.A. and Ebert, M.H. (1981) Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science 211: 1437-1438.

23. Meyerhof, P.G. and Masui, Y. (1979) Properties of a cytostatic factor from Xenopus eggs. Dev. Biol. 72: 182-187.

24. Miake-Lye, R., Newport, J.W. and Kirschner, M.W. (1983) Maturation promoting factor induces nuclear envelope breakdown in cycloheximide-arrested embryos of Xenopus laevis. J. Cell Biol. 97: 81-91.

25. Moll, E. and Paweletz, N. (1980) Membranes of the mitotic apparatus of mammalian cells. Eur. J. Cell Biol. 21: 280-287.

26. Murray, R.G., Murray, A.S. and Pizzo, A (1965) The fine structure of mitosis in rat thymic lymphocytes. J. Cell Biol. 26: 601-619.

27. Nelkin, B., Nichols, C. and Vogelstein, B. (1980) Protein factor(s) from mitotic CHO cells induce meiotic maturation in Xenopus laevis oocytes. FEBS Lett. 109: 233-238.

28. Newport, J.W. and Kirschner, M.W. (1982) A major developmental transition in early Xenopus embryos: I. Characterization and timing of cellular changes at the midblastula stage. Cell 30: 675-686.

29. Newport, J.W. and Kirschner, M.W. (1984) Regulation of the cell cycle during early Xenopus development. Cell 37: 731-745.

30. O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. (1977) High resolution 2-D electrophoresis of basic as well as acidic proteins. Cell 12: 1133-1142.

31. Rao, P.N. and Johnson, R.T. (1970) Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. Nature 225: 159-164.

32. Smith, L.D. and Ecker, R.E. (1971) The interaction of steriods with Rana pipiens oocytes in the induction of maturation. Dev. Biol. 25: 232-247.

33. Stafstrom, J.P. and Staehlin, L.A. (1984) Dynamics of the nuclear envelope and of nuclear pore complexes during mitotis in the Drosophila embryo. Eur. J. Cell Biol. 34: 179-189.

34. Sunkara, P.S., Wright, D.A. and Rao, P.N. (1979) Mitotic factors from mammalian cells: a preliminary characterization. J. Supramol. Struct. 11: 189-195.

35. Wasserman, W.J. and Smith, L.D. (1978) The cyclic behavior of a cytoplasmic factor controlling nuclear membrane breakdown. J. Cell Biol. 78: R15-R22.

36. Wilson, E.B. (1925) The Cell in Development and Heredity (New York: The Macmillan Co.) p. 85, 149.

37. Wolfe, S.L. (1972) Biology of the Cell (Belmont, CA: Wadsworth Publishing Co, Inc.).

38. Wu, M. and Gerhart, J.C. (1980) Partial purification and characterization of the maturation promoting factor from eggs of Xenopus laevis. Dev. Biol. 79: 465-477.

Chapter 4

1. Bonner, W.M. and Laskey, R.A. (1974) A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46: 83-88.

2. Gerhart, J., Wu, M. and Kirschner, M.W. (1984) Cell cycle dynamics of an M-phase specific cytoplasmic factor in Xenopus laevis oocytes and eggs. J. Cell Biol. 98: 1247-1255.

3. Heidemann, S.R. and Kirschner, M.W. (1975) Aster formation in eggs

of Xenopus laevis: induction by isolated basal bodies. J. Cell Biol. 57: 105-117.

4. Heidemann, S.R. and Kirschner, M.W. (1978) Induced formation of asters and cleavage furrows in oocytes of Xenopus laevis during in vitro maturation. J. Exp. Zool. 206: 431-444.

5. Heidemann, S.R. and Gallas, P.T. (1980) The effect of taxol on living eggs of Xenopus laevis. Dev. Biol. 80: 489-494.

6. Karr, T.L., Kristofferson, D. and Purich, D.L. (1980) Mechanism of microtubule depolymerization. J. Biol. Chem. 255: 8560-8566.

7. Karsenti, E., Newport, J., Hubble, R. and Kirschner, M. (1984) Interconversion of metaphase and interphase microtubule arrays, as studied by the injection of centrosomes and nuclei into Xenopus eggs. J. Cell Biol. 98: 1730-1745.

8. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

9. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

10. MacNeal, R.K., Webb, B.C. and Purich, D.L. (1977) Neurotubule assembly at substoichiometric nucleotide levels using a GTP regenerating system. Biochem. Biophys. Res. Comm. 74: 440-447.

11. Margolis, R.L. and Wilson, L. (1978) Opposite end assembly and disassembly of microtubules at steady state in vitro. Cell 13: 1-8.

12. Murphy, D.B. and Borisy, G.G. (1975) Association of high-molecular-weight proteins with microtubules and their role in microtubule assembly in vitro. Proc. Natl. Acad. Sci. 72: 2696-2700.

13. Olmsted, J.B. and Borisy, G.G. (1975) Ionic and nucleotide requirements for microtubule polymerization in vitro. Biochemistry 14: 2996-3005.

14. Spiegelman, B.M., Penningroth, S.M., Kirschner, M.W. (1977) Turnover of tubulin and the N site GTP in Chinese hamster ovary cells. Cell 12: 587-600.

15. Wasserman, W.J. and Masui, Y. (1975) Effects of cycloheximide on a cytoplasmic factor initiating meiotic maturation in Xenopus oocytes. Exp. Cell Res. 91: 381-388.

16. Weingarten, M.D., Suter, M.M., Littman, D.R. and Kirschner, M.W. (1974) Properties of the depolymerization products of microtubules from mammalian brain. Biochemistry 13: 5529-5537.

17. Weingarten, M.D., Lockwood, A.H., Hwo, S. and Kirschner, M.W. (1975) A protein factor essential for microtubule assembly. Proc. Natl. Acad. Sci. 72: 1858-1862.

18. Wilson, L., Snyder, K.B., Thompson, W.C. and Margolis, R.L. (1982) A rapid filtration assay for analysis of microtubule assembly, disassembly, and steady-state tubulin flux. Methods in Cell Biol. 24:159-169.

19. Witman, G.B., Cleveland, D.W., Weingarten, M.D. and Kirschner, M.W. (1976) Tubulin requires tau for growth onto microtubule initiating sites. Proc. Natl. Acad. Sci. 73: 4070-4074.

Chapter 5

1. Agutter, P.S., Cockrill, J.B., McCaldin, B. and Sim, R.B. (1979)

Properties of mammalian nuclear-envelope nucleoside triphosphatase. Biochem. J. 181: 647-658.

2. Ajiro, K., Nishimoto, T. and Takahaski, T. (1983) Histone-H-1 and histone-H-3 phosphorylation during premature chromosome condensation in a temperature-sensitive mutant (tsBN2) of baby hamster-kidney cells. J. Biol. Chem. 258: 4534-4538.

3. Bornens, M. (1977) Is the centriole bound to the nuclear membrane? Nature 270: 80-82.

4. Bostock, C.J. and Sumner, A.T. (1978) The eukaryotic chromosome (New York: North-Holland), pp. 202-206.

5. Charp, P.A., Kinders, R.J. and Johnson, T.C. (1983) G2 cell cycle arrest induced by glycopeptides isolated from the bovine cerebral cortex. J. Cell Biol. 97: 311-316.

6. Comings, D.E. and Okada, T.A. (1971) Condensation of chromosomes onto the nuclear membrane during prophase. Exp. Cell Res. 63: 471-473.

7. Davis, F.M., Tsao, T.Y., Fowler, S.K. and Rao, P.N. (1983) Monoclonal antibodies to mitotic cells. Proc. Natl. Acad. Sci. 80: 2926-2930.

8. DeRobertis, E.M. (1983) Nucleocytoplasmic segregation of proteins and RNAs. Cell 32: 1021-1025.

9. Dingwall, C., Sharnick, S.V. and Laskey, R.A. (1982) A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. Cell 30: 449-458.

10. Eckstein, F. (1975) Investigation of enzyme mechanisms with nucleoside phosphorothioates. Angew. Chem. 14: 160-166.

11. Forbes, D.J., Kirschner, M.W. and Newport, J.W. (1983) Spontaneous

formation of nucleus-like structures around bacteriophage DNA microinjectd into Xenopus eggs. Cell 34: 13-23.

12. Franke, W.W. and Scheer, U. (1974) Structures and functions of the nuclear envelope. In The Cell Nucleus, H. Busch, ed. (New York, Academic Press) pp. 219-347.

13. Fries, E. and Rothman, J.E. (1980) Transport of vesicular stomatitis virus glycoprotein in a cell-free extract. Proc. Natl. Acad. Sci. 77: 3870-3874.

14. Fry, D.J. (1976) The nuclear envelope in mammalian cells. In Mammalian Cell Membranes, G.A. Jamieson and D.M. Robinson, eds. Vol. 2 (Boston, Butterworth Group) pp. 197-265.

15. Fuchs, J.-P., Giloh, H., Kuo, C-.H., Saumweber, H. and Sedat, J. (1983) Nuclear structure: determination of the fate of the nuclear envelope in Drosophila during mitosis using monoclonal antibodies. J. Cell Sci. 64: 331-349.

16. Fussell, C.P. (1975) The position of interphase chromosomes and late replicating DNA in centromere and telomere regions of Allium cepa L. Chromosoma 50: 201-210.

17. Gerace, L. and Blobel, G. (1980) The nuclear envelope lamina is reversibly depolymerized during mitosis. Cell 19: 277-287.

18. Gerace, L. and Blobel, G. (1982) Nuclear lamina and the structural organization of the nuclear envelope. Cold Spring Harbor Symp. 46: 967-978.

19. Gerhart, J., Wu, M. and Kirschner, M. (1984) Cell cycle dynamics of an M-phase-specific cytoplasmic factor in Xenopus laevis oocytes and eggs. J. Cell Biol. 98: 1247-1255.

20. Gurdon, J.B. (1968) Changes in somatic cell nuclei inserted into

growing and maturing amphibian oocytes. J. Embryol. Exp. Morph. 20: 401-414.

21. Gschwendt, M., Horn, F., Kittstein, W., and Mark, F. (1983) Inhibition of the calcium-dependent and phospholipid dependent protein-kinase activity from mouse-brain cytosed by quercetin. Biochem. Biophys. Res. Comm. 117: 444-447.

22. Gurley, L.R., Walter, R.A. and Tobey, R.A. (1978) Sequential phosphorylation of histone subfractions in the Chinese hamster cell cycle. J. Biol. Chem. 250: 3936-3944.

23. Hall, M.N., Hereford, L. and Herskowitz, I. (1984) Targeting of E. coli β-galactosidase to the nucleus in yeast. Cell 36: 1057-1065.

24. Heath, I.B. (1980) Variant mitoses in lower eukaryotes: indicators of the evolution of mitosis. Int. Rev. Cytol. 64: 1-80.

25. Imoh, H., Okamoto, M. and Eguchi, G. (1983) Accumulation of annulate lamellae in the subcortical layer during progesterone-induced oocyte maturation in Xenopus laevis. Develop. Growth. Differ. 25: 1-10.

26. Jost, E., d'Arcy, A. and Ely, S. (1979) Transfer of mouse nuclear envelope specific proteins to nuclei of chick erythrocytes during reactivation in heterokaryons with mouse A9 cells. J. Cell Sci. 37: 97-107.

27. Jost, E. and Johnson, R.T. (1981) Nuclear lamina assembly, synthesis and disaggregation during the cell cycle in synchronized HeLa cells. J. Cell Sci. 47: 25-53.

28. Kalderon, D., Richardson, W.D., Markham, A.F. and Smith, A.E. (1984) Sequence requirements for nuclear location of simian virus 40 large-T antigen. Nature 311: 33-38.

29. Karsenti, E., Newport, J., Hubble, R. and Kirschner, M. (1984) The interconversion of metaphase and interphase microtubule arrays, as studied by the injection of centrosomes and nuclei into Xenopus eggs. J. Cell Biol. 98: 1730-1745.

30. Kay, R.R. and Johnston, I.R. (1973) The nuclear envelope: current problems of structure and function. Subcell. Biochem. 2: 127-166.

31. Kessel, R.G. (1983) The structure and function of annulate lamellae: porous cytoplasmic and intranuclear membranes. Int. Rev. Cytol. 82: 181-303.

32. Kishimoto, T., Kuriyama, R., Kondo, H. and Kanatani, H. (1982) Generality of the action of various maturation-promoting factors. Exp. Cell Res. 137: 121-126.

33. Krohne, G., Franke, W.W., Ely, S., D'Arcy, A. and Jost, E. (1978) Localization of a nuclear-envelope-associated protein by indirect immunofluorescence microscopy using antibodies against a major polypeptide from rat liver enriched in nuclear envelope-associated material. Cytobiologie 18: 22-38.

34. Kuriyama, R. and Borisy, G.G. (1981) Centriole cycle in Chinese hamster ovary cells as determined by whole-mount electron microscopy. J. Cell Biol. 91: 814-821.

35. Lam, K.S. and Kasper, C.B. (1979) Selective phosphorylation of a nuclear envelope polypeptide by an endogenous protein kinase. Biochemistry 18: 307-311.

36. Lebkowski, J.S. and Laemmli, U.K. (1982) Non-histone proteins and long range organization of HeLa interphase DNA. J. Mol. Biol. 156: 325-344.

37. Lohka, M.J. and Masui, Y. (1984a) Effects of Ca^{+2} ions on the

formation of metaphase chromsomes and sperm pronuclei in cell-free preparations from unactivated Rana pipiens eggs. Dev. Biol. 103: 434-442.

38. Lohka, M.J. and Masui, Y. (1984b) Roles of cytosol and cytoplasmic particles in nuclear envelope assembly and sperm pronuclear formation in cell-free preparations form amphibian eggs. J. Cell Biol. 98: 1222-1230.

39. Masui, Y. and Markert, C.L. (1971) Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J. Exp. Zool. 177: 129-146.

40. Meyerhof, P.G. and Masui, Y. (1979) Properties of a cytostatic factor from Xenopus eggs. Dev. Biol. 72: 182-187.

41. McKeon, F.D., Tuffanelli, D.L., Kobayashi, S. and Kirschner, M.W. (1984) The redistribution of a conserved nuclear envelope protein during the cell cycle suggests a pathway for chromosome condensation. Cell 36: 83-92.

42. Moens, P.B. (1969) The fine structure of meiotic chromosome polarization and pairing in Locusta migratoria spermatocytes. Chromosoma 28: 1-25.

43. Moll, E. and Paweletz, N. (1980) Membranes of the mitotic apparatus of mammalian cells. Eur. J. Cell Biol. 21: 280-287.

44. Nelkin, B., Nichols, C. and Vogelstein, B. (1980) Protein factor(s) from mitotic CHO cells induce meiotic maturation in Xenopus laevis oocytes. FEBS Lett. 109: 233-238.

45. Newport, J.W. and Kirschner, M.W. (1984) Regulation of the cell cycle during early Xenopus development. Cell 37: 731-742.

46. Paine, P.L., Moore, L.C. and Horowitz, S.B. (1975) Nuclear envelope

permeability. Nature 254: 159-164.

47. Paulson, J.R. and Taylor, S.S. (1982) Phosphorylation of histones 1 and 3 and nonhistone high mobility group 14 by an endogenous kinase in HeLa metaphase chromosomes. J. Biol. Chem. 257: 6064-6072.

48. Rao, P.N. and Johnson, R.T. (1970) Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. Nature 225: 159-164.

49. Smith, L.D. and Ecker, R.E. (1971) The interaction of steroids with Rana pipiens oocytes in the induction of maturation. Dev. Biol. 25: 232-247.

50. Stafstrom, J.P. and Staehelin, L.A. (1984a) Dynamics of the nuclear envelope and of nuclear pore complexes during mitosis in the Drosophila embryo. Eur. J. Cell Biol. 34: 179-189.

51. Stafstrom, J.P. and Staehelin, L.A. (1984b) Are annulate lamellae in the Drosophila embryo the result of overproduction of nuclear pore components? J. Cell Biol. 98: 699-708.

52. Sunkara, P.S., Wright, D.A., Rao, P.N. (1979) Mitotic factors from mammalian cells induce germinal vesicle breakdown and chromosome condensation in amphibian oocytes. Proc. Natl. Acad. Sci. 76: 2799-2802.

53. Tuffanelli, D.L., McKeon, F., Kleinsmith, D., Burham, T.K. and Kirschner, M. (1983) Anticentrosome and anticentriole antibodies in the scleroderma spectrum. Arch. Derm. 119: 560-566.

54. Unwin, P.N.T. and Milligan, R.A. (1982) A large particle associated with the perimeter of the nuclear pore complex. J. Cell Biol. 93: 63-75.

55. Wasserman, W.J. and Masui, Y. (1975) Effects of cycloheximide on a

cytoplasmic factor controlling nuclear membrane breakdown. Exp. Cell Res. 91: 381-388.

56. Wasserman, W.J. and Smith, L.D. (1978) The cyclic behavior of a cytoplasmic factor controlling nuclear membrane breakdown. J. Cell Biol. 78: R15-R22.

57. Wu, M. and Gerhart, J.C. (1980) Partial purification and characterization of the maturation-promoting factor from eggs of Xenopus laevis. Dev. Biol. 79: 465-477.

58. Zatsepina, O.V., Polyakov, V.Y. and Chentsov, Y.S. (1977) Some structural aspects of the fate of the nuclear envelope during mitosis. Cytobiologie 16: 130-144.