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Mechanism of Induction of M-phase in Meiotic Maturation of *Xenopus laevis* oocyte

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CONTENTS

General Introduction

Cell division is one of the most fundamental process in life for single cell and multicellular organisms. The pathway from one cell division to the next is called cell cycle.

The cell cycle consists of at least four functionally distinct phases. DNA synthesis occurs during S phase, mitosis during M phase, and each of these two phases is generally separated by period of time during which neither DNA synthesis nor mitosis is occurring. These gap periods between M and S phase and between S and M phase are called G_1 phase and G_2 phase respectively.

Once cell enters the division cycle, interruption at random points in the cycle causes usually cell death. However, two physiological check points have been identified in the cell cycle [1], one is governing entry into S phase and the other is in the G_2 phase governing entry into M phase. The former restriction point determines cells whether to enter the next cell division cycle or to keep quiescent state, G_0 state, in which cell can remain viable and healthy for long periods without dividing. The later restriction point guarantees the successful division of the cell. This point monitors whether numerous biochemical prerequisites for mitosis, such as cell growth, centriole duplication, and replication of genomic information, have taken place normally [2]. Only when these conditions have been fulfilled, the cell begins to enter M phase. Once cell enters M phase, in contrast to the onset of S phase, a lot of changes in morphology occur irreversibly. The drastic changes in M-phase have attracted many researchers for a long time. Recently, some

biochemical aspects of the events within the M phase came into sight. However, the control mechanism in the onset of M phase is largely unknown. In this thesis, I focus on the control mechanism in G_2-M transition in the cell cycle.

In order to investigate the mechanism of G_2-M transition, it has great advantage to use the cell which paused their cell cycle in the G_2-M border. In this point of view, maturation of amphibian oocyte is a good model system for studing the regulatory mechanism of M-phase induction. Fully grown amphibian oocytes are arrested physiologically in late G_2 of meiosis I and they must progress to the second meiotic metaphase before the fertilization [3]. This process of the oocyte preparing for fertilization is called 'oocyte maturation'. In amphibian oocyte, it is easy to manipulate thawing the pause in the cell cycle to induce M-phase both *in vivo* and *in vitro*. The resumption of meiosis *in vivo* is brought by injection with a gonadotropic hormone which acts on ovarian follicle cells, causing them to produce progesterone which acts directly on the oocyte to initiate the process of oocyte maturation [4] (Fig. 1). Similarly, addition of progesterone to the medium induces maturation *in vitro* in oocytes dissected from their ovarian follicles [4]. In addition to the physiological inducer, insulin and insulin-like growth factor can also induce oocyte maturation *in vitro* [5,6].

Oocyte maturation has been studied in a variety of amphibians, but the process has been investigated most intensively in *Xenopus laevis*, the South African clawed frog.

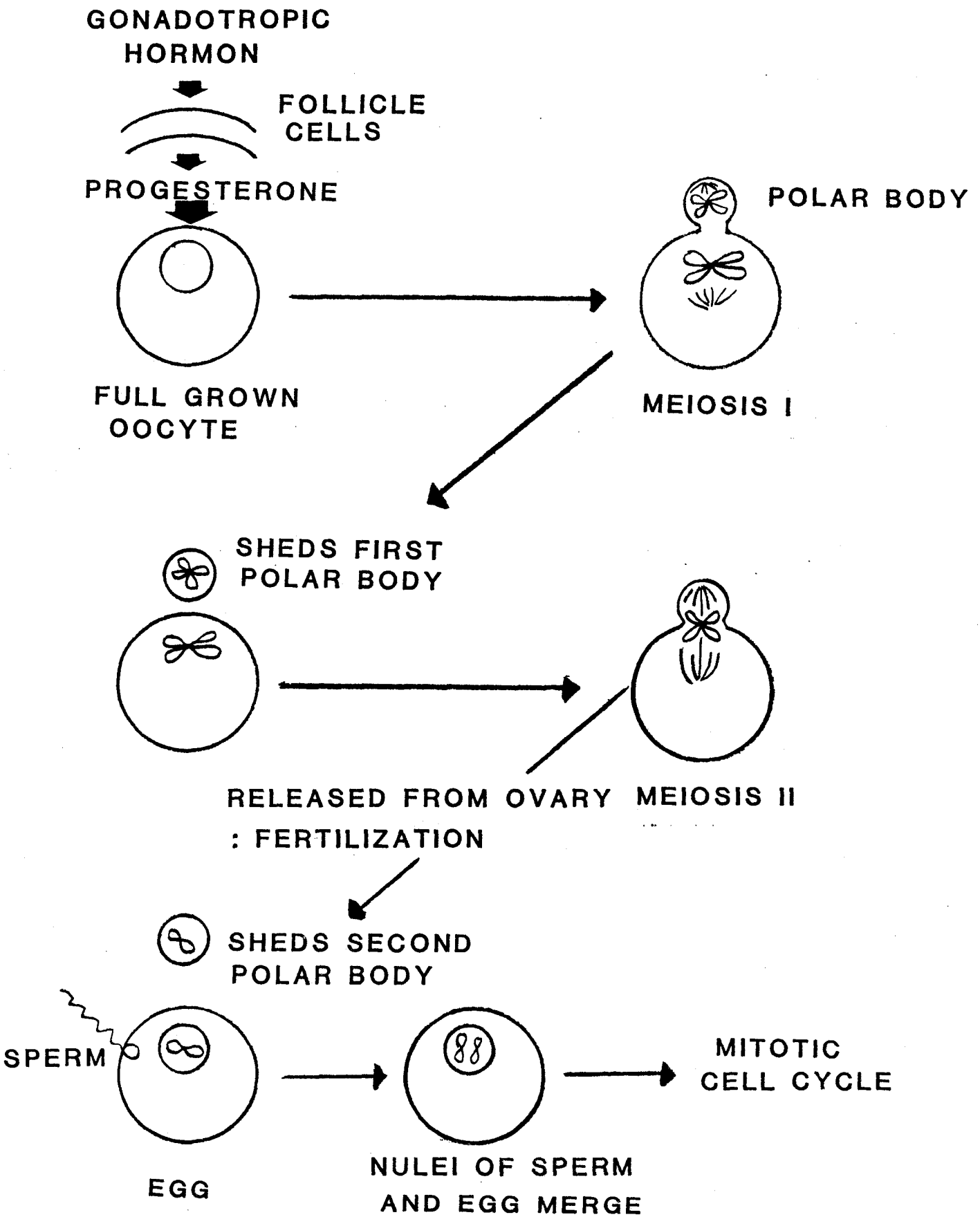


Fig. 1. Schematic diagram of oocyte maturation

Xenopus oocytes are extremely large cells. Fully grown oocytes have 1.2 mm in diameter [7]. This size facilitates the use of semi-quantitative microinjection as a powerful tool for the study of fundamental questions on the cell cycle control and on the development of animal. In addition, a female of *Xenopus laevis* has a large quantity of oocytes almost throughout the year. These merits in this animal facilitate the biochemical approaches to the control mechanism in G_2-M transition in the cell cycle.

The mechanism of progesterone action on the oocytes is different from that of steroid action on somatic target tissues. In somatic tissues, steroids are thought to bind to specific cytosolic receptors and this is followed by migration of the steroid-receptor complex to the nucleus where binding of the complex to particular DNA sequences presumably causes specific changes in gene transcription [8,9]. In *Xenopus* oocyte maturation, it was shown that progesterone binds to a receptor which is located on the surface of oocytes [10-12]. In addition, neither actinomycin D nor α-amanitin, which is an inhibitor of gene transcription, could inhibit the progesterone action to induce maturation [13,14]. However, progesterone-induced maturation was totally inhibited by cycloheximide and puromycine [13,15]. The mechanism of this control in translational level by progesterone is largely unknown.

About 6-8 hours after progesterone treatment, the oocyte nucleus (germinal vesicle), situated near the center of the oocyte, starts to migrate towards the animal hemisphere surface and begin the process of dissolution. The arrival of the

germinal vesicle at the cortex causes pigment to be displaced, producing a white circular spot which is later delineated by a dark ring of the displaced pigment (see Fig. 2). This white spot is the first visible indication that oocyte maturation is proceeding. After dissolution of the nuclear membrane (germinal vesicle break down, GVBD), the condensed chromosome align on the first metaphase spindle where they remain until the mature egg is fertilized.

In addition to these morphological changes, a transferable activity is known to appear in the cytoplasm of metaphase that, when microinjected into immature oocytes, can induce them to engage in meiotic maturation in the absence of progesterone (Fig. 3). This cytoplasmic activity has been called maturation-promoting factor or MPF [11]. MPF activity has been revealed to be universal in oocytes undergoing maturation or in somatic cells in mitosis [16]; extracts from maturing amphibian oocytes can induce maturation in immature starfish oocytes [17], and extracts from synchronized yeast can induce maturation in amphibian oocytes [18]. Furthermore, cytoplasmic extracts from mature oocytes were shown to induce mitotic events in somatic nuclei [19-21].

As mentioned above, when oocytes are treated with progesterone in the presence of cycloheximide, neither morphological change [22] nor production of MPF [23] occurs. However, cytoplasm containing MPF can induce recipient oocytes to complete meiotic maturation even in the presence of cycloheximide [23]. These results have been interpreted to mean that

Fig. 2. Morphological appearance of oocyte exposed to progesterone. (A) Appearance of full grown oocyte with no treatment. (B) Appearance of oocytes incubated in progesterone (10 μM) for 6 h. (C) A section of boiled oocyte of (A). There is a large germinal vesicle. (D) A section of boiled oocyte of (B). There is no germinal vesicle.

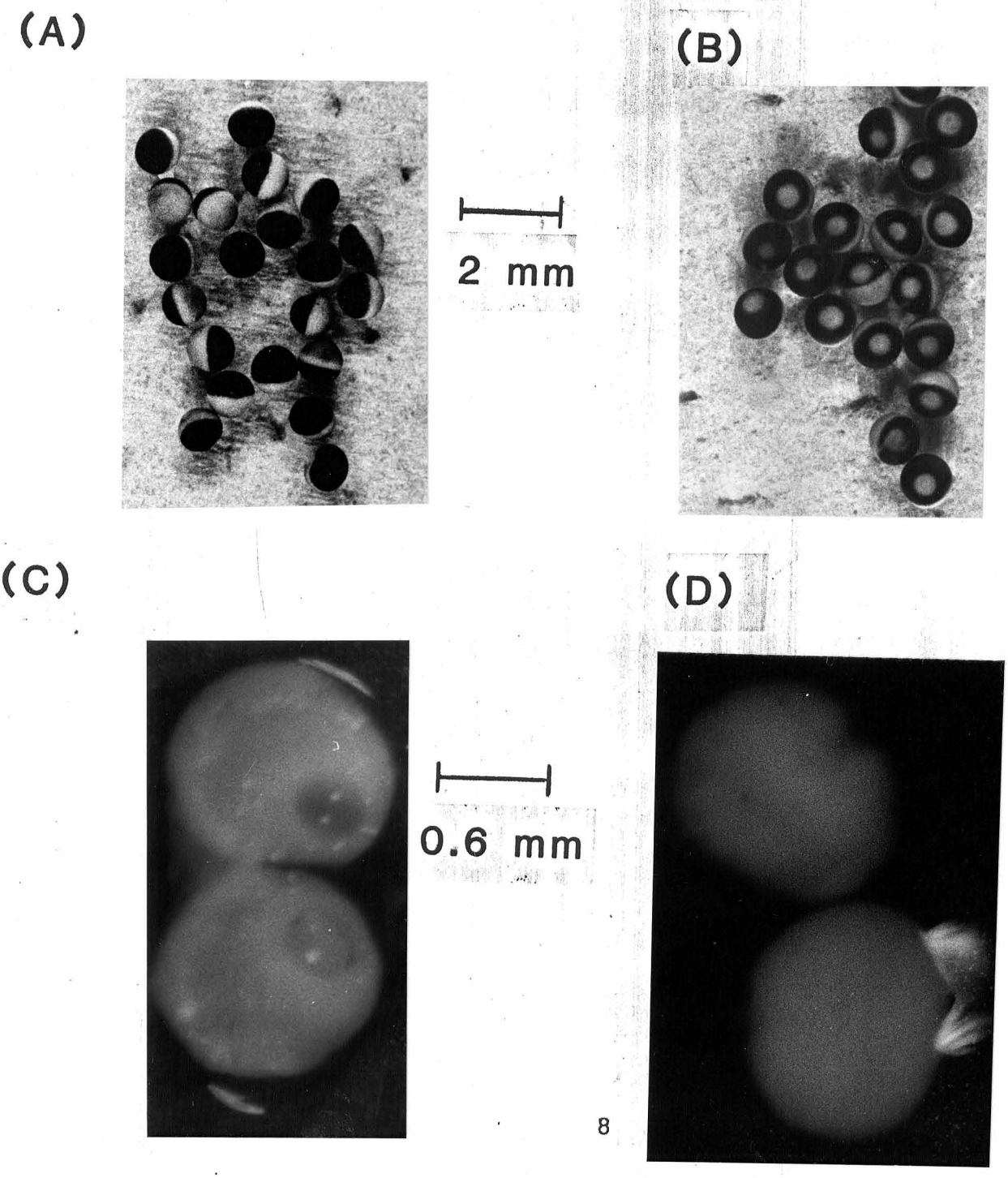
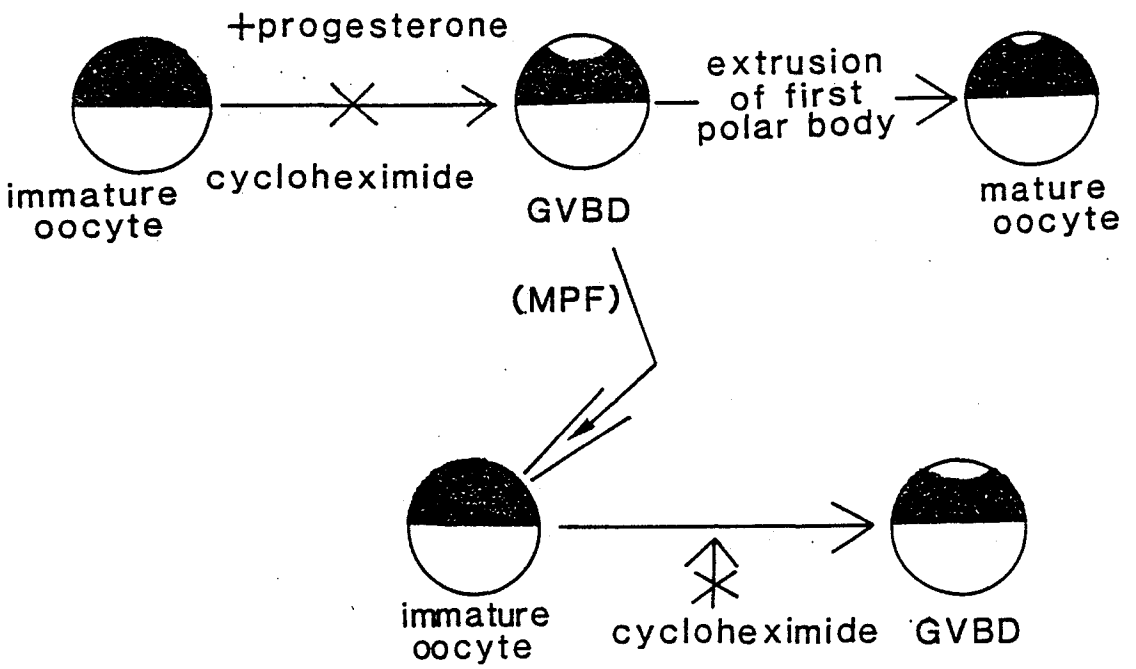


Fig. 3. Schematic diagram of MPF activity. When oocytes are treated with progesterone, a transferable activity (MPF) appears in the cytoplasm of metaphase that, when microinjected into immature oocytes, can induce them into M-phase in the absence of progesterone. Cycloheximide inhibits the production of MPF by progesterone. However, cytoplasm containing MPF can induce recipient oocytes to complete meiotic maturation even in the presence of cycloheximide.



progesterone induces oocytes to synthesize an initiator protein(s) that activates MPF and then MPF triggers meiotic events without a requirement of protein synthesis.

In recent years, the molecular nature of MPF came into sight. It was shown that the entity of MPF is activated form of histone H1 kinase which is a complex of $p34^{cdc2}$ and a cyclin protein family [24-30] and that during meiotic maturation of *Xenopus* oocytes the cyclin B-$p34^{cdc2}$ complex is activated by dephosholylation of $p34^{cdc2}$ [31].

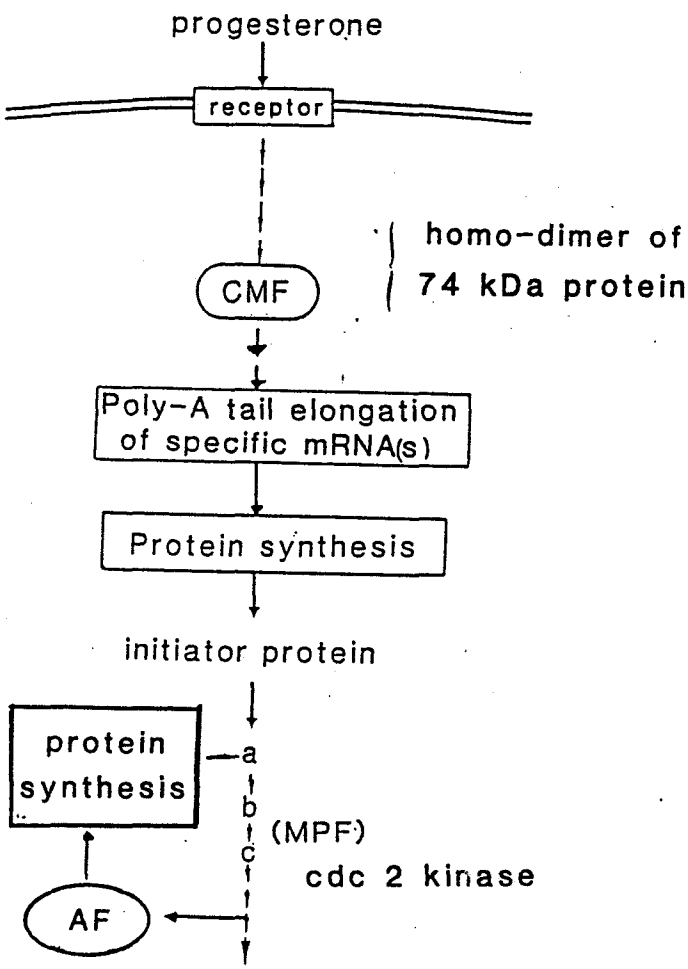
In contrast, the steps before the synthesis of initiator proteins remain largely unknown. Because transcription of the genome is dispensable for induction of meiotic maturation in *Xenopus*, the proteins required for induction of meiotic maturation are translational products of stored maternal mRNA [32]. However, mechanism of this translational regulation remains unclear.

In this thesis, I showed in chapter I that progesterone induces the activation of a protein factor called CMF which induces the meiotic maturation through protein synthesis when injected into immature oocytes. Then, in chapter II, I showed the involvement of poly-A tail elongation of mRNA for the process of oocyte maturation, and how the inhibition of poly-A tail elongation influences the translational products of *Xenopus* oocytes (see fig. 4).

Onset of M-phase is the start of irreversible process toward new generation of the cell. There must be an elaborate control mechanism to guarantee that various changes in M phase should

take place irreversibily and contemporary. A sudden increase of MPF activity occurs surely in cytoplasm in the onset of M phase. In addition, injection of a small amount of MPF activity triggers the production of a much larger amount of MPF activity by the recipient oocyte. This result indicates that a positive feedback control exist in the regulation mechanism of MPF activity. However, the mechanism of the amplification of MPF activity is largly unknown. In chapter III, I examined the involvement of protein synthesis in the process of MPF amplification and found that M-phase extract contain an activity to increase MPF activity even in the absence of protein synthesis. However, this activity disappeared by a serial transfer in the presence of cycloheximide. A large increase in the MPF activity requires protein synthesis. I found the existence of an active factor (amplification factor) which induce an amplification of MPF activity through protein synthesis (Fig. 4). These positive feedback control of MPF activity guarantee the drastic activation of MPF activity and irreversible transition to M phase at G_2-M border.

Fig. 4. Scheme of cytoplasmic mechanism for induction of meiosis I. Meiosis I is induced by the following steps. (1) Progesterone acts on the receptor localized on the surface of the oocyte. (2) Concentration of cyclic AMP is decreased. (3) CMF is activated via the decrease in the concentration of cAMP (4) CMF induces poly(A) tail elongation of some specific mRNA. (5) Initiator protein is translated from activated mRNA. (6) MPF is activated by initiator protein. (7) A drastic increase of MPF activity is induced by positive feedback through protein synthesis. (8) GVBD is induced by MPF.



REFERENCES

1. Pardee, A.B., Durbrow, R., Hamlin, J.L. & Kletzien, R.F. (1978) *Annu. Rev. Biochem.* **47**, 715-750
2. Hartwell, L., and Weinert, T. (1989) *Science* **246**, 629-634.
3. Subtelny, S., and Bradt, C. (1961) *Develop. Biol.* **3**, 96-114.
4. Smith, L.D., Ecker, R.E., and Subtelny, S. (1968) *Develp. Biol.*, **17**, 627-643.
5. El-Etr, M. Schorderet-Slatkine, S., and Baulieu, E. (1979) *Sience* **205**, 1397-1399.
6. Maller, J.L., and Koontz. A. (1981) *Develop. Biol.* **85**, 309-316.
7. Dumont, J.N. (1972) *J. Morphol.* **136**, 153-180.
8. Gorski, J., and Gannon, F. (1976) *Annu. Rev. Physiol.* **38**, 425-450.
9. Yamamoto, K.R., Gehring, V., Stampfer, M.R., and Sibley, C.H. (1976) *Rec. Prog. Horm. Res.* **32**, 3-32.
10. Smith, L.D., and Ecker, R.E. (1971) *Develop. Biol.* **25**, 233-247.
11. Masui, Y., and Markert, C.L. (1971) *J. Exp. Zool.* **177**, 129-146.
12. Ishikawa, K., Hanaoka, Y., Kondo, Y., and Imai, K. (1977) *Mol. Cell Endocr.* **9**, 91-100.12.
13. Smith, L.D., and Ecker, R.E. (1969) *Develop. Biol.* **19**, 281-309
14. Baltus, E., Brachet, J., Hanocq-Quertier, J., and Hubert, E. (1973) *Differentiation* **1**, 127-143.

15. Brachet, J. (1967) *Exp. Cell Res.* **48**, 233-236

16. Sunkara, P.S., Wright, D.A., and Rao, P.N. (1979) *Proc. Natl. Acad. Sci. USA* **76**,2799-2802

17. Kishimoto, T., Kuryama, R., Kondo, H., and Kanatani, H. (1982) *Exp. Cell Res.* **137**, 121-126.

18. Weintraub, H., Buscaglia, M., Ferrez, M., Weiller, S., Boult, A., Fabrie, F., and Baulieu, E.E. (1984) *Gen. Comp. Endocrinol.* **53**, 461.

19. Lohka, M.J., and Masui, Y. (1984) *Develop. Biol.* **101**, 518-523.

20. Lohka, M.J., and Maller, J.L. (1985) *J. Cell Biol.* **101**, 518-523.

21. Miake-Lye, R., and Kirschner, M. W. (1985) *Cell* **41**, 165-175.

22. Merrian, R.W. (1972) *J. Exp. Zool.* **180**, 421-426.

23. Wasserman, W.J., and Masui, Y. (1975) *Exp. Cell Res.* **91**, 381-388.

24. Lohka, M.J. Hayes, M.K., and Maller, J.L. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3009-3019.

25. Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J.L. (1988) *Cell* **54**, 433-439.

26. Arion, D., Meijer, L., Brizuela, L., and Beach, D. (1988) *Cell* **55**, 371-378.

27. Labbe, J.C., Picard, A., Peaucellier, G., Cavadore, J.C., Nurse, P., and Doree, M. (1989) *Cell* **57**, 253-263.

28. Meijer, L., Arion, D., Golsteyn, R., Pines, J., Brizuela, L., Hunt, T., and Beach, D. (1989) *EMBO J.* **8**, 2275-2282.

29. Dunphy, W.G., and Newport, J.W. (1988) *Cell* **55**, 925-928.

30. Murray, A.W., and Kirschner, M.W. (1989) *Science* **246**, 614-621.

31. Jessus, C., Rime, H., Haccard, O., Lint, J.V., Goris, J., Merlevede, W., and Ozon, R. (1991) *Development* **111**, 813-820.

32. Newport, J., and Kirschner, M. (1982) *Cell* **30**, 687-696.

Abbreviations

GVBD -- germinal vesicle breakdown

CMF -- cycloheximide-sensitive meiotic maturation-inducing factor

MPF -- maturation promoting factor

CSF -- cytostatic factor.

EGTA -- glycoletherdiaminetetraacetic acid

DTT -- dithiothreitol

PMSF -- phenylmethylsulfonylfluoride

Chapter I.

Meiotic Maturation of *Xenopus laevis* Oocytes by Progesterone Occurs through an Activation of 74 kDa Protein, CMF, Which Induces Germinal Vesicle Breakdown of Immature Oocytes *via* Protein Synthesis.

SUMMARY

When *Xenopus laevis* oocytes were pretreated with progesterone in the presence of cycloheximide, neither germinal vesicle breakdown (GVBD) nor production of maturation promoting factor occurs. However, the extract of such oocytes induced germinal vesicle breakdown, dependent on protein-synthesis, when injected into immature oocytes. The active factor (cycloheximide-sensitive meiotic maturation-inducing factor, CMF) was found to be proteinous. The CMF activity appeared in the cytoplasm about 3 hrs after the treatment of oocytes with progesterone, while GVBD occurred about 6 hrs after the treatment. The time required for GVBD by CMF injection was 2-3 hrs which is about twice that by MPF. Therefore, we concluded that the activation of CMF is the intermediate step in the meiotic maturation of oocytes. The CMF activity was also appeared when oocytes were treated with insulin in the presence of progesterone. CMF was purified 10000-fold to near homogeneity by precipitation with ammonium sulfate and five successive chromatographic steps. During purification, CMF activity was eluted in a single position. Analysis by SDS-PAGE of the most purified preparation showed that a protein with a molecular weight of 74 kDa was the only major component. The mobility of CMF by gel filtration corresponded to approximately 190 kDa, suggesting that CMF is a homodimer. The availability of the purified preparations of CMF described herein should help in the elucidation of the way in which progesterone induces the meiotic maturation of oocytes.

INTRODUCTION

Meiotic maturation of amphibian oocytes has drawn attention as a good model for the study of cell cycle regulation. Fully grown *Xenopus laevis* oocytes are physiologically arrested in the first meiotic prophase or late G2 phase. When the oocytes are exposed to progesterone, they complete meiotic maturation, undergoing breakdown of the nuclear envelope (germinal vesicle breakdown, GVBD), chromosome condensation, spindle formation, and extrusion of the first polar body [1,2]. It has been inferred that progesterone acts on the surface of the oocyte from the fact that injection of the hormone into oocyte does not induce GVBD, while exposure of the oocyte surface to the hormone does [3-5]. Therefore, the internal cytoplasm should act as a mediator of the hormone stimulus transmitting the surface reaction to the nuclear event.

The existence of cytoplasmic mediator was demonstrated by experiment in which a transferable activity is appeared in the cytoplasm that, when microinjected into immature oocytes, can induce them to engage in meiotic maturation in the absence of progesterone. This cytoplasmic activity has been called maturation-promoting factor or MPF [3].

When oocytes are treated with progesterone in the presence of cycloheximide, neither morphological change [6] nor production of MPF [7] occurs. However, cytoplasm containing MPF can induce recipient oocytes to complete meiotic maturation even in the presence of cycloheximide [7]. These results have been

interpreted to mean that progesterone induces oocytes to synthesize an initiator protein(s) that activates MPF and then MPF triggers meiotic events without a requirement for protein synthesis.

In recent years, the character of MPF and the mechanism of its activation after the completion of protein synthesis came into sight [8-12]. In contrast, the stages prior to the synthesis of relevant proteins remain largely unknown. When the oocytes are exposed to progesterone, an immediate and transient increase in the activity of free calcium [15] and a drop in the concentration of cAMP [16,17] within the cytoplasm are known to occur. However, the biochemical pathway from these two events to meiotic maturation remains unclear.

It is considered that the treatment of oocytes with progesterone in the presence of cycloheximide may induce activation of steps that occur prior to the synthesis of relevant proteins. In this report, we showed that the cytoplasm of oocytes treated in this way induces GVBD, depending on protein synthesis, when it is injected into immature oocytes. The active factor in the cytoplasm, which is named 'cycloheximide-sensitive meiotic maturation-inducing factor (CMF)', was purified by precipitation with ammonium sulfate and five chromatographic steps, and found to be a homo-dimer of 74 kDa protein.

MATERIALS AND METHODS

Materials - Cycloheximide and progesterone were obtained from Nacalai Tesque (Kyoto). Hydroxylapatite and the silver-staining kit were from Bio-Rad (Richmond, U.S.A.).

Animals - Adult *Xenopus laevis* females were obtained from Hamamatsu Seibutsu Kyouzai (Shizuoka). The ovaries were removed surgically into OR-2* (OR-2 medium [18] (82.5 m*M* NaCl, 2.5 m*M* KCl, 1.0 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 1.0 m*M* Na_2HPO_4, 5.0 m*M* Hepes at pH 7.8 with NaOH) with 10 m*M* glucose) from animals that had been anesthetized by immersion in ice-cold water. The lobes of the ovaries were washed in OR-2 medium and dissected into clumps of 100 to 200 oocytes in OR-2*. For assays of CMF activity, oocytes were removed manually from ovaries, and kept in OR-2*. Oocytes at stage IV [19] were selected for microinjection, having a diameter of more than 1.2 mm.

Assay of CMF Activity by Microinjection into Xenopus Oocytes - To determine CMF activity, oocytes were injected with 80 nl each of sample. Injected oocytes were incubated for 3 hours at 25° C, and the fraction that underwent GVBD was determined by scoring for 'white spot' formation in the pigment hemisphere. CMF induces GVBD only in the absence of cycloheximide, while MPF activity is not affected by treatment of recipient oocytes with cycloheximide. Therefore, the following two experiments were carried out in parallel as negative controls. Injected oocytes were incubated in the presence of 50 μg/ml cycloheximide, or oocytes were injected with the extraction buffer (buffer A: 80 m*M*

2-glycerophosphate, 5 m*M* NaF, 20 m*M* EGTA, 15 m*M* $MgCl_2$, 1 m*M* DTT, 300 μM PMSF, 1 m*M* ATP, 20 m*M* HEPES at pH 7.5 with NaOH). The normal maturation *in vitro* that is induced by exposure to 100 μM progesterone was also examined. For each experiment the activities of various fractions were tested with oocytes from the same animal and more than 20 oocytes were injected for each fraction.

CMF activity of the crude extract was measured after removal of cycloheximide from the extract by gel filtration on Sephadex G-25 or by precipitation of proteins by the addition of ammonium sulfate to 70% saturation. CMF activity was quantified by stepwise dilution. The concentration that caused GVBD in just 50% of the oocytes was estimated with due consideration of the dependence of CMF activity on dilution (see Fig. 1). One unit of activity was arbitrarily defined as the amount of activity that causes GVBD in 50% of the oocytes injected with a volume of 80 nl of the sample.

Concentration of Protein - The concentration of protein in the various fractions was determined by Bradford's procedure [20], with bovine serum albumin as a standard. Since the amounts of protein in the fractions from the second Phenyl-Sepharose column and the second DEAE-cellulose column were low, the amounts of protein in the active fractions were estimated from the intensity of bands after SDS-PAGE and staining with Coomassie brilliant blue R-250 [21].

SDS-PAGE - SDS-polyacrylamide gel electrophoresis was carried out essentially as described by Laemmli [22]. An aliquot

of each fraction was concentrated to 20 μl using swelling gel (Ms.BTAURY-KN, Atto Co.,Tokyo), and was mixed with equal volume of concentrated sample buffer (2% SDS, 2% 2-mercaptoethanol, 20 m*M* Tris-HCl at pH 6.8, 8 *M* urea, 0.004% bromophenol blue). After boiling for 2 min, samples were subjected to electrophoresis through 5% stacking gels and 10% SDS-polyacrylamide resolving gels. Gels were stained with Coomassie brilliant blue R-250 and further analyzed by silver staining.

Purification of CMF from Xenopus Oocytes - CMF was purified from the oocytes of a total of 50 female frogs. All procedures after the initial extraction were carried out at 4° C. Column chromatography was performed using Hitachi L-6200 liquid chromato-system.

Step 1. Extraction of CMF from *Xenopus* Oocytes: The clumps of oocytes (75-100 ml) from 10 females were treated with 100 μM progesterone and 50 μg/ml cycloheximide in 300 ml of OR-2* medium at 20° C for 5 h. These oocytes were then rinsed three times with buffer A, and suspended in 80 ml of the same buffer. The oocytes were homogenized by several passages of a loose-fitting pestle in a Dounce homogenizer. The homogenate was centrifuged at 70,000 x g for 1 h and the translucent layer between the cap of fat and the pellet of yolk was recovered, having a volume of approximately 90 ml.

Step 2. Ammonium Sulfate Fractionation: The extract (90 ml) was mixed with 0.82 volume of saturated ammonium sulfate (final concentration 45% of saturation), and the precipitate, after centrifugation at 10,000 x g for 20 min, was discarded. The

supernatant solution was mixed with 0.52 volume of saturated ammonium sulfate (final concentration 65% of saturation) and centrifuged at 10000 x g for 20 min. The pellet was dissolved in 60 ml of 25% buffer B and dialyzed against the same buffer for 12 h.

Step 3. Chromatography on DEAE-Cellulose: The dialyzed solution was applied to a column of DEAE-cellulose (2.6 x 25 cm), pre-equilibrated with 25% buffer B (buffer A but 1 m*M* EGTA and -ATP) at a flow rate of 1 ml/min. The column was washed with 130 ml of the same buffer. Proteins were eluted with a linear gradient of 25-100% buffer B at a flow rate of 1 ml/min, and fractions of 10 ml were collected. The fractions containing CMF activity (fractions 18-32) were pooled and stored at -30° C.

Step 4. Chromatography on Phenyl-Sepharose: The active fractions after step 3 from five preparations (total 750 ml) were pooled. The solution was adjusted to 10% saturation with ammonium sulfate in buffer B and applied to a column of Phenyl-Sepharose (2 x 9 cm), pre-equilibrated with buffer B at 10% saturation with ammonium sulfate, at a flow rate of 2 ml/min. Proteins were eluted with a linear decreasing-concentration gradient of ammonium sulfate (10-0% saturation) in buffer B and then a similar gradient of buffer B (100-0%) at a flow rate of 2 ml/min. Fractions of 10 ml were collected.

Step 5. Chromatography on Hydroxylapatite: The active fractions from chromatography on Phenyl-Sepharose (fractions 20-26) were pooled and applied directly to a column of hydroxylapatite (2 x 7 cm), pre-equilibrated with buffer C

(buffer A, without NaF, EGTA and ATP) at a flow rate of 0.4 ml/min. Proteins were eluted with a linear gradient of sodium phosphate at pH 7.5 (0-250 m*M*) in buffer C at a flow rate of 0.4 ml/min. Fractions of 10 ml were collected, and 2 ml of each fraction was analyzed by SDS-PAGE. Because calcium ions liberated from hydroxylapatite caused the death of recipient oocytes or abnormally large white spots, the CMF activity of each fraction was measured after a ten-fold dilution with buffer A, which contains 20 m*M* EGTA.

Step 6. Chromatography on Phenyl-Sepharose: The active fractions from hydroxylapatite chromatography (fractions 12-18) were pooled and mixed with saturated ammonium sulfate (final concentration, 10% of saturation). The sample was applied to a column of Phenyl-Sepharose (1 x 0.6 cm), pre-equilibrated with a 10% saturated solution of ammonium sulfate in buffer D (buffer B without NaF and $MgCl_2$). Proteins were eluted with a linear decreasing-concentration gradient of ammonium sulfate in buffer D (10-0% saturation) and then with a decreasing-concentration gradient of buffer D (100-0%) at a flow rate of 0.2 ml/min. Fractions of 1 ml were collected.

Step 7. Chromatography on DEAE-Cellulose: The active fractions from the second chromatographic fractionation on Phenyl-Sepharose (fractions 13-18) were pooled, diluted four-fold with deionized water and applied to a column of DEAE-cellulose (1 x 0.6 cm) pre-equilibrated with 25% buffer D. Proteins were eluted with a linear gradient of 25-100% buffer D at a flow rate of 0.2 ml/min. Fractions of 1 ml were collected, and 0.6 ml of

each fraction was analyzed by SDS-PAGE.

Estimation of Molecular Mass of CMF - Active fractions eluted from the second column of Phenyl-Sepharose (600 μl) were concentrated to 20 μl using swelling gel (Ms.BTAURY-KN, Atto Co.). The sample was loaded on a column of Sephacryl S-300 (0.56 x 20 cm), pre-equilibrated with buffer D, and fractions of 125 μl were collected. An aliquot (12.5 μl) of each fraction was used for the determination of activity. The remainder (100 μl) of each fraction was concentrated to 20 μl and samples were subjected to electrophoresis in an SDS-10% polyacrylamide gel, and stained with the silver-staining kit.

RESULTS

Induction of Meiotic Maturation by a Protein Factor, CMF-

When*Xenopus* oocytes were treated with progesterone in the presence of cycloheximide neither germinal vesicle breakdown, GVBD, nor the activation of maturation promoting factor, MPF, was induced (Table 1). We expected that the treatment of oocytes with progesterone in the presence of cycloheximide may activate the steps that occur prior to the protein synthesis. Then, we prepared the extract of these oocytes (see "MATERIAL and METHODS"). The extract was injected into immature oocytes after removal of low molecular weight component (cycloheximide) from the extract. When 80 nl of the extract (x6 dilution) was injected into immature oocytes, more than 60% of oocytes shows GVBD within 3 hrs after the injection. However, when cycloheximide was added to the injected oocytes, the fraction of oocytes that underwent GVBD decreased to less than 10% (see Table 1).

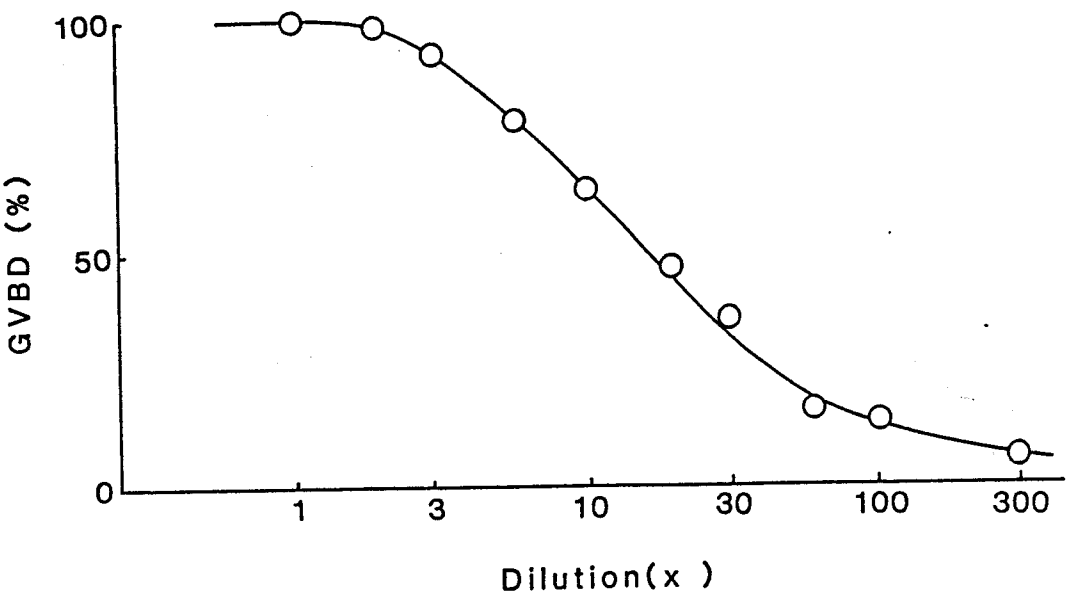
Figure 1 shows the relationship between the extent of GVBD against the dilution of the extract. The fraction of oocytes that underwent GVBD decreased with dilution of the extract. We defined one unit of the activity arbitrarily as the amount of activity that causes GVBD in 50% of the oocytes. Therefore, the extract contains 2×10^5 units/ml. The active factor for induction of GVBD which was named 'cycloheximide-sensitive meiotic maturation-inducing factor (CMF)'.

Table 1. Induction of germinal vesicle breakdown by the cytoplasm of oocytes treated with progesterone or insulin with cycloheximide. Oocytes were treated with 100 μM progesterone or 20 μg/ml insulin in OR-2* medium for 6 hrs. The extract from each batch of oocytes was injected into immature oocytes. GVBD of each oocytes were scored after 3 hrs.

Treatment	GVBD(%)	
	-cycloheximide	+cycloheximide (50 μg/ml)
progesterone	100	5[a]
insulin	95	10[b]
injection of extract of oocytes (b)*	75	10
injection of extract of oocytes (c)*	60	5

* x 6 dilution.

Fig. 1. Dependence of the fraction of oocytes that undergo the germinal vesicle breakdown, GVBD, on the concentration of oocyte-extract. Oocytes were treated with progesterone in the presence of cycloheximide and the extract was prepared as described in the text. 80 nl of the extract was injected after dilution with buffer B, and the fraction that underwent GVBD at 3 hrs were scored.



Kinetics of CMF in Meiotic Maturation of Oocytes

When *Xenopus laevis* oocytes were treated with progesterone or insulin, GVBD occurred about 6 hrs after the hormone treatment, while MPF activity appeared 1-2 hrs before GVBD [3,7]. Then, we studied the time course of appearance of CMF activity in the oocyte after the hormone treatment to examine whether the activation of CMF is the intermediate step of meiotic maturation. As shown in Fig. 2, the oocytes used in this experiment required about 6 hrs to induce GVBD by progesterone treatment. When oocytes were treated with progesterone in the presence of cycloheximide no GVBD was induced. The CMF activity increased around 3 hrs after the progesterone treatment. The maximal activity was about 200 units/oocyte.

Figure 3 shows the kinetics of induction of GVBD after injection of oocytes with the above extract (CMF), with M-phase extract (MPF) and that of progesterone-treatment. The oocytes from the same female were used for these experiments. M-phase extract (MPF, 20 units) induced GVBD at about 1 hr after the injection, whereas progesterone induced GVBD at about 6 hrs after the treatment. On the other hand, CMF-extract (20 units CMF) induced at about 3 hrs after the injection. The time required for the induction of GVBD was not decreased even when the activity of CMF increased from 20 to 50 units.

It was shown that GVBD by progesterone was inhibited by isobutylmethylxantin (IBMX), an inhibitor of phosphidiesterase [23]. Then we examined whether CMF was produced by progesterone

Fig. 2. Time course of appearance of cycloheximide-sensitive meiotic maturation inducing factor (CMF) in oocytes after treatment of oocytes with progesterone in the presence of cycloheximide. (a), CMF activity in the oocytes treated with 100 μM progesterone and 50 μg/ml cycloheximide. The CMF activity after extraction at different times were plotted. The time course of progesterone-induced GVBD in the absence (b) or presence (c) of cycloheximide was shown together.

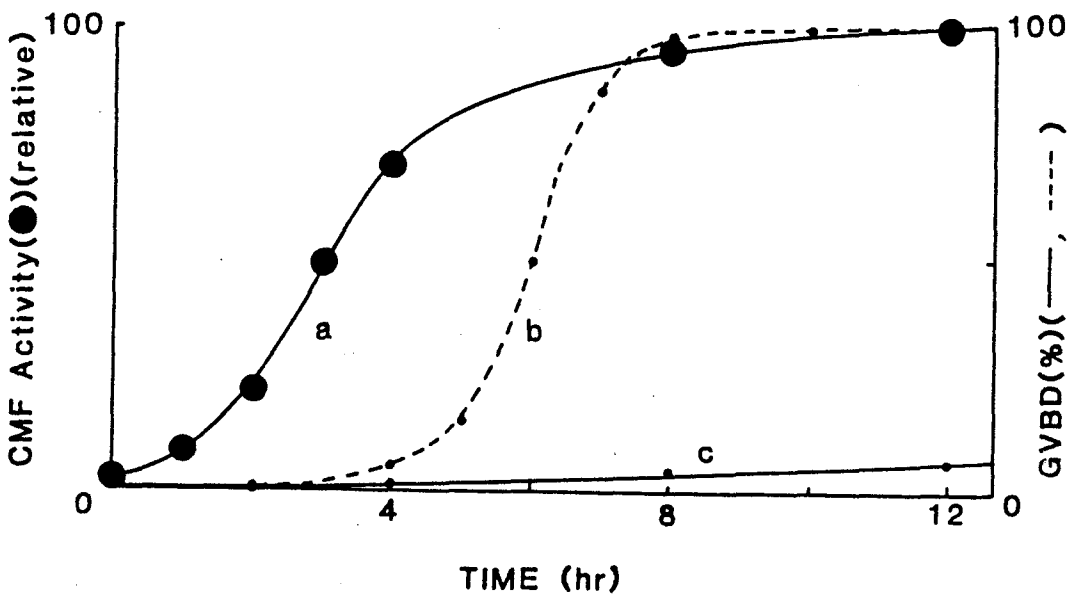
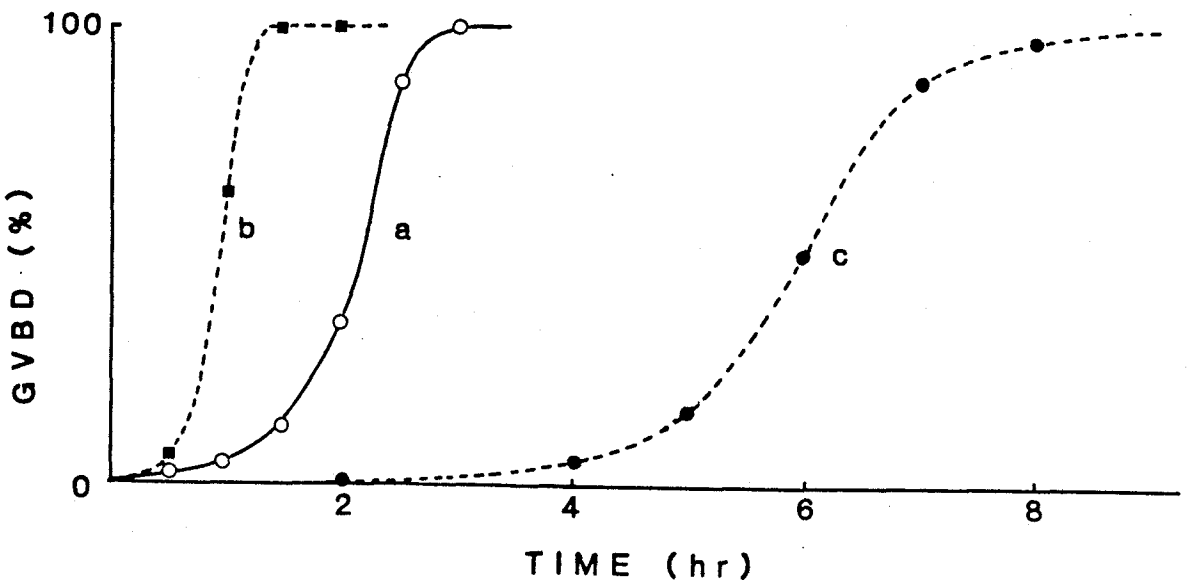


Fig. 3. Kinetics of induction of GVBD after treatment of oocytes with progesterone and those after injection with CMF or MPF. In all cases pools of 50 oocytes from the same female were used. a, GVBD after injection of 80 nl of the MPF extract (Extract from progesterone-treated oocytes undergoing GVBD) into immature oocytes. b, GVBD after injection of 80 nl of the CMF extract (Extract from the oocytes treated with progesterone and cycloheximide). c, GVBD after treatment of oocytes with 100 μg/ml progesterone.



in the presence of IBMX. IBMX (100 μM) inhibit GVBD, but no detectable CMF activity (less than 40 unit/oocyte) was observed.

Purification of CMF

CMF was extracted from the oocyte as described in "Material and Methods". The active factor was assumed to be proteinous since it disappeared by heating at 70° C or by treatment with trypsin or chymotrypsin. CMF activity also disappeared by N-ethylmaleimide. CMF activity of the extract was not affected by repeated freezing and thawing, so that the samples throughout the purification procedure were frozen on occasion.

When CMF activity was examined after fractionation of the extract into ammonium sulfate, more than 90% of the activity after the fractionation was recovered in the protein precipitated by 45-65% ammonium sulfate. Therefore, the extract was precipitated by 45-65% saturated ammonium sulfate and the resultant pellet of protein was dissolved in the same volume of 25% buffer B as the initial volume of the sample, and then the solution was dialyzed against the same buffer. This step gave a 2.6-fold purification with a yield of 75%.

CMF was further purified by chromatography on five successive columns. The dialyzed preparation after fractionation with ammonium sulfate was applied to a column of DEAE-cellulose, and proteins were eluted with a linear gradient of buffer B from 25% to 100% (Fig. 4). CMF activity was eluted as a single peak at around 40% buffer B. The fractions containing CMF activity

Fig. 4. Chromatography on DEAE-cellulose of the preparation of CMF that was obtained by precipitation of the crude extract with ammonium sulfate. The precipitated fraction (45-65% saturation) of the crude extract was dissolved in 25% buffer B and dialyzed against 25% buffer B at 4° C. Then it was applied to a column of DEAE-cellulose (2.6 x 25 cm) equilibrated with 25% buffer B. The column was washed with the same buffer and proteins were eluted with a linear gradient of 25-100% buffer B (——) at a flow rate of 1 ml/min. Fractions (10 ml) were collected and assayed for CMF activity. The bar indicates the fractions with CMF activity.

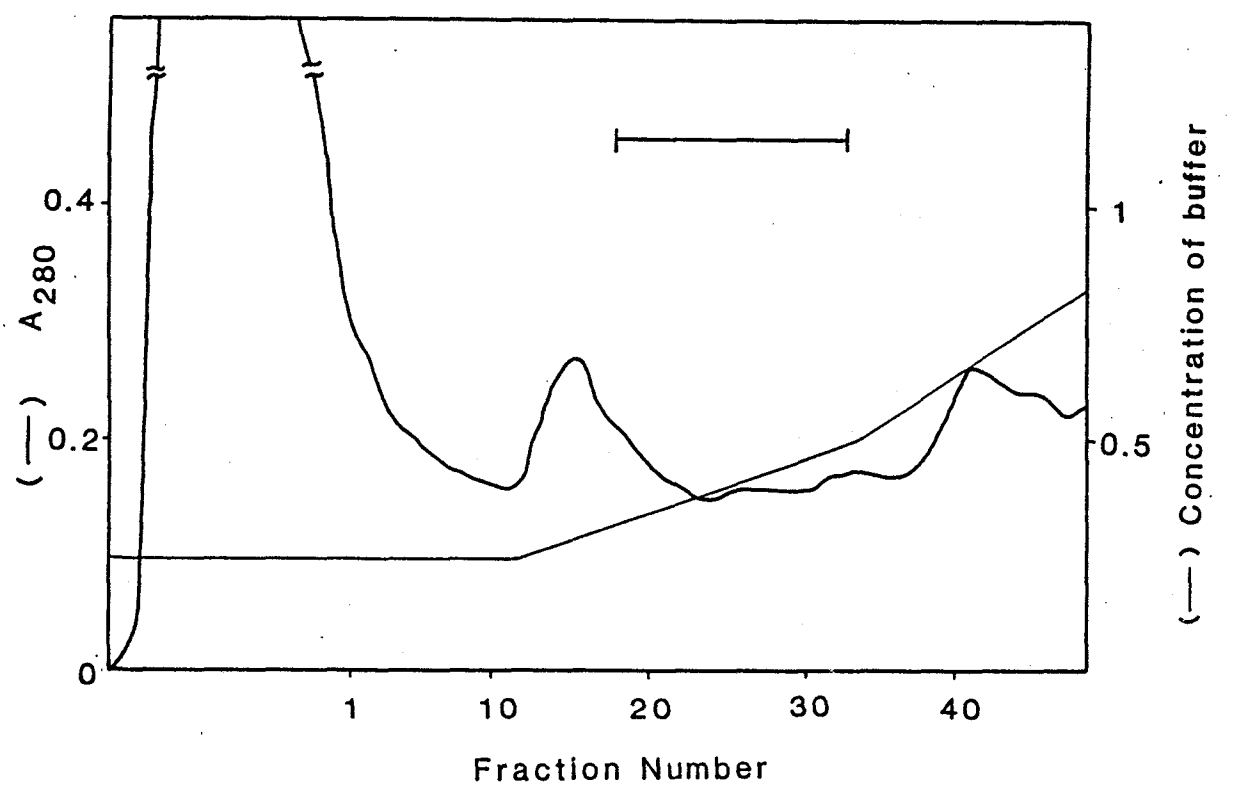
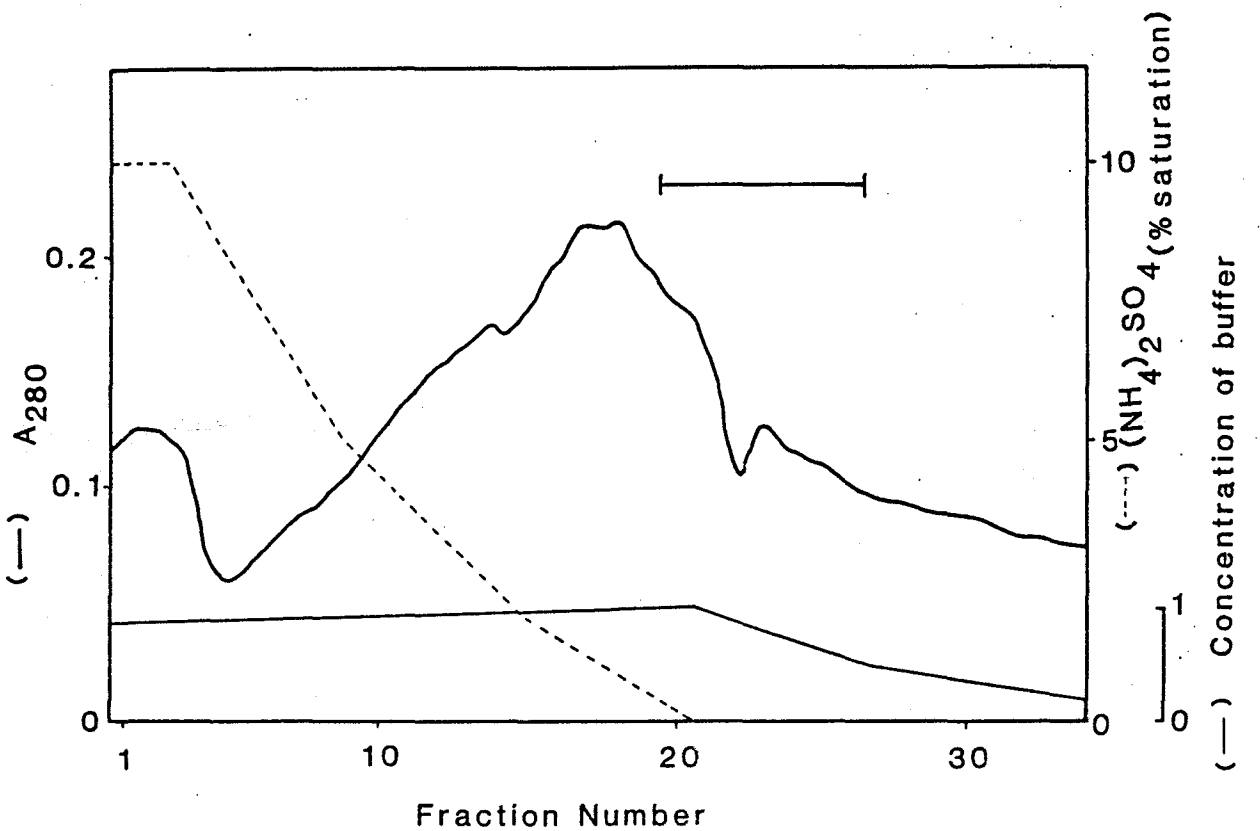


Fig. 5. Chromatography on Phenyl-Sepharose of the preparation of CMF obtained by chromatography on DEAE-cellulose. The active fractions from chromatography on DEAE-cellulose (fractions 18-32) were pooled, adjusted to 10% saturation with ammonium sulfate in buffer B and applied to a column of Phenyl-Sepharose (2 x 7 cm), equilibrated with a 10% saturated solution of ammonium sulfate in buffer B, at a flow rate of 2 ml/min. Proteins were eluted with a linear decreasing-concentration gradient of ammonium sulfate (10-0% saturation) (---), and then with a similar gradient of buffer B (100-0%) (——), at flow rate of 2 ml/min. Fractions (10 ml) were collected and assayed for CMF activity. The bar indicates the fractions with CMF activity.



(fractions 18-32) were pooled. This step afforded a 7.7-fold purification with a yield of 50%. The eluate from DEAE-cellulose was adjusted to 10% saturation of ammonium sulfate in buffer B and applied to a Phenyl-Sepharose column. Proteins were eluted with a linear decreasing-concentration gradient of ammonium sulfate (10-0%) and then with a similar gradient of buffer B (100-0%) (Fig. 5). CMF activity was eluted as a single peak at around 80% buffer B. The fractions containing CMF activity (fractions 20-26) were pooled. This step gave a 5-fold purification with a yield of 33%. The eluate from the Phenyl-Sepharose column was loaded directly onto a hydroxylapatite column. Proteins were eluted with a linear gradient of sodium phosphate (0-250 m*M* in buffer C). As shown in Fig. 6, most of proteins were eluted before fraction 5, while CMF activity was eluted as a single peak around 80 m*M* sodium phosphate (fractions 12-18). This step resulted in a 20-fold purification with a yield of 70%. Purification of the activity by a factor of about 2000 was achieved by chromatography on these three supports with a relatively high yield (8.8%). Figure 7 shows the silver-stained polyacrylamide gel after electrophoresis of fractions 10-20 from the hydroxylapatite column. Proteins of 74 kDa, 65 kDa and several minor proteins are visible in the fractions with CMF activity.

Further purification of CMF was accompanied by loss of activity, probably due to adsorption to the chromatographic media, since the concentration of proteins was very low. We purified CMF by rechromatography on Phenyl-Sepharose and DEAE-

Fig. 6. Chromatography on hydroxylapatite of the preparation of CMF obtained by chromatography on Phenyl-Sepharose. The active fractions from chromatography on Phenyl-Sepharose (fractions 20-26) were pooled and applied to a column of hydroxylapatite (2 x 7 cm) at a flow rate of 0.4 ml/min. Proteins were eluted with a linear gradient of sodium phosphate in buffer C at pH 7.5 (0-250 m*M*) at a flow rate of 0.4 ml/min ( ). Fractions of 10 ml were collected. The CMF activity of each fraction was measured after a ten-fold dilution of each sample with buffer A. The bar indicates the fractions with CMF activity.

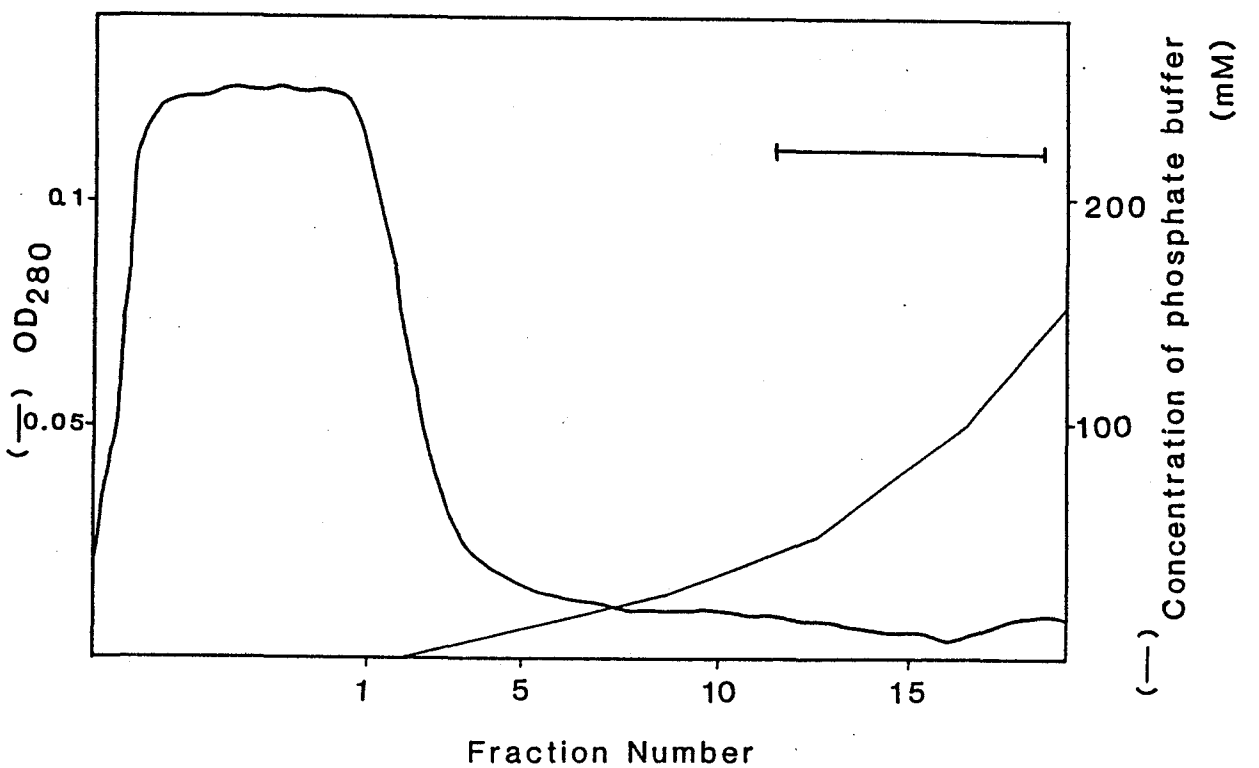
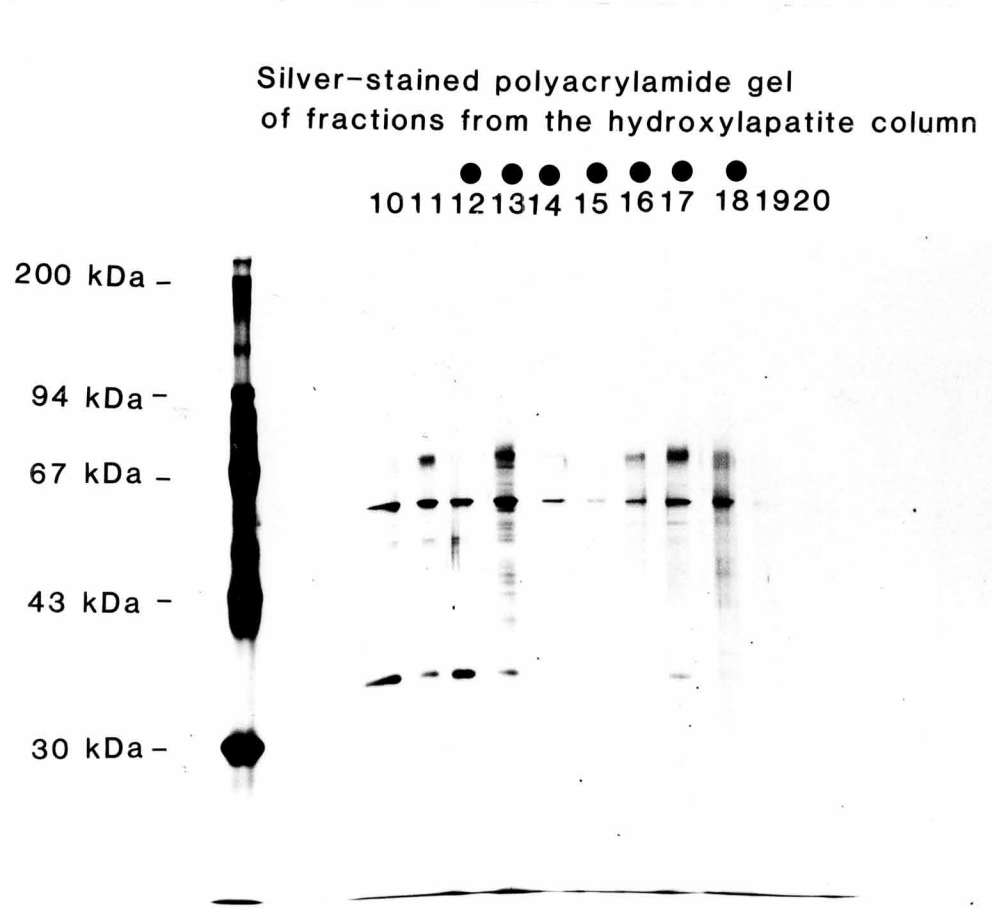


Fig. 7. Silver-stained polyacrylamide gel after electrophoresis of fractions from the hydroxylapatite column. Proteins in 2 ml of each fraction eluted from the hydroxylapatite column were concentrated and fractionated by electrophoresis on an SDS-10% polyacrylamide gel and analyzed by silver staining. Fractions 12-18 induced GVBD when injected into oocytes. Fraction numbers are indicated at the top of the gel.



cellulose. The eluate from the hydroxylapatite column was mixed with saturated ammonium sulfate to give a final concentration equivalent to 10% saturation, and applied to a column of Phenyl-Sepharose. From this second column of Phenyl-Sepharose, CMF activity was eluted by the same concentration of buffer B (80%) as from the first one. The second fractionation on Phenyl-Sepharose gave a 4-fold purification with a yield of 9%. The eluate from the second fractionation on Phenyl-Sepharose was diluted four-fold with water and applied to a column of DEAE-cellulose. CMF activity was eluted in fractions 3-4, and the concentration of buffer D (around 40%) required for elusion of CMF was the same as that during the first fractionation on this resin. Figure 8 shows a polyacrylamide gel after electrophoresis of fractions after the second fractionation on DEAE-cellulose. Fractions 3-4 have CMF activity and a 74-kDa protein was only the component of the active fractions. Thus, CMF was purified by precipitation with ammonium sulfate and five chromatographic steps. Table 2 summarized the purification of CMF by each steps. The purification of CMF by a factor of about 10,000 was achieved.

Molecular Size of CMF

As shown in Fig. 8, CMF has an molecular weight of 74K by SDS-PAGE. Then, we examined the molecular weight of native protein by a gel filtration method. A small amount (600 μl) of the pooled active fractions after the second column of Phenyl-Sepharose was concentrated and then loaded on a column of

Table 2. Purification of CMF from *Xenopus* oocytes.

Step of purification	Volume (ml)	Protein (mg)	Total* activity (unit)	Recovery (%)	Specific activity (units/ug)
Sup. 10^5 x g	400	1.7×10^4	8×10^7	100	5
Am.Sulf.(45-65%)	400	4.8×10^3	6×10^7	75	13
DEAE-cellulose	750	3.0×10^2	3×10^7	38	100
Phenyl-Sepharose	70	2.2×10	1×10^7	13	500
Hydroxylapatite	70	0.6	7×10^6	8.8	10000
2nd Phenyl-Sepharose	6	0.02	6×10^5	0.75	30000
2nd DEAE-cellulose	2	0.004	2×10^5	0.25	50000

* CMF activity was quantified by stepwise dilution. A unit of activity is defined arbitrarily as the amount of activity that causes GVBD in 50% of the oocytes injected with a volume of 80 nl of the fraction.

Fig. 8. Silver-stained polyacrylamide gel of fractions 8-19 after column chromatography on Sephacryl S-300 . The active fractions from the second fractionation on Phenyl-Sepharose (fractions 13-18) were pooled and 600 μl of the sample were concentrated to 20 μl. This sample was loaded on a column of S-300 (0.56 x 20 cm), equilibrated with buffer D, and fractions of 125 μl were collected. An aliquot (12.5 μl) of each fraction was used for the assay of CMF activity. The remainder (100 μl) of each fraction was concentrated to 20 μl and proteins were fractionated by electrophoresis on an SDS-10% polyacrylamide gel and analyzed by silver-staining. Ferritin (440 kDa) and hemoglobin (65 kDa) were eluted at the positions indicated by arrows. CMF activity was eluted in the eleventh and the twelfth fractions.

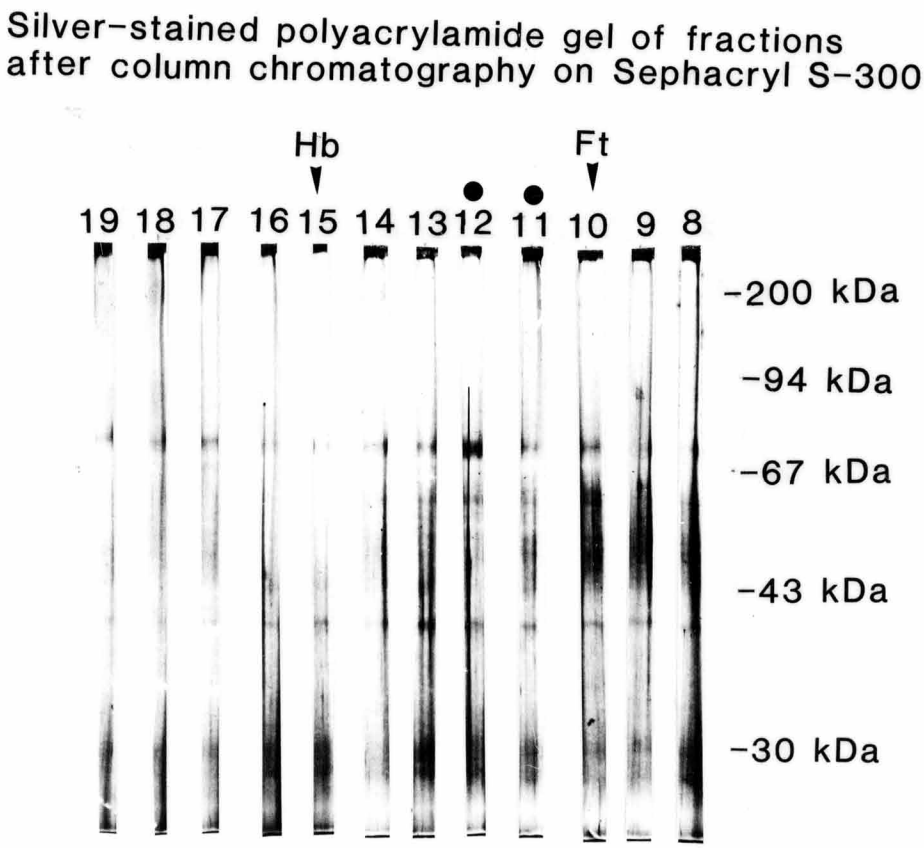
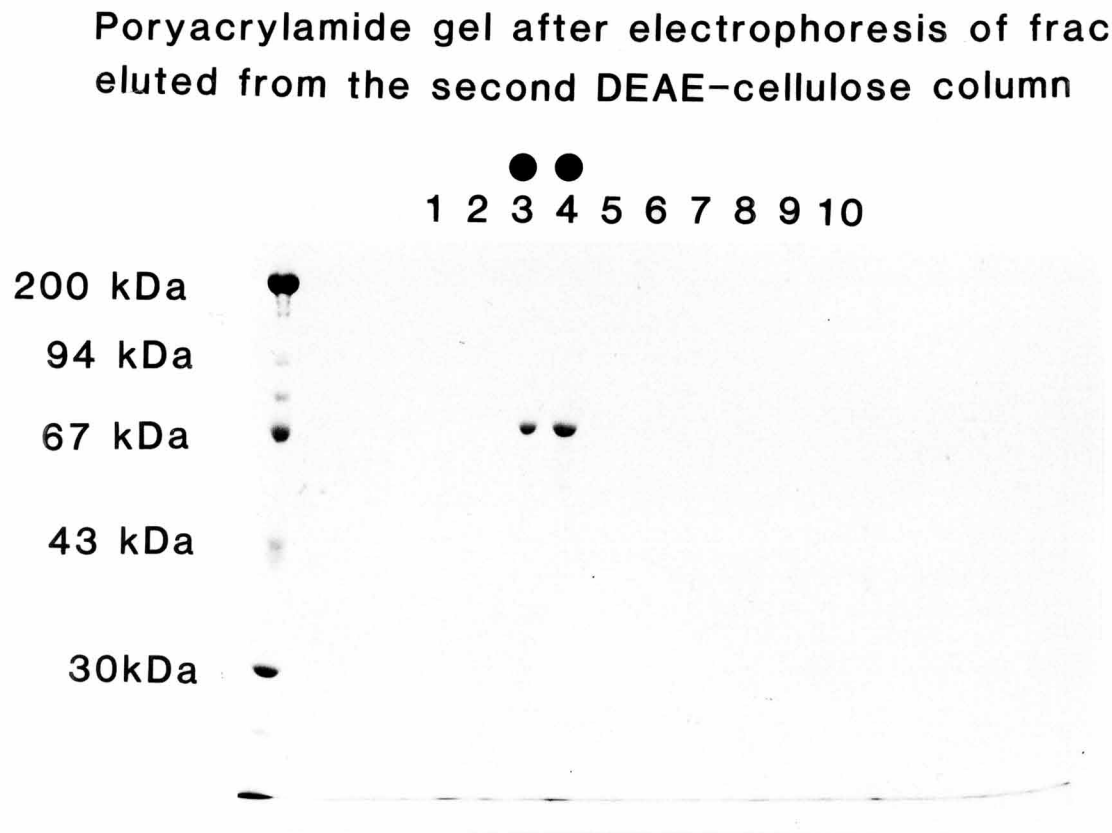


Fig. 9. Polyacrylamide gel after electrophoresis of fractions eluted from the second DEAE-cellulose column. Proteins in fractions 1-10, eluted from the column were concentrated by exposure to swelling gels. Then these concentrated samples were fractionated by electrophoresis on an SDS-10% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Fractions 3-4 induced cycloheximide-sensitive GVBD when injected into oocytes.



Sephacryl S-300. CMF activity was eluted in fractions 11 and 12, with a mobility that corresponded to an apparent molecular weight of about 190 kDa. The eluted fractions were concentrated and proteins were separated by electrophoresis on an SDS-10% polyacrylamide gel. This gel was analyzed by silver-staining (Fig. 9). Only the band of protein of 74 kDa was associated with CMF activity.

Ca-Sensitivity of CMF

CMF in the crude extract was sensitive to calcium ions: when oocytes were homogenized in the absence of EGTA, the resulting extract exhibited no CMF activity. We examined the effect of Ca^{2+} on the CMF activity by mixing 1 m*M* $CaCl_2$1 to the crude extract for 10 min, then with 20 m*M* EGTA. CMF activity in the crude extract was inactivated by this treatment. However, the CMF activity in the 45-65% ammonium sulfate fraction or that in the highly purified preparation was insensitive to the presence of 1 m*M* $CaCl_2$.

CMF Activity in the M-phase Extract

We examined whether the existence of cycloheximide is required for the appearance of CMF activity. When cycloheximide is absent oocytes shows GVBD and have high activity of MPF which induce GVBD even in the presence of cycloheximide in the recipient oocytes. Then the extract of oocytes treated by progesterone was

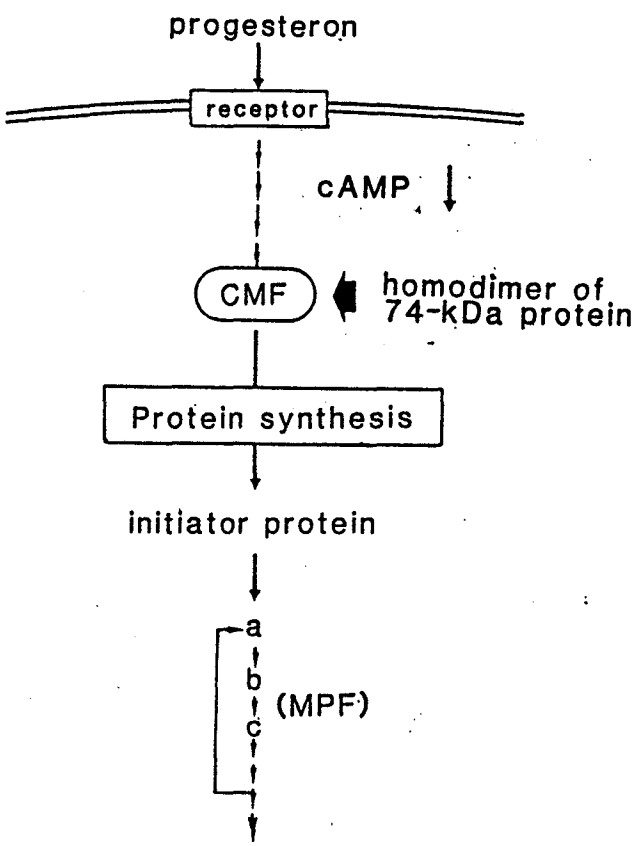
purified with by ammonium sulfate fractionation and the column chromatography on DEAE-cellulose and Phenyl-cellulose. The CMF fraction after phenyl-sephalose column chromatography did not induce GVBD when cycloheximide was added to the recipient oocyte but induce GVBD in the absence of cycloheximide when injected into immature oocyte.

DISCUSSION

When *Xenopus* oocytes were treated with progesterone in the presence of cycloheximide, neither GVBD nor MPF activity was induced, however, such oocytes contained an active factor (CMF) which induced GVBD with a requirement for protein synthesis (Table 1). We considered that activation of CMF is the intermediate step for induction of M-phase (see Fig. 10), since (1) CMF was activated before GVBD and also before MPF production (Fig. 2). (2) Injection of CMF took about 3 hrs for induction of GVBD (Fig. 3) which is about a half that for progesterone-treatment, but twice that for MPF-injection. We found that even in the presence of cycloheximide CMF was activated (see "RESULTS").

CMF was purified 10000-fold to near homogeneity. During five successive fractionation by column chromatography, CMF activity was always eluted as a single peak. Furthermore, when the 2000-fold purified preparation of CMF was rechromatographed on a column of Phenyl-Sepharose and then on a column of DEAE-cellulose, the activity was eluted at the same positions as it was from the first columns. This result indicates that a single macromolecule possesses CMF activity and that purified CMF is equivalent the main fraction of CMF in the crude extract. Analysis by SDS-PAGE of the purest preparation revealed that a protein with a molecular weight of 74 kDa was the only major component of the active fraction. The active fraction after gel filtration on Sephacryl S-300 also contained a 74-kDa protein,

Fig. 10. Scheme of cytoplasmic mechanism for induction of meiosis I. Meiosis I is induced by the following steps. (1) Progesterone acts on the receptor localized on the surface of the oocyte. (2) Concentration of cyclic AMP is decreased. (3) CMF is activated via the decrease in the concentration of cAMP (4) Initiator protein is synthesized by active CMF. (5) MPF is activated by initiator protein. (6) GVBD is induced by MPF.



while the mobility of CMF during gel filtration corresponded to approximately 190 kDa. Therefore, it appears that CMF is a homo-dimer of the 74 kDa protein.

Several proteins, including the regulatory subunit of cAMP-dependent protein kinase (55 kDa), the heat-stabile kinase inhibitor (26 kDa) [24], and the human H-ras protein (21 kDa) [25] are known to induce GVBD in a manner that is dependent on protein synthesis. However, CMF has different properties from those of the above mentioned proteins with respect to the induction of GVBD. First, the maturation of oocytes injected with these proteins proceeds more slowly than in the case of progesterone-treated oocytes. Injected CMF induces GVBD within a half of the time that is required for progesterone-treated oocytes to start GVBD. Second, in the case of the most purified preparation of CMF, only about 20 pg of protein per recipient oocyte was able to induce GVBD. The regulatory subunit of cAMP-dependent protein kinase and the H-ras protein must be injected as 5-10 ng of protein per recipient oocyte if GVBD is to be induced [24,25].

It is unknown how progesterone causes the production of active CMF in the oocyte cytoplasm. Since CMF is activated even in the absence of protein synthesis, there must be precursor of CMF. Several lines of evidence indicate that progesterone induces the maturation of oocytes by causing a decrease in intraoocyte levels of cAMP *via* inhibition of the oocyte adenylate cyclase [23,26,27]. The decrease in levels of cAMP may precede the production of active CMF, since isobutylmetylxanthine, an

inhibitor of phosphodiesterase, inhibits both the progesterone-induced maturation of oocytes [23] and the production of CMF (see "RESULTS").

Insulin also induces the maturation of oocytes in a series of events that requires protein synthesis [2]. Treatment with insulin also results in the production of CMF in cycloheximide-treated oocytes (see Table 1). However, the injection of a monoclonal antibody against ras p21 prevented insulin-induced but not progesterone-induced maturation [28]. Therefore, we speculate that both treatment with progesterone and with insulin causes production of active CMF that induces meiotic maturation but the biochemical pathways for the activation of CMF are different.

It is expected that CMF activity is regulated in association with the cell cycle. We found that CMF activity in the crude extract is abolished rapidly in the presence of calcium ions, whereas the purified preparation of CMF is insensitive to calcium ions. Other activities specific to the M phase such as those of MPF and CSF [3], are also destroyed by calcium ions, the level of which is increased as a result of fertilization [29-34]. We suspect that the activity of CMF is diminished by a similar mechanism to that which results in reduction in the activities of MPF and CSF.

MPF induces GVBD without a requirement for protein synthesis, whereas CMF requires protein synthesis for its action. Therefore, it appears that the 74-kDa protein induces the synthesis of 'initiator' protein(s) that activate MPF. The

purified preparation of CMF described herein may be useful for the elucidation of the mechanism of activation of CMF and the role of CMF in the maturation of oocytes.

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REFERENCES

1. Schuetz, A.W. (1974) *Biol. Reprod.* **10**, 150-178
2. El-Etr, M. Schorderet-Slatkine, S., & Baulieu, E. (1979) *Science* **205**, 1397-1399
3. Masui, Y. & Markert, C.L. (1971) *J. Exp. Zool.* **177**, 129-146
4. Ishikawa, K., Hanaoka, Y., Kondo, Y., and Imai, K. (1977) *Mol. Cell. Endocinol.* **9**, 91-100
5. Sadller, S.E. and Maller, J.L. (1982) *J. Biol. Chem.* **257**, 355-361
6. Merrian, R.W. (1972) *J. Exp. Zool.* **180**, 421-426
7. Wasserman, W.J. & Masui, Y. (1975) *Exp. Cell Res.* **91**, 381-388
8. Lohka, M.J. Hayes, M.K., & Maller, J.L. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3009-3019
9. Gautier, J., Norbury, C., Lohka, M., Nurse, P., & Maller, J. (1988) *Cell* **54**, 433-439
10. Arion, D., Meijer, L., Brizuela, L., & Beach, D. (1988) *Cell* **55**, 371-378
11. Labbe, J.C., Picard, A., Peaucellier, G., Cavadore, J.C., Nurse, P., & Doree, M. (1989) *Cell* **57**, 253-263
12. Meijer,L., Arion, D., Golsteyn, R., Pines, J., Brizuela, L., Hunt, T., & Beach, D. (1989) *EMBO J.* **8**, 2275-2282
13. Dunphy, W.G. & Newport, J.W. (1988) *Cell* **55**, 925-928
14. Murray, A.W. & Kirschner, M.W. (1989)*Science* **246**, 614-621
15. Moreau, M., Vilain, J.P., & Guerrier, P. (1980) *Develop. Biol.* **78**, 201-214
16. Speaker, M.G. & Butcher, F.R. (1977) *Nature* **267**, 848-849

17. Maller, J.L., Butcher, F.R., & Krebs, E.G. (1979) *J. Biol. Chem.* **254**, 579-582

18. Wallace, R.A., Jared, D.W., Dumont, J.N., & Sega, M.W. (1973) *J. Exp. Zool.* **184**, 321-334

19. Dumont, J.N. (1972) *J. Morphol.* **136**, 153-180

20. Bradford, M.M. (1976) *Anal. Biochem.* **72**, 248-254

21. Graziani, Y., Erikson, E., & Erikson, R.L. (1983) *J. Biol. Chem.* **258**, 6344-6351

22. Laemmli, U.K. (1970)*Nature* **227**, 680-685

23. Sadler, S.E. & Maller, J.L. (1987) *J. Biol. Chem.* **262**, 10644-10650

24. Maller, J.L. & Krebs, E.G. *J. Biol. Chem.* **252**, 1712-1718

25. Birchmeier, C., Broek, D., & Wigler, M. (1985) *Cell* **43**, 615-621

26. Sadler, S.E. & Maller, J.L. (1981) *J. Biol. Chem.* **256**, 6368-6373

27. Finidori-Lepicard, J., Schorderet-Slatkine, S., Hanoune, J., & Baulieu, E. (1981) *Nature* **292**, 255-256

28. Korn, L.J., Siebel, C.W., McCormick, F., & Roth, R.A. (1987) *Science* **236**, 840-843

29. Busa, W.B. & Nuccitelli, R. (1985) *J. Cell Biol.* **100**, 1325-1329

30. Wasserman, W,J. & Masui, Y. (1976) *Science* **191** 1266-1268

31. Meyerhof, P.G. & Masui, Y. (1977) *Develop. Biol.* **61**, 214-229

32. Newport, J.W. & Kirschner, M.W. (1984) *Cell* **37**, 731-742

33. Watanabe, N., Vande Woude, G.F., Ikawa, Y., & Sagata, N. (1989) *Nature* **342**, 505-511

34. Sagata, N., Watanabe, N., Vande Wounde, G.F., & Ikawa, Y. (1989) *Nature* **342**, 512-518

Chapter II.

Maturation of *Xenopus laevis* Oocyte by Progesterone Requires Poly(A) Tail Elongation of mRNA.

SUMMARY

Meiotic maturation of *Xenopus laevis* oocytes by progesterone requires translation of stored maternal mRNAs. We investigated the role of poly(A) tail elongation of mRNAs during this process using cordycepin which inhibits poly(A) tail elongation of mRNAs. When oocytes were treated with the buffer containing 10 m*M* cordycepin for 12 h, concentration of 3'-dATP in cytosol of oocytes increased to 0.7 m*M*, while that of ATP remained constant at around 1.2 m*M*. Incorporation of [^{32}P]AMP into poly(A)mRNA was inhibited almost completely by this treatment. Progesterone-induced germinal vesicle breakdown (GVBD) and CMF-induced GVBD were also abolished. Dose dependence of inhibition of progesterone-induced GVBD on cordycepin was similar to that of [^{32}P]AMP incorporation into poly(A)mRNA. However, MPF-induced GVBD was unaffected by treatment of oocytes with cordycepin. Furthermore, the inhibition of GVBD by cordycepin was rescued by removal of cordycepin even in the presence of actinomycin D. Therefore, we concluded that poly(A) tail elongation of mRNA is required for induction of meiotic maturation of *Xenopus laevis* oocytes. In addition, progesterone induced a 2.7-fold activation of [^{32}P]AMP incorporation into the poly(A) tail of mRNA after a lag period of 3 h whereas GVBD was induced after 6-8 h from the progesterone treatment. Syntheses of most of the proteins were unaffected by treatment of oocytes with progesterone or cordycepin. However, syntheses of several proteins were increased or decreased by progesterone and cordycepin treatment.

INTRODUCTION

Fully grown *Xenopus laevis* oocytes are physiologically arrested in the first meiotic prophase. When oocytes are exposed to progesterone, they resume meiotic maturation to complete undergoing breakdown of the nuclear envelope (germinal vesicle breakdown, GVBD), chromosome condensation, spindle formation, and extrusion of the first polar body [1,2]. In addition to these morphological changes, a transferable activity is known to appear in the cytoplasm that, when microinjected into immature oocytes, can induce them to engage in meiotic maturation in the absence of progesterone. This cytoplasmic activity has been called maturation-promoting factor or MPF [3]. When oocytes are treated with progesterone in the presence of cycloheximide, neither morphological change [4] nor production of MPF [5] occurs. However, cytoplasm containing MPF can induce recipient oocytes to complete meiotic maturation even in the presence of cycloheximide [5]. These results have been interpreted to mean that progesterone induces oocytes to synthesize an initiator protein(s) that activates MPF and then MPF triggers meiotic events without a requirement of protein synthesis.

In recent years, the character of MPF and the mechanism of its activation after the completion of protein synthesis came into sight. It was shown that the entity of MPF is activated form of histone H1 kinase which is a complex of $p34^{cdc2}$ and a cyclin protein family [6-12] and that during meiotic maturation of *Xenopus* oocytes the cyclin B-$p34^{cdc2}$ complex is activated by

dephospholylation of $p34^{cdc2}$ [13]. In contrast, the steps before the synthesis of relevant proteins remain largely unknown. It was shown that progesterone binds to a receptor which is located on the surface of oocytes [14,15]. When oocytes are exposed to progesterone, an immediate and transient drop in the concentration of cAMP [16,17] within the cytoplasm is known to occur. Moreau *et al.* [18] reported that the activity of free calcium in the cytoplasm was increased by progesterone, though their result was not confirmed [19, 20]. Furthermore, we showed that progesterone induces the activation of a protein factor called CMF which induces the meiotic maturation through protein synthesis when injected into immature oocyte in chapter I [21].

In *Xenopus*, transcription of the genome is dispensable for induction of meiotic maturation and for progression of development until the midblastula stage. Therefore, the proteins required for induction of meiotic maturation and of the early development are translational products of stored maternal mRNA [22]. However, the mechanism of this translational regulation remains unclear. In the early development of many animal species, it has been reported that poly(A) tail elongation of a particular mRNA is correlated with activation of its translation [23-31]. During maturation of *Xenopus* oocytes, the majority of mRNAs undergo deadenylation in their tail and total cell poly(A) content decreases by about 50% [32], while a subset of mRNAs is known to be elongated in their poly(A) tail [33-36]. However, the role of these changes in polyadenylation status for the process of oocyte maturation has not yet ascertained. In this

study, we examined the involvement of poly(A) tail elongation for the process of oocyte maturation using cordycepin; an inhibitor of poly(A) tail elongation, and how the inhibition of poly(A) tail elongation influences the translational products of *Xenopus* oocytes.

MATERIALS AND METHODS

Materials. Cycloheximide and progesterone were obtained from Nacalai Tesque (Kyoto, Japan). Oligotex-dT30 resin was from Takara Shuzo (Kyoto, Japan). Charcoal (Norit A) was purchased from Wako Chemical (Osaka, Japan). PEI-cellulose (POLYGRAM CEL300PEI) was the product of Macherey-Nagel (Germany). Cordycepin and 3'-deoxycytidine were obtained from Sigma.

Animals. Adult *Xenopus laevis* females were obtained from Nihon Seibutsu Zairyou (Osaka, Japan). The ovaries were removed surgically into OR-2 medium (82.5 m*M* NaCl, 2.5 m*M* KCl, 1.0 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 1.0 m*M* Na_2HPO_4, 5.0 m*M* Hepes at pH 7.8) [34] from animals that had been anaesthetized by immersion in ice-cold water. The lobes of the ovaries were washed in OR-2 medium and dissected into clumps of 100 to 200 oocytes in OR-2. Then, oocytes were removed manually from ovaries, and kept in OR-2. Oocytes at stage VI [37], having a diameter of more than 1.2 mm, were selected for the study.

Measurement of 3'-deoxyadenosine-5'-triphosphate content in the oocyte. Batches of 100 oocytes were preincubated with the indicated concentration of cordycepin in OR-2 for 6 or 12 h. Then each batch of oocytes was homogenized in 0.75 ml of 5% TCA. The cell lysate was centrifuged at 10,000 x *g* for 5 min and the supernatant was taken and brought to pH 7 by the addition of 2 *M* Tris. Nucleotides in the supernatant were isolated using charcoal as described by Smith and Khorana [38]. Separation of ATP and 3'-dATP in the sample was performed by PEI-cellulose thin

layer chromatography. After an aliquot of sample was applied (30 μl each), the layer was washed with methanol for 10 min and dried intensely. Then, the layer was developed with the medium prepared as described by Neuhard *et al*: 1.0 *M* LiCl was saturated with boric acid at 25 ℃ and brought to pH 7.0 by the addition of ammonia [39]. In this system, *R* values of ATP and 3'-dATP were 0.17 and 0.25, respectively. Nucleotides were detected under short-wave uv light. PEI-cellulose including each nucleotide was scratched off into a 1.5 ml tube. Eluent (250 μl of 1 N HCl) was added to the tube and vigorously stirred for 15 min at 4°C. After the sample was centrifuged at 10000 x *g* for 10 min, the supernatant was taken to measure the concentration of nucleotides by absorbance at 259 nm against a blank elution from an adjacent area.

Measurement of [^{32}P]AMP incorporation into poly(A) tail of mRNA . Oocytes were preincubated with various concentrations of cordycepin or 3'-deoxycytidine in OR-2 medium for 12 h at 25 C. To eliminate DNA dependent RNA synthesis, 20 μg/ml actinomycin D was also added to the medium at the beginning of the incubation. Then, batches of 20 oocytes were injected with α-[^{32}P]ATP (3000 Ci/m*M*, 10 μCi/μl, 80 nl/oocyte) by glass needle. After incubation at 25 C for various periods, the reaction was terminated by freezing the oocytes at -70° C. Total RNA was extracted from the frozen oocytes by the phenol/chloroform method as described by Dworkin and Dawid[40]. Aliquots of RNA samples were taken to quantify the content of injected α-[^{32}P]ATP. Messenger RNA with a long poly(A) tail was isolated with

oligotex-dT30 resin [41]. The solution of poly(A) RNA was diluted to 500 μl with the buffer (10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 0.1% SDS). Then, mRNA was precipitated by addition of an equal volume of 10% TCA with 40 m*M* NaPPi and 1 mg/ml bovine serum albumin as carrier. The precipitate was transferred to a Millipore membrane (pore size, 0.45 μm) and was washed twice with 10% TCA and 40 m*M* NaPPi and once with 0.1 *N* HCl. After drying the membrane, the radioactivity of the precipitate was measured using a xylene scintillation cocktail.

Preparation of MPF cytosol. Cytosol preparation containing active MPF was prepared from *Xenopus* eggs using a procedure of Lohka and Maller [42]. To examine the response of oocytes to MPF, 80 nl of the cytosolic fraction was injected into the each oocyte.

Measurement of inhibition of GVBD by cordycepin. Oocytes were preincubated with various concentrations of cordycepin or 3'-deoxycytidine for 12 h prior to the addition of 100 μ*M* progesterone or injection with MPF fraction. Twelve hours after the activation, GVBD of the oocytes was ascertained by dissecting them after boiling. To ascertain the reversibility of the inhibition, oocytes treated with 10 m*M* cordycepin for 12 h were incubated with 20 μg/ml actinomycin D in the absence of cordycepin for 12 h and then the response to progesterone was examined in this medium.

Analysis of protein synthesis in oocytes treated with cordycepin. Control and cordycepin-treated oocytes were incubated with [^{35}S]methionine (500 μCi/ml) in OR-2 for 4 h. In the case

of progesterone treatment, 100 μM progesterone was added simultaneously with [^{35}S]methionine. MPF activation was performed by injecting 80 nl of cytosolic fraction 2 h after the addition of [^{35}S]methionine and incubating a further 2 h with [^{35}S]methionine. After the oocytes were washed extensively with OR-2 medium supplemented with 1 mM unlabeled methionine, each of 20 oocytes was homogenized in 200 μl of the buffer (0.1 M NaCl, 20 mM Hepes, pH 7.8, 1 mM methionine). An aliquot of each extract was taken to measure the uptake of [^{35}S]methionine into the oocytes. The amount of [^{35}S]methionine incorporated into protein was measured by trapping protein onto a Millipore membrane (pore size, 0.8 μm) after the extract was mixed with 10% TCA containing 1 mM methionine [43]. The rest of each extract was centrifuged at 10,000 x g for 5 min and protein in the supernatant (10 μl) was precipitated by adding 9 vol. of acetone. Protein precipitate was separated by two-dimensional gel electrophoresis. The first-dimension gel, which was separated by isoelectric focusing according to O'Farrell [43], was applied to a second-dimension 10% Laemmli gel [44]. The autoradiograph was prepared by Fuji Bio-Image analyzer system (Tokyo).

RESULTS

Inhibition of Poly(A) Tail Elongation by Cordycepin

We investigated the correlation between progesterone-induced oocyte maturation and poly(A) tail elongation of mRNA using cordycepin (3'-deoxyadenosine). It was reported that Ehrlich ascites-tumor cells take up cordycepin from the culture medium and then cordycepin is converted into 3'-dATP in the cell [46]. 3'-dATP inhibits DNA-dependent RNA synthesis and poly(A) tail elongation as a chain terminator [47]. We measured the amount of 3'-dATP and of ATP in oocytes when they were treated with various concentrations of cordycepin. As shown in Table 1, the concentration of 3'-dATP in the cytoplasm increased with the concentration of cordycepin in the culture medium. As a result of treatment of oocytes with 10 m*M* cordycepin for 12 h the concentration of 3'-dATP in the cytoplasm increased to 0.7 m*M*, while the concentration of ATP in the cytoplasm remained at a constant level (from 1.2 $\pm$ 0.2 to 1.18 $\pm$ 0.06 m*M*). The concentration of ATP in the control oocytes decreased from 1.2 $\pm$ 0.2 to 0.8 $\pm$ 0.2 m*M*.

We measured the effect of cordycepin on the rate of incorporation of [^{32}P]AMP into poly(A) mRNA when α-[^{32}P]ATP was injected into oocytes (Fig. 1). If poly(A) tail elongation was inhibited, the rate of incorporation of [^{32}P]AMP into poly(A) mRNA might be reduced. DNA dependent RNA synthesis was inhibited by the addition of 20 μg/ml actinomycin D to the culture medium.

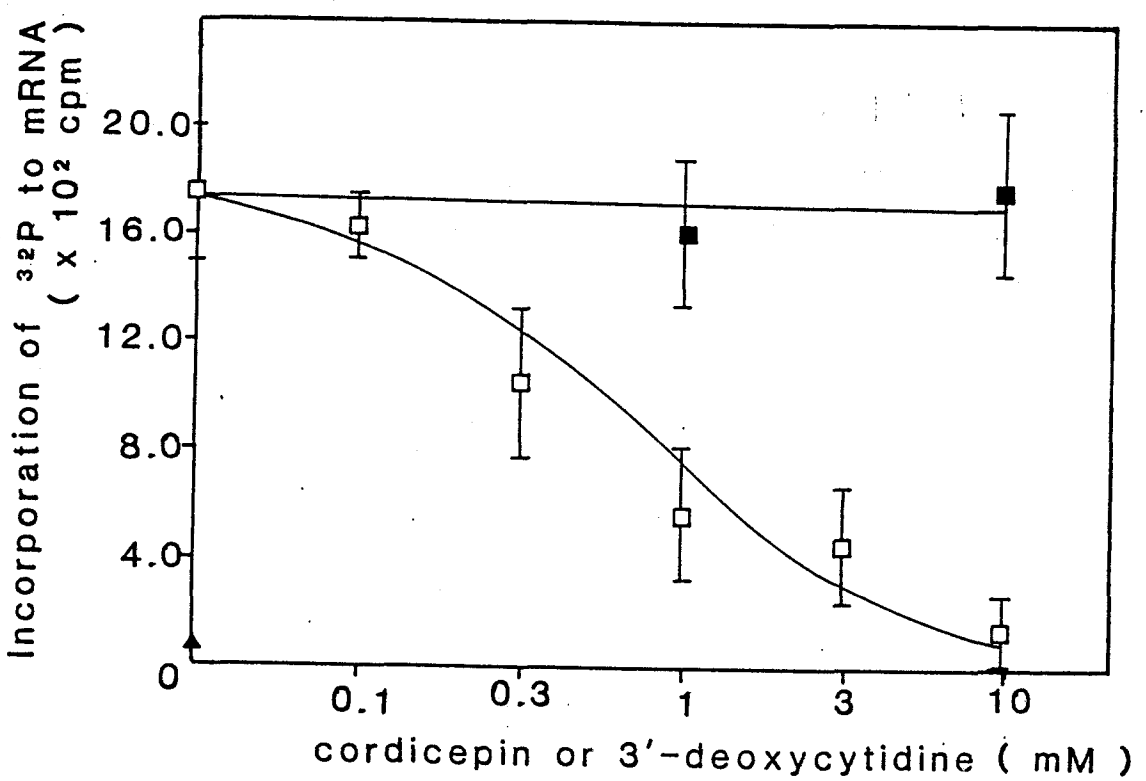
Table 1

Amounts of ATP and of 3'-dATP in Oocytes Treated with Cordycepin

Treatment		Amount of Nucleotides	
Concentration of cordycepin in the medium (m*M*)	Incubation time (h)	3'-dATP (nmol/oocyte)	ATP (nmol/oocyte)
0	0	0.0	1.2 ± 0.2
0	12	0.0	0.8 ± 0.2
1	12	0.20 ± 0.05	0.5 ± 0.1
3	12	0.35 ± 0.06	0.8 ± 0.2
10	12	0.7 ± 0.1	1.18 ± 0.06
10	6	0.21 ± 0.04	0.94 ± 0.08

Note. Batches of 100 oocytes were incubated in OR-2 medium with indicated concentrations of cordycepin for 6 or 12 h. The amounts of 3'-dATP and ATP were measured as described under Materials and Methods.

FIG. 1. Inhibition of incorporation of [^{32}P]AMP into the poly(A) tail of mRNA by cordycepin. Batches of 20 oocytes were preincubated for 12 h in the indicated concentrations of cordycepin (□) or 3'-deoxycytidine (■) in the presence of 20 μg/ml actinomycin D, and then microinjected with 1.0 x 10^7 cpm of [α-^{32}P]ATP (□, ■) or [α-^{32}P]UTP (▲). Oocytes were then incubated in the presence of 100 μM progesterone and 3'-deoxynucleosides for 4 h and then the amount of ^{32}P incorporated into mRNA was measured as described under Materials and Methods.

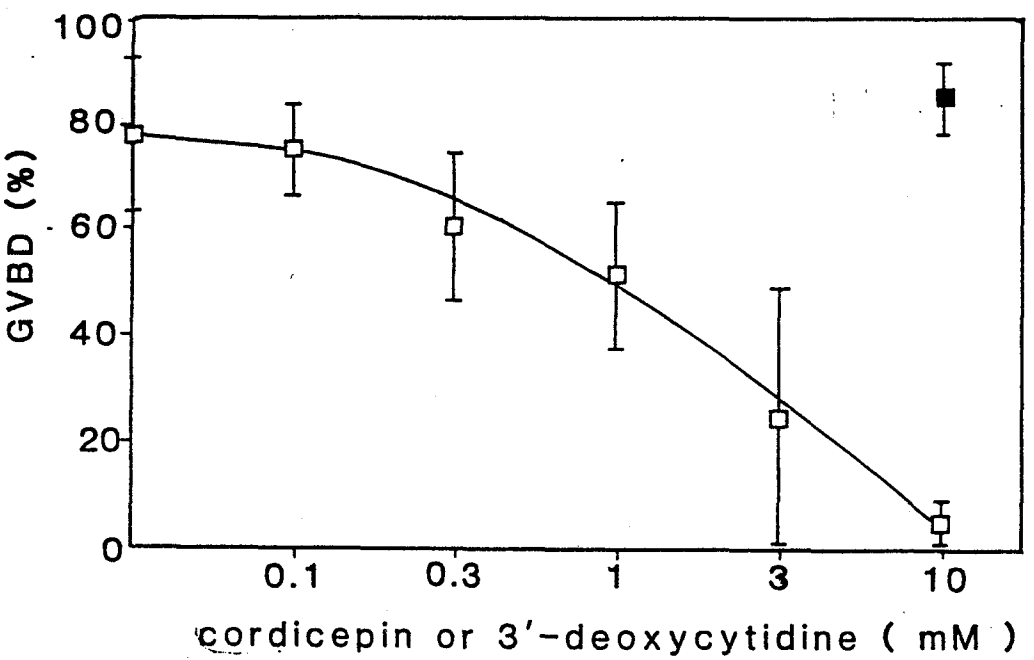


The incorporation of [^{32}P]UMP into mRNA after injection of α-[^{32}P]UTP was small compared to that of [^{32}P]AMP after injection of α-[^{32}P]ATP (Fig. 1). Therefore, the content of ^{32}P in mRNA after α-[^{32}P]ATP injection is considered to be the content of [^{32}P]AMP incorporated into the poly(A) tail of mRNA. As shown in Fig. 1, the [^{32}P]AMP incorporation was inhibited by cordycepin in a dose dependent manner and disappeared when oocytes were treated with 10 m*M* cordycepin for 12 h. The rate of [^{32}P]AMP incorporation into the poly(A) mRNA decreased to a half that of the maximum level at 0.9 m*M* cordycepin. On the other hand, the [^{32}P]AMP incorporation was unaffected by treatment of oocytes with 10 m*M* 3'-deoxycytidine for 12 h.

Inhibition of Progesterone-Induced Oocyte Maturation by Cordycepin

We investigated the effect of cordycepin on progesterone-induced GVBD. Oocytes were treated with various concentrations of cordycepin as described in the legend to Fig. 1. As shown in Fig. 2, progesterone-induced GVBD was inhibited by treatment of oocytes with cordycepin. The dose dependence of inhibition of GVBD on cordycepin was quite similar to that of [^{32}P]AMP incorporation into the poly(A) mRNA (Fig. 1); the frequency of GVBD was decreased to 50% at 1.5 m*M* cordycepin and was decreased to 5% at 10 m*M* cordycepin. Furthermore, the treatment of oocytes with 10 m*M* 3'-deoxycytidine for 12 h had no effect on the progesterone-induced GVBD (Fig. 2).

FIG. 2. Inhibition of progesterone induced GVBD by cordycepin. Oocytes were preincubated for 12 h in the indicated concentrations of cordycepin (□) or 3'-deoxycytidine (■) and then treated with 100 μM progesterone. After 12 h, germinal vesicle breakdown was ascertained by dissection of oocytes.



We examined whether progesterone induced GVBD was recovered by washing out cordycepin: Oocytes treated with 10 m*M* cordycepin for 12 h were washed and incubated in the absence of cordycepin for 12 h. DNA dependent RNA synthesis was inhibited by adding 20 μg/ml actinomycin D to the medium. The fraction of oocytes that underwent GVBD by progesterone was increased from 5 to 60% by these procedures (Table 2).

In chapter I, I described CMF activity which can induce GVBD through protein synthesis when injected into oocyte. We investigated the effect of cordycepin on CMF induced GVBD (Table 2). CMF induced GVBD was inhibited by treatment of recipient oocytes with cordycepin but was not affected by treatment with 3'-deoxycytidine.

It is established that MPF-induced GVBD does not require protein synthesis, though progesterone-induced GVBD requires it [5]. Therefore, if cordycepin has no cytotoxity, it is expected that MPF-induced GVBD is not inhibited by cordycepin. As shown in Table 2, progesterone-induced GVBD was inhibited almost completely by treatment of oocytes with 10 m*M* cordycepin for 12 h. However, MPF-induced GVBD was unaffected by the same treatment.

Activation of [^{32}P]AMP incorporation into poly(A) tail of mRNA by Progesterone

We examined the effect of progesterone treatment on the rate of [^{32}P]AMP incorporation into the poly(A) mRNA. The time course

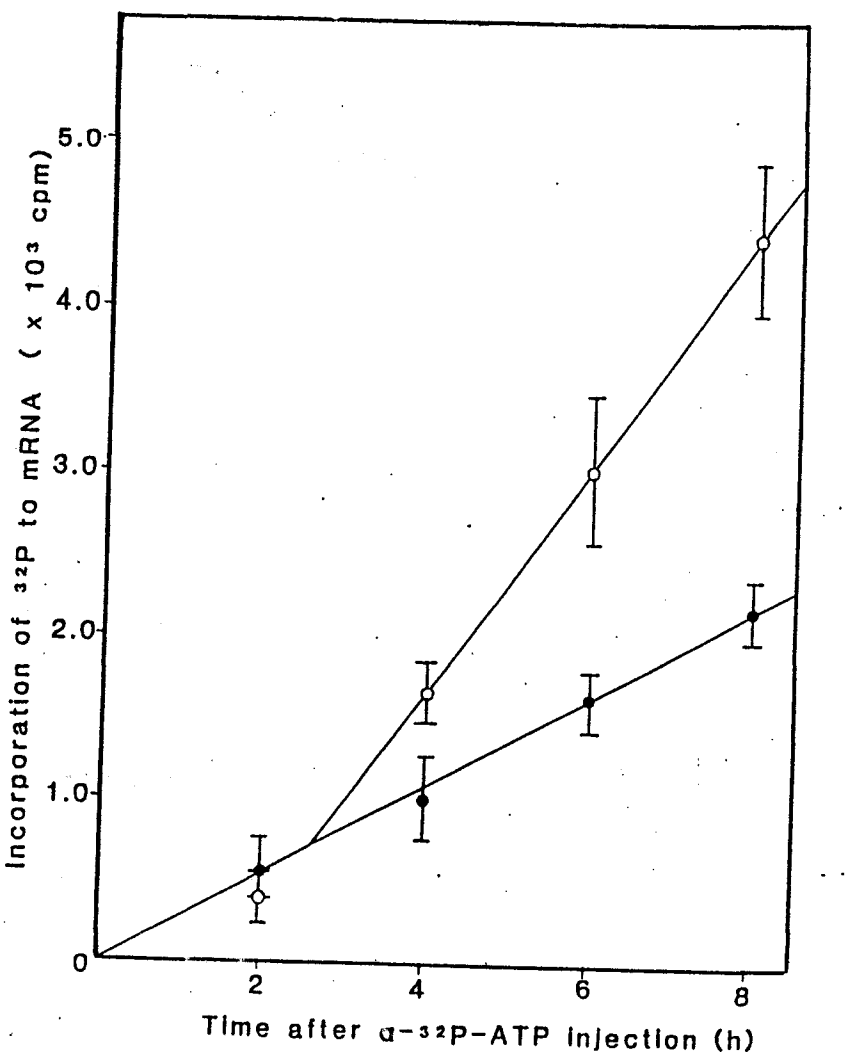
Table 2

Effect of Cordycepin on MPF-Induced GVBD and the Reversibility of Inhibition of Progesterone-Induced GVBD by Cordycepin

Treatment	Activation	GVBD (%)
3'-deoxycytidine (10 m*M* for 12 h)	1. Pogesterone	100
	2. MPF	100
	3. CMF	100
Cordycepin (10 m*M* for 12 h)	4. Progesterone	5
	5. MPF	100
	6. CMF	25
Cordycepin (10 m*M*, 12h) then in the absence of cordycepin (12h)	7. Progesterone	60

Note. Lane 1-6; batches of 40 oocytes were incubated with 10 m*M* cordycepin or 10 m*M* 3'-deoxycytidine in OR-2 for 12 h. Oocytes were then activated by 100 μM progesterone (lane 1) or microinjection of cytosolic fraction containing MPF activity (lane 2,5) or ammonium sulfate fraction containing CMF actibity (lane 3,6). Lane 7; batches of oocytes were treated with 10 m*M* cordycepin for 12 h. Then, they were incubated in the absence of cordycepin in 20 μg/ml actinomycin D for 12 h and then activated by progesterone. The fractions that underwent GVBD were scored by dissection of boiled oocytes 12 h after the activation.

FIG. 3. Time course of [^{32}P]AMP incorporation into mRNA. Oocytes were preincubated for 12 h with 1 m*M* 3'-deoxycytidine and 20 μg/ml actinomycin D. Then batches of 20 oocytes were microinjected with 1.0 x 10^7 cpm of α-[^{32}P]ATP and incubated in the presence (○) or absence (●) of 100 μM progesterone for the indicated period of time. Then the amounts of ^{32}P incorporated into mRNA were measured.

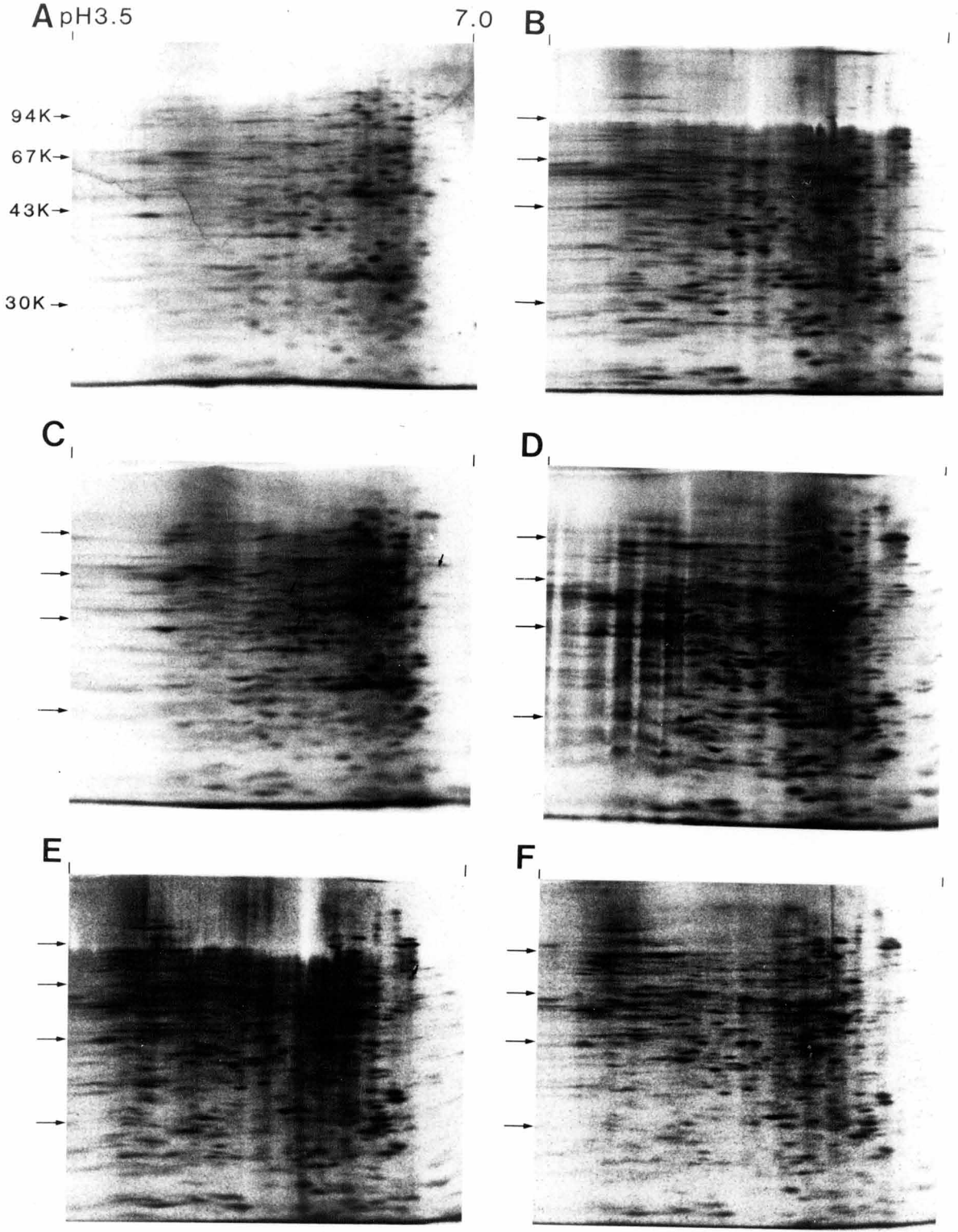


of incorporation of [^{32}P]AMP into the mRNA of control and progesterone-treated oocytes is shown in Fig. 3. In control oocytes, the incorporation was linear for at least 8 h after the injection of α-[^{32}P]ATP. The mean rate of [^{32}P]AMP incorporation in control oocytes is 2.7 x 10^2 cpm h^{-1} per 20 oocytes. The absolute rate of the incorporation of AMP, calculated using the size of the ATP pool (Table 1), was 3.3 x 10^{-14} mol AMP h^{-1} per oocyte. In contrast, the rate of [^{32}P]AMP incorporation in progesterone-treated oocytes diverged from the control rate to the higher value (6.7 x 10^2 cpm h^{-1} per 20 oocytes, 8.2 x 10^{-14} mol AMP h^{-1} per oocyte) at approximately 3 h after the progesterone-treatment. Thus, progesterone induces the 2.7-fold activation of [^{32}P]AMP incorporation into poly(A) tail of mRNA. These oocytes induce GVBD 6-8 h after the progesterone treatment. Therefore, increase in the rate of incorporation of [^{32}P]AMP occurs before the GVBD.

Translation Products in Oocytes Treated with Cordycepin

Synthesis of protein during meiotic maturation of *Xenopus* oocytes has been studied by many workers [48-53]. However, no clear result has been obtained. Since progesterone-induced GVBD and incorporation of [^{32}P]AMP into the poly(A) tail of mRNA were abolished by treatment of oocytes with cordycepin, we investigated the effect of cordycepin on the protein synthesis of progesterone treated or MPF-injected oocytes. The rate of uptake

FIG. 4. Translation products in oocytes treated with cordycepin. Oocytes were preincubated with (B, D, and F) or without (A, C and E) 10 m*M* cordycepin in OR-2 for 12 h. Then, oocytes were incubated with [^{35}S]methionine in the presence (C and D) or absence (A and B) of progesterone for 4 h. In E and F, oocytes were injected with MPF 2 h after incubation in [^{35}S] methionine and then incubated a further 2 h. The oocytes were homogenized in 0.1 *M* NaCl, 20 m*M* Hepes (pH 7.8) and 1 m*M* methionine. Proteins in the soluble fraction were analyzed by two-dimensional gel electrophoresis. Arrows indicate where the modifications occur by progesterone, MPF, and cordycepin.



of [^{35}S]methionine into cytoplasm was increased 20% by treatment of oocytes with 10 m*M* cordycepin for 12 h and the rate of incorporation of [^{35}S]methionine into protein was increased 40% by the same treatment (data not shown). Then, we analyzed the incorporation of [^{35}S]methionine into proteins after two-dimensional gel electrophoresis. Similar protein bands after autoradiography were observed even in the presence of progesterone or cordycepin. However, as shown in Fig. 4, there were some qualitative changes in translation products between the presence and absence of cordycepin. In the absence of cordycepin, we found at least three species of protein (38, 54, and 66 kDa) whose syntheses were increased remarkably in response to progesterone (arrows in Fig. 4C), and the syntheses of these proteins were abolished by the presence of cordycepin. In contrast, in the presence of cordycepin, we cannot find any qualitative changes in translation products in response to progesterone.

We studied the effect of cordycepin on the protein synthesis in MPF-injected oocytes. Oocytes were incubated in the presence or absence of cordycepin for 12 h, and then incubated in the medium containing [^{35}S]methionine for 2 h. After MPF was injected, they were incubated for 2 h in the presence of [^{35}S] methionine. We analyzed the incorporation of [^{35}S]methionine into proteins after two-dimensional gel electrophoresis (Fig. 4E and 4F). New bands (50 and 76 kDa) were observed by MPF-injection, and they were suppressed by the presence of cordycepin.

DISCUSSION

Induction of meiotic maturation of *Xenopus* oocytes provoked by progesterone requires protein synthesis [4]. However, it is unknown how the synthesis of the relevant proteins is controlled in the cytoplasm. In this study, we investigated the role of poly(A) tail elongation in the induction of oocyte maturation by the use of cordycepin. It was reported that when cordycepin is added to the culture medium, it is taken into cytoplasm and converted into 3'-dATP which acts as a poly(A) chain terminator [46,47]. In fact, we found that the content of 3'-dATP in oocytes is increased when cordycepin is added to the medium (Table 1).

We measured the extent of poly(A) tail elongation of mRNA from the incorporation of [^{32}P]AMP into poly(A) mRNA after injection of α-[^{32}P]ATP into oocytes. To simplify the system, DNA-dependent RNA synthesis was eliminated by addition of 20 μg/ml actinomycin D, since DNA-dependent RNA synthesis is dispensable for the progesterone-induced maturation [22]. The abolishment of DNA-dependent RNA synthesis was confirmed by the absence of incorporation of [^{32}P]UMP into mRNA. In this system, incorporation of [^{32}P]AMP was inhibited in a dose dependent manner by treatment of oocytes with cordycepin (Fig. 1). Incorporation of [^{32}P]AMP into poly(A) mRNA was inhibited almost completely by treatment of oocytes with 10 m*M* cordycepin for 12 h. Likewise, progesterone-induced GVBD was inhibited by treatment of oocytes with cordycepin in a dose dependent manner

and treatment of oocytes with 10 m*M* cordycepin for 12 h prevented GVBD almost completely (Fig. 2). Good similarity in dose dependence of inhibition on cordycepin between the incorporation of [^{32}P]AMP into poly(A) mRNA and that of progesterone-induced GVBD suggests that the process of poly(A) tail elongation is required for induction of meiotic maturation of oocytes by progesterone.

The inhibitory effect of cordycepin on the oocyte maturation does not appear to be the result of cytotoxity of 3'-deoxynucleoside since treatment of oocytes with 10 m*M* 3'-deoxycytidine for 12 h, which did not affect incorporation of [^{32}P]AMP, also did not prevent progesterone-induced maturation at all (Fig. 2,3). Furthermore, MPF-induced maturation was not inhibited by treatment of oocytes with 10 m*M* cordycepin for 12 h. This result agrees with the previous report that MPF obviates the requirement for protein synthesis to provoke GVBD. In addition, CMF induced GVBD is also inhibited by the treatment of recipient oocyte with cordycepin. These results suggests that cordycepin prevents only the process(es) before active MPF is formed during oocyte maturation.

There remains a possibility that the treatment with cordycepin might cause not only abolishment of poly(A) tail elongation but also shortening of the tail and this might result in destabilization of their coding or regulatory regions [54], depressing their translation efficiency. However, the possibility of destabilization of mRNA by the treatment with cordycepin did not appear to be the case since the translational

efficiency of most transcripts was not altered by treatment of oocytes with cordycepin (Fig. 4) and the inhibition of maturation by cordycepin was rescued by removal of cordycepin even in the presence of actinomycin D (Table 2).

We examined whether incorporation of [^{32}P]AMP was activated in response to progesterone. Progesterone induced a 2.7-fold activation of [^{32}P]AMP incorporation into the poly(A) tail of mRNA after a lag period of 3 h, whereas GVBD was induced 6-8 h after progesterone treatment under this condition. The fact that activation of incorporation of [^{32}P]AMP preceded the induction of GVBD was not contradictory with the possibility that part of poly(A) tail elongation participated an induction of GVBD. We conclude that elongation of the poly(A) tail of mRNA is necessary to induce meiotic maturation of *Xenopus* oocytes.

We found that syntheses of most of the proteins were unaffected even by the complete absence of poly(A) tail elongation (Fig. 4). This result suggests that translation of most of the mRNA in stage VI oocytes was not regulated by poly(A) tail elongation. However, synthesis of several protein bands was activated by progesterone and abolished by treatment of oocytes with cordycepin. We also found that cordycepin suppresses the MPF-induced changes in translation. We have not yet determined which protein is required for the induction of oocyte maturation. It was shown by Sagata *et al.* that oocyte maturation is induced by c-mos mRNA and that c-mos protein is synthesized in response to progesterone [56]. Paris and Richter [57] showed that c-mos RNA has an element which induces maturation specific

polyadenylation when fused to *Xenopus* β-globin RNA. However, it has not yet been shown whether c-mos protein directly activates MPF. The application of cordycepin as a specific inhibitor of poly(A) tail elongation will offer a novel approach to identifying other key proteins in the process of the oocyte maturation.

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REFERENCES

1. Schuetz, A.W. (1974) *Biol. Reprod.* **10**, 150-178.
2. El-Etr, M. Schorderet-Slatkine, S., and Baulieu, E. (1979) *Science* **205**, 1397-1399.
3. Masui, Y., and Markert, C.L. (1971) *J. Exp. Zool.* **177**, 129-146.
4. Merrian, R.W. (1972) *J. Exp. Zool.* **180**, 421-426.
5. Wasserman, W.J., and Masui, Y. (1975) *Exp. Cell Res.* **91**, 381-388.
6. Lohka, M.J. Hayes, M.K., and Maller, J.L. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3009-3019.
7. Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J.L. (1988) *Cell* **54**, 433-439.
8. Arion, D., Meijer, L., Brizuela, L., and Beach, D. (1988) *Cell* **55**, 371-378.
9. Labbe, J.C., Picard, A., Peaucellier, G., Cavadore, J.C., Nurse, P., and Doree, M. (1989) *Cell* **57**, 253-263.
10. Meijer, L., Arion, D., Golsteyn, R., Pines, J., Brizuela, L., Hunt, T., and Beach, D. (1989) *EMBO J.* **8**, 2275-2282.
11. Dunphy, W.G., and Newport, J.W. (1988) *Cell* **55**, 925-928.
12. Murray, A.W., and Kirschner, M.W. (1989) *Science* **246**, 614-621.
13. Jessus, C., Rime, H., Haccard, O., Lint, J.V., Goris, J., Merlevede, W., and Ozon, R. (1991) *Development* **111**, 813-820.
14. Ishikawa, K., Hanaoka, Y., Kondo, Y., and Imai, K. (1977) *Mol. Cell Endocr.* **9**, 91-100.

15. Sadler, S.E., and Maller, J.L. (1982) *J. Biol. Chem.* **257**, 355-361.

16. Speaker, M.G., and Butcher, F.R. (1977) *Nature* **267**, 848-849.

17. Maller, J.L., Butcher, F.R., and Krebs, E.G. (1979) *J. Biol. Chem.* **254**, 579-582.

18. Moreau, M., Vilain, J.P., and Guerrier, P. (1980) *Dev. Biol.* **78**, 201-214.

19. Cork, R.J., Cicirelli, M.F., and Robinson, K.R. (1987) *Dev. Biol.* **121**, 41-47.

20. Cicirelli, M.F. and Smith, L.D. (1987) *Dev. Biol.*, **121**, 48-57.

21. Kuge, H., and Inoue, A. (1990) *J. Cell Biol.* **111**, 91a

22. Newport, J., and Kirschner, M. (1982) *Cell* **30**, 687-696.

23. Galili, G., Kawata, E.E., Smith, L.D., and Larkins, B.A. (1988) *J. Biol. Chem.* **263**, 5764-5770.

24. Huarte, J., Belin, D., Vassalli, A., Strickland, S., and Vassalli, J.-D. (1987) *Genes Dev.* **1**, 1201-1211.

25. Hyman, E.H., and Wormington, W.M. (1988) *Genes Dev.* **2**, 598-605.

26. McGrew, L.L., Dworkin-Rastl, E., Dworkin, M.B., and Richer, J.D. (1989) *Genes Dev.* **3**, 803-815.

27. Munroe, D., and Jacobson, A. (1990) *Mol. Cell. Biol.* **10**, 3441-3455.

28. Rosenthal, E.T., Tansey, T.R., and Ruderman, J.V. (1983) *J. Mol. Biol.* **166**, 309-327.

29. Rosenthal, E.T., and Ruderman, J.V. (1987) *Dev. Biol.* **121**, 237-246.

30. Strickland, S., Huarte, J., Belin, D., Vassalli, A., Rickles, R.J., and Vassalli, J.-D. (1988) *Science* **241**, 680-684.

31. Wickens, M. (1990) *Trends Biochem. Sci.* **15**, 320-324.

32. Sagata, N., Shiokawa, K., and Yamana, K. (1980) *Dev. Biol.* **77**, 431-448.

33. Colot, H.V., and Rosbash, M. (1982) *Dev. Biol.* **94**, 76-86.

34. Dworkin, M.B., and Dworkin-Rastl, E. (1985) *Dev. Biol.* **112**, 451-457.

35. Wallace, R.A., Jared, D.W., Dumont, J.N., and Sega, M.W. (1973) *J. Exp. Zool.* **184**, 321-334.

36. Paris, J., Guellec, R.L., Couturrier, A., Guellec, K.L., Omilli, F., Caminos, J., Macneill, S., and Phillipe, M. (1991) *Proc. Natl Acad. Sