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

RYN MIAKE-LYE, JOHN NEWPORT, and MARC KIRSCHNER
Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

ABSTRACT   We have studied the effect of maturation-promoting factor (MPF) on embryonic nuclei during the early cleavage stage of *Xenopus laevis* development. When protein synthesis is inhibited by cycloheximide during this stage, the embryonic cell cycle arrests in an artificially produced G_2 phase-like state, after completion of one additional round of DNA synthesis. Approximately 100 nuclei can be arrested in a common cytoplasm if cytokinesis is first inhibited by cytochalasin B. Within 5 min 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 antilamin antiserum. The breakdown of the nuclear envelope occurs at levels of MPF comparable to or slightly lower than those required for oocyte maturation. Amplification of MPF activity, however, does not occur in the arrested egg as it does in the oocyte. 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.

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 ~30 min, and the cells divide synchronously. There are effectively no G_1 and G_2 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 that 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 suggest that even the replication of injected prokaryotic DNA comes under the control of this early cell cycle (17).[1] 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

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[1] Newport, J., K. Butner, and M. Kirschner, manuscript in preparation.

an immature oocyte, which is naturally arrested in G_2 phase 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 Chinese hamster ovary 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 before 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),[2] 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 that 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 before nuclear envelope breakdown and mitosis (M phase). Although the normal embryo passes rapidly from S to M phase, with no detectable G_2 phase, the cycloheximide-arrested embryos are blocked at the transition from S to M phase in an artificial G_2 phase-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 90 min between the time of MPF injection and the breakdown of the oocyte nucleus, but the arrested embryonic nuclei respond to MPF injection within 5 min. Typically, the transition from the G_2 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: (a) MPF purified from meiotic cells is competent to induce a

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[2] Gerhart, J., M. Wu, and M. Kirschner, manuscript in preparation.

part of the mitotic cell cycle, and (b) nuclear envelope breakdown is a rapid response to partially purified MPF in vivo.

MATERIALS AND METHODS

MPF: MPF was generously provided by Michael Wu and John Gerhart, U of California, Berkeley. It was purified 30-fold 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 min after fertilization, they were transferred into a modified amphibian Ringer's (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 Chemical Co., St. Louis, MO) to facilitate later injections and 5 μg/ml of 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 min 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. 4 or 5 h 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 ml of MMR containing 5% Ficoll and 10 μg/ml cycloheximide (Sigma Chemical Co.). (Cytochalasin B was no longer necessary to inhibit cytokinesis at this point; after 2–3 h postfertilization, cytochalasin B-treated embryos will not cleave, even if cytochalasin B is rinsed out of the medium.) Embryos were completely arrested in a G_2 phase-like state (as judged by the appearance of their nuclei) 55–60 min 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 1 h of real time corresponded to 20 s of recording.

DNA Synthesis: Fertilized eggs were pulse-labeled 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 10 min. At the end of the labeling 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 of Proteinase K (EM Biochemicals, Darmstadt, Federal Republic of Germany) was added, and the homogenate incubated at 37°C for 1 h. 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 × 15 cm) and electrophoresed overnight at 35 V. The gel was dried onto a paper backing and autoradiographed for 2 d 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 milliliters of Tellysnicky's modification of Smith's fixative (0.5 g of potassium dichromate, 2.5 ml of glacial acetic acid, and 10 ml of formaldehyde solution, diluted to 100 ml with water) between 2 and 16 h. 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, American Hospital Supply Corp., Gibbstown, NJ). After drying, the slides were stained with Mayer's acid hematoxylin (hematoxylin powder from Chroma-Gesellschaft, Stuttgart, Federal Republic of 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., American Hoechst 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 with nail polish to prevent dehydration. These squashes were scanned by fluorescence microscopy with a Zeiss photomicroscope to locate nuclei; the state of the nuclear envelope was observed with 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 ~15s, 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 min onto acid-cleaned coverslips coated with cytochrome c. The coverslips were washed once with 1% bovine serum albumin (BSA, Sigma Chemical Co.) and 0.1% Triton X-100 in PBS (2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 137 mM NaCl, 8.1 mM NaH$_2$PO$_4$, 0.7 mM CaCl$_2$, and 0.5 mM MgCl$_2$). The primary antiserum was human serum from a patient with linear scleroderma.[3] This serum contains antibodies to lamins, the major proteins of the nuclear envelope lamina (10, 21). Sections were incubated in a 1:1,000 dilution of this serum for 15 min. 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 min. The secondary antibody 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 Chemical Co.) 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 min. 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% (vol/vol) Triton X-100 was added to a final concentration of 0.1%. Cells were extracted for 5 min on ice, then repelleted (1,000 g, 5 min). 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 U/ml. The cells were digested for 15 min 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 min.

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 Laboratories, Richmond, CA) or transferred to nitrocellulose (Schleicher & 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 min 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 min in a Beckman Microfuge B (Beckman Instruments, Inc., Fullerton, CA). 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.[2]

RESULTS

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,[1] and can be visualized by time-lapse cinematography

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[3] McKeon, F. D., D. Tuffanelli, K. Fukuyama, and M. W. Kirschner. 1983. Autoimmune response directed against conserved determinants of nuclear envelope proteins in a patient with linear scleroderma. *Proc. Natl. Acad. Sci. USA.* In press.

(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 min 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 30 min. 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
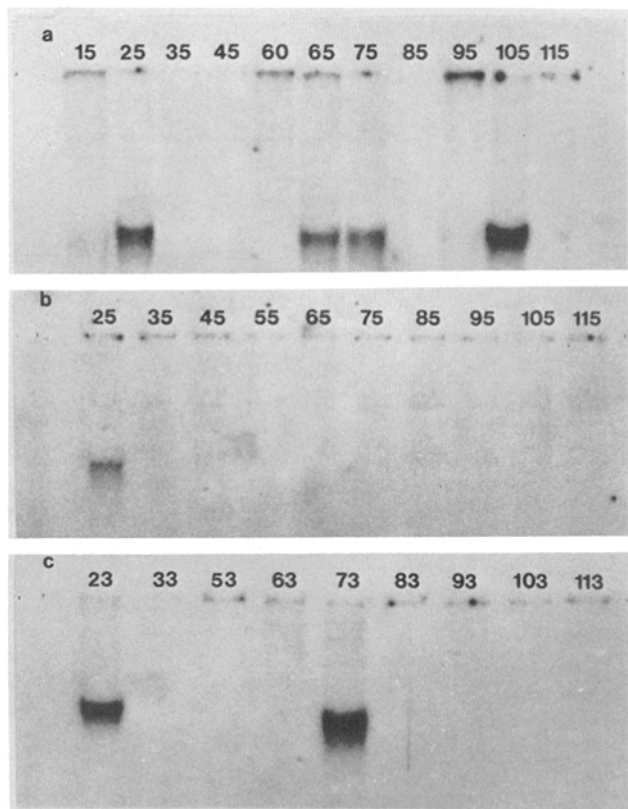

FIGURE 1 Timing of DNA synthesis in normal and cycloheximide-treated fertilized eggs. Each lane on the gel is the nucleic acid extracted from a group of three fertilized eggs that have been pulse-labeled with α-^{32}P-dCTP. Labeling was started at the times indicated (shown as minutes after fertilization) by injection of 50 nl of α-^{32}P-dCTP. After 10 min, eggs were lysed in buffer containing 1% SDS to terminate the labeling. Nucleic acid isolated from these cells was run on a 1% agarose gel and autoradiographed for 2 d. 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 (>55 kb), and is the only band labeled on the gel. (a) normal fertilized eggs, (b) cycloheximide (50 nl of 200 μg/ml) injected 12–20 min postfertilization, before first round of DNA synthesis, and (c) cycloheximide injected 38–48 min postfertilization, before second round of DNA synthesis.

for 10-min 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-labeled 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 Fig. 1 co-migrate with a 55-kb fragment, above a 17-kb fragment.) The labeled bands are completely DNase-sensitive, and resistant to digestion by RNase. Fig. 1 a shows the normal times of occurrence of the first three rounds of DNA synthesis in *Xenopus* embryos. If these times are normalized to the average time of first cleavage (90 min after fertilization, at 21°C), then the average periods of DNA synthesis are between 24 and 35 min, 71 and 85 min, and 105 and 120 min after fertilization. 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 before the first S phase still allows the initiation and termination of the first round of DNA synthesis at the normal time (Fig. 1 b). The first S phase occurs even though protein synthesis, as assayed by ^{35}S-methionine incorporation into TCA-precipitable material, is inhibited more than 95% (data not shown) before initiation of DNA synthesis (within 5 min 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 38–48 min postfertilization (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 (Fig. 1 c). (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 5 h 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 4 h 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 G_2 phase-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 4–5 h after fertilization, ~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 Fig. 2 a, 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. 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% formal-
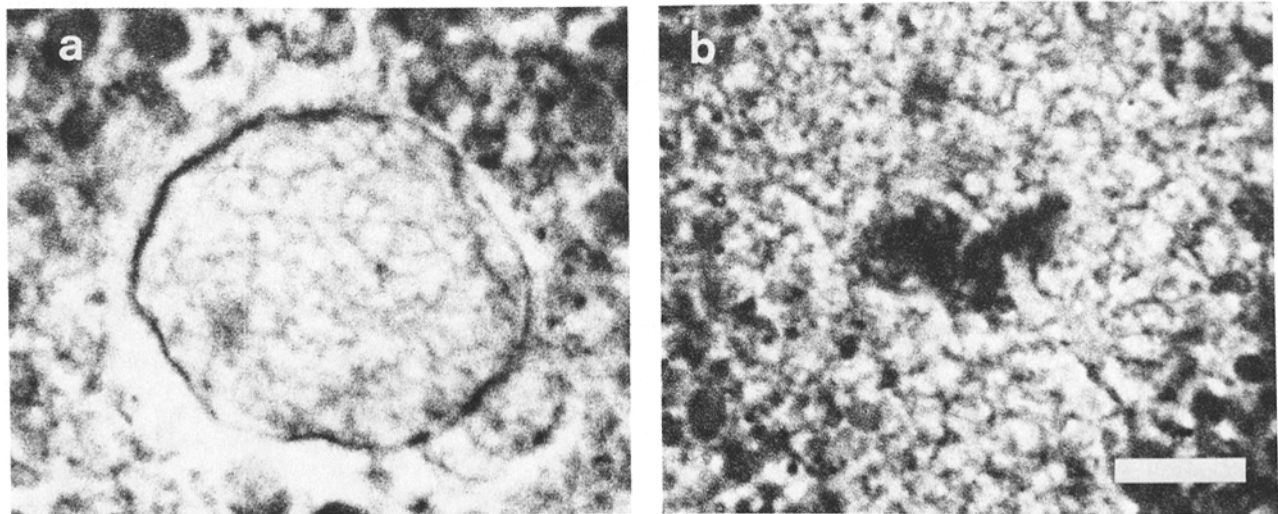

FIGURE 2 Detail of paraffin section of arrested cell showing nucleus before and after injection of MPF. Cells were blocked with cycloheximide as described in Materials and Methods. 1 h after injection of cycloheximide, cells were either fixed (a), or (b) injected with 50 nl of partially purified MPF, incubated for an additional 30 min, and then fixed. After fixation, both samples were embedded in paraffin, sectioned, and stained with hematoxylin and chlorazol black E. Bar, 10 μm. × 1,725.
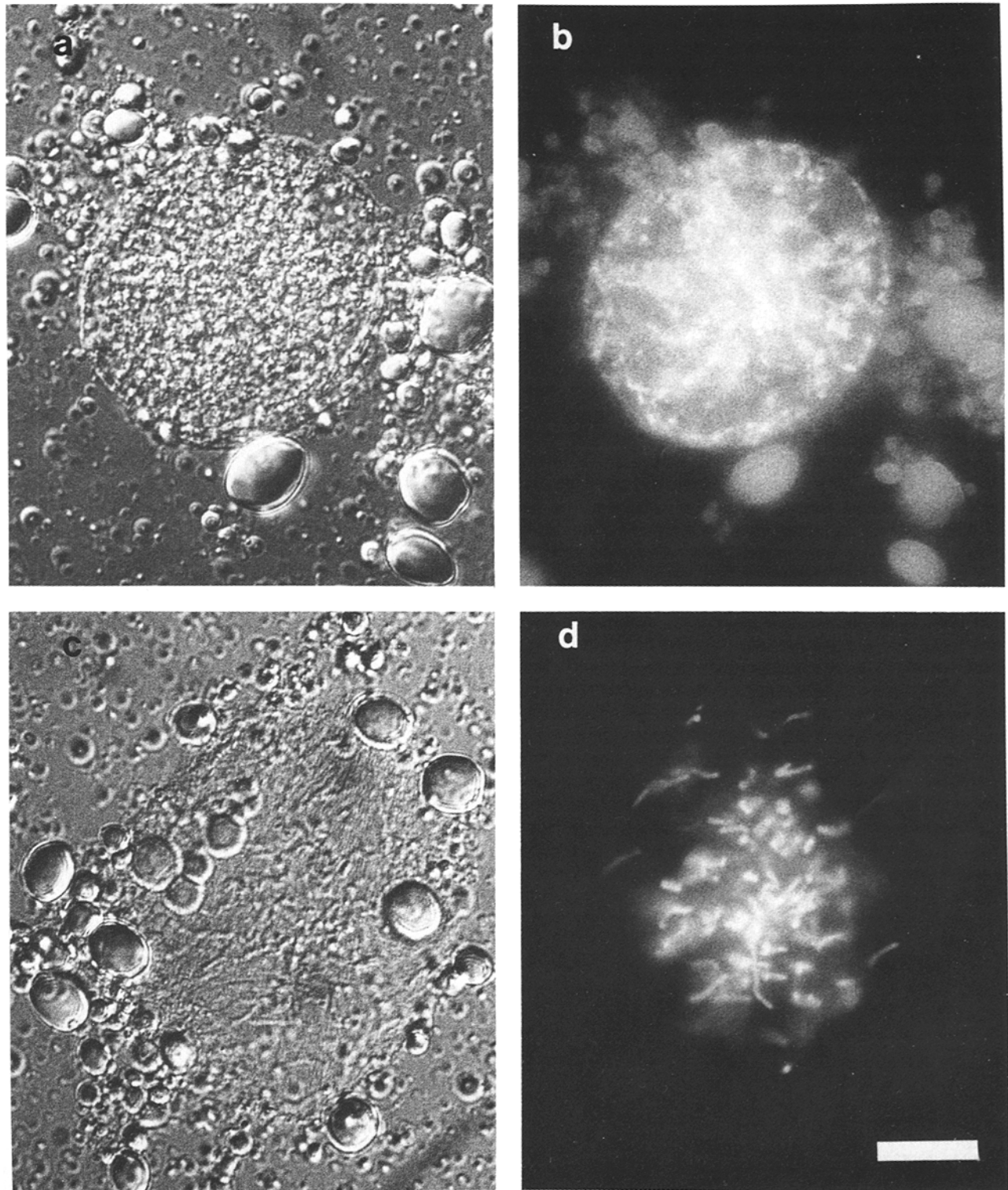
FIGURE 3 Morphology of nuclei in a squash of a cycloheximide-arrested embryo before and after injection of MPF. Cells were blocked with cycloheximide as described in Materials and Methods. Either before (a and b) or after (c and d) injection of MPF, 10 embryos were very gently lysed into 100 μl of buffer containing 1% formaldehyde, allowed to fix for 20 s, 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 μm. × 1,725.

dehyde; this has no effect upon their morphology. Fig. 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 (Fig. 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.[3] This antiserum reacts with lamins in a variety of species, including a cultured *Xenopus* epithelial cell line, A6 (Fig. 4 *a*). 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 (Fig. 4 *b*). (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.)[3]

Nuclei in cycloheximide-arrested embryos (identified by Hoechst staining, Fig. 5 *b*), which have been stained with this antiserum have a distinct and continuous lamina surrounding the chromatin, seen as strong perinuclear staining in Fig. 5 *a*. A continuous lamina is the configuration normally seen in interphase cells (10, 21), and similar to what we observe in interphase *Xenopus* cultured cells (Fig. 4 *a*). 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 motosis, we conclude that they are in an artificially extended G_2 phase, which does not normally occur during the early cleavage stage.

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 μm. × 1,725. (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 (primary) and ^{125}I goat anti-human immunoglobulin (secondary). (c) Identical gel stained with Coomassie Blue. Molecular weight markers are shown on right in kilodaltons.

Effect of MPF on Nuclei in Cycloheximide-arrested Embryos

When MPF partially purified from *Xenopus* eggs is injected into the cycloheximide-arrested embryos (having ~100 nuclei in a common cytoplasm), a radical change in nuclear morphology is observed. Within 5 min after injection, the nuclear envelope breaks down, and chromatin in these embryos is no longer contained within a discrete nucleus. In Fig. 2 *b*, the light microscopic image of paraffin-sectioned material shows a yolk-excluding region that contains darkly staining chromatin. However, the chromatin is no longer dispersed within a clearly defined nuclear envelope (as in Fig. 2 *a*). In Fig. 3 *c*, 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 (Fig. 3 *d*). No changes in the
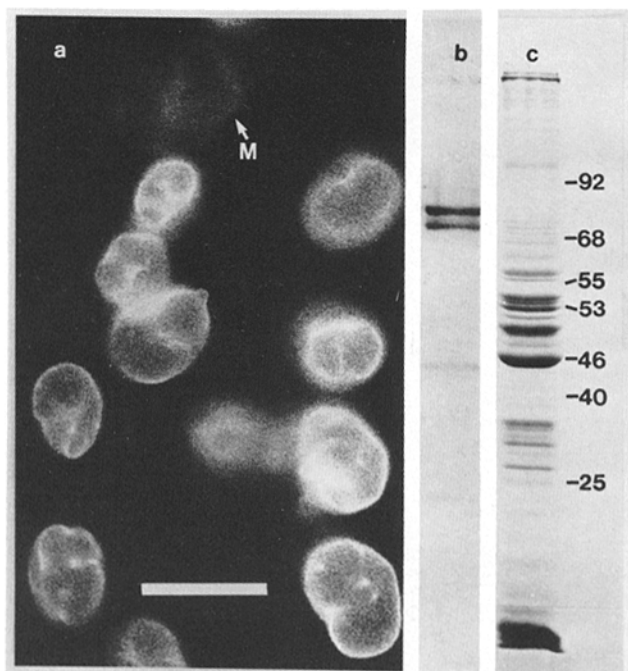
nuclei are observed if the embryos are injected with extraction buffer only.

The breakdown of the nuclear envelope is accompanied by a dissolution of the nuclear lamina. Before injection of MPF, all Hoechst-staining chromatin is surrounded by immunofluorescent staining of the nuclear lamina (Fig. 5, *a* and *b*). 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 (Fig. 5, *c* and *d*). 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 lamina

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 with normal human serum. Bar, 10 μm. × 1,225.
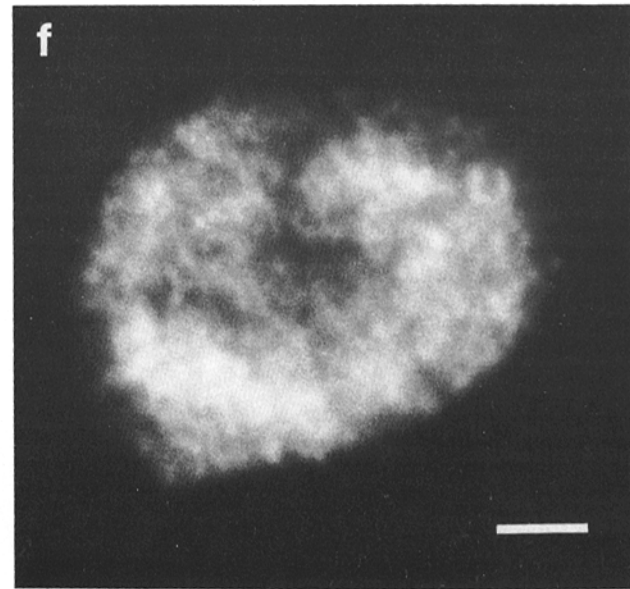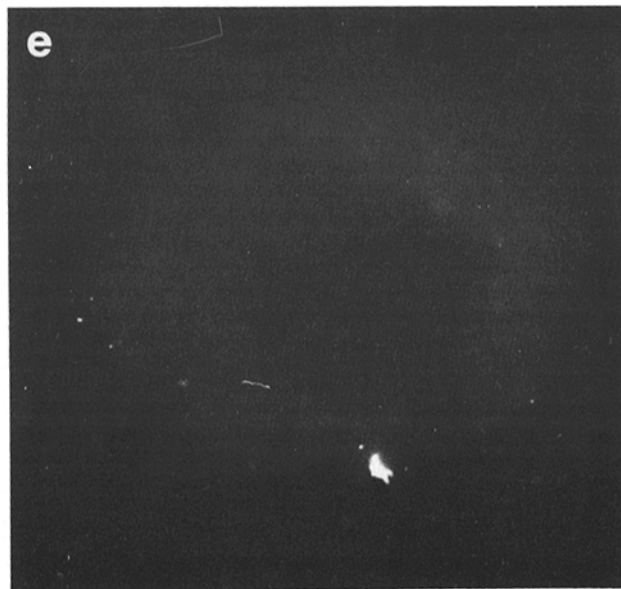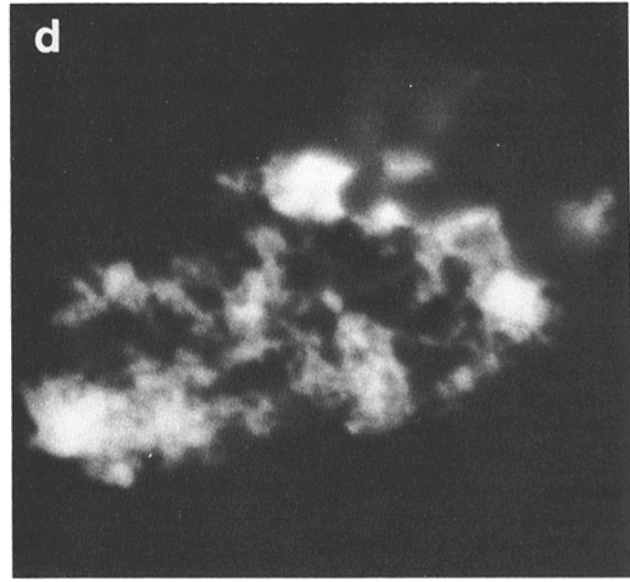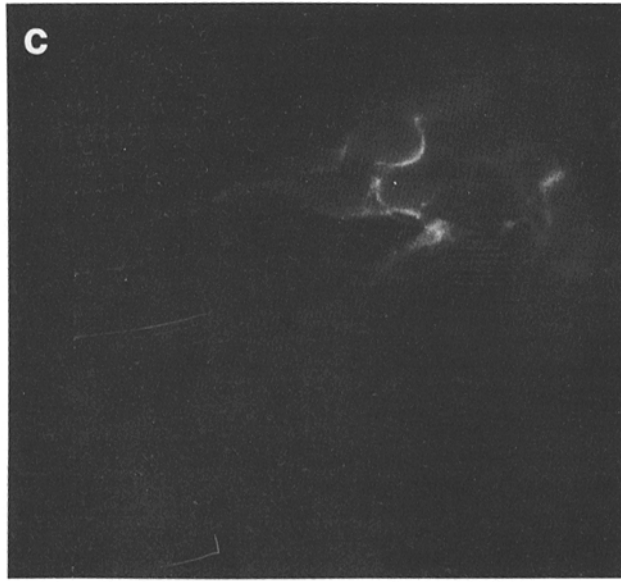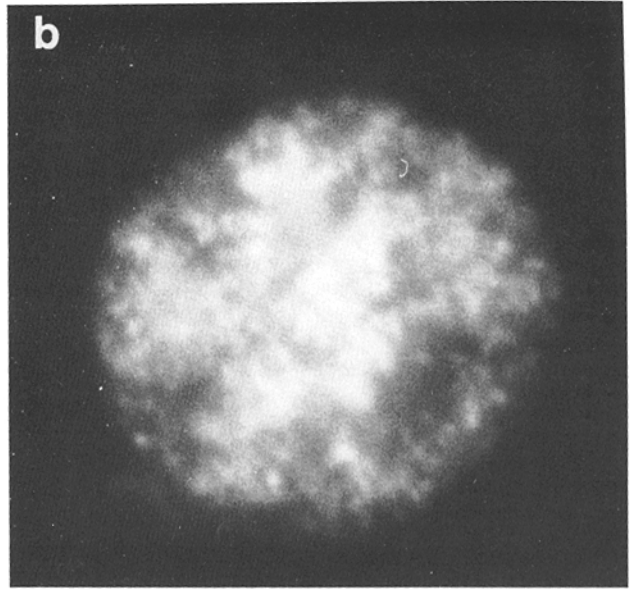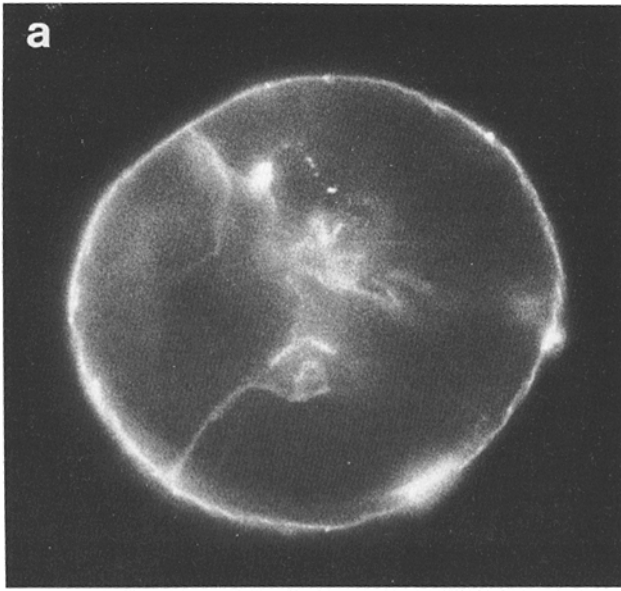
staining is not an artifact of the lysis procedure described above (data not shown). Just as in Fig. 5a, intact nuclei in sections show continuous staining at their periphery. After MPF treatment, no staining is observed in the area surrounding the Hoechst-stained chromatin.

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 that 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 15 min after activation.[2] 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 I, 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 that is 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 min postinjection (56%) as at 68 min postinjection (54%). Similar results are found for MPF at a 1:200 dilution: At 5 min after injection, 20% of the nuclei had broken down, compared with 19% at 45 min.

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. Fig. 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 with 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 Pharmaceutica, Beerse, Belgium]), when any of these are coinjected with MPF.

## Lack of Amplification of MPF in Cycloheximide-arrested Embryos.

Before 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.

Table I

MPF Is Responsible for Nuclear Envelope Breakdown

	Fraction of nuclei or germinal vesicles broken down		
	Source of donor extract		
Recipients	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 6 Dependence of nuclear envelope breakdown on the concentration of MPF injected. Partially purified MPF was injected at various dilutions into arrested embryos (●) or immature oocytes (O). 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 30 min after injection of MPF. Immature oocytes were scored for germinal vesicle breakdown 3 h after injection of MPF. At each concentration, six oocytes were injected with 30 nl of the appropriate dilution of MPF.
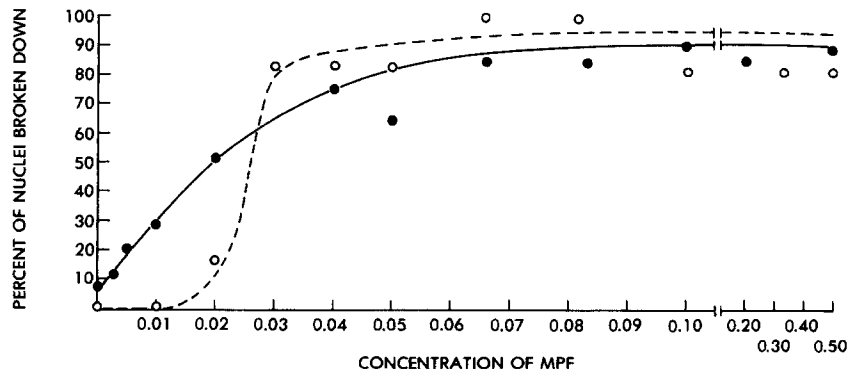
An extract is made of the recipient embryos using conditions that stabilize MPF. This extract, in turn, is injected into immature oocytes to test for the presence of amplified MPF. To be able to detect if MPF activity is transiently amplified and subsequently lost, extracts were made at various times after injection of MPF. No maturation is observed in oocytes injected with extracts made 5, 10, 20, or 30 min 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.[2] 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., 2 h), the nuclei appear as masses of small Hoechst-staining vesicles. This morphology is typical of nuclei that are in the process of re-forming. 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 >95% within 5 min 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 G_2 phase-like state.

The state of this arrest is consistent with observations made using sea urchin eggs. H. Shimada (Zoological Institute, University of Tokyo, personal communication) has shown that sea urchin eggs always initiate and complete one round of DNA synthesis subsequent to inhibition of protein synthesis. 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 mol-

ecules" (such as histones [39], DNA polymerases [1], tubulin [29], small nuclear RNA[4] 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 that 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 that 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 that 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 with 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 ∼30-fold. Although it is not purified to homogeneity, we have provided evidence that it is MPF, and not some protein that 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 that 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 that break down. This relationship does not have a sharp threshold, which is puzzling in view 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

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[4] Forbes, D. J., T. B. Kornberg, and M. W. Kirschner. 1983. Small nuclear RNA transcription and ribonucleoprotein assembly in early *Xenopus* development. *J. Cell Biol.* 97:62–72.

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 (Fig. 6), raises interesting questions about the role of MPF amplification. Injection of MPF in the oocyte can result in amplification of 150–300-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 30 min 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 posttranslational 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 that stabilize MPF activity also stabilize phosphoproteins and/or inhibit phosphatases. For example, both γ-thio-ATP (a good kinase substrate that can be hydrolyzed very poorly by phosphatases [6]) and β-glycerophosphate stabilize MPF and also competitively inhibit phosphatases. Furthermore, Maller et al. (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 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 indicate the possibility that the same response may 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.

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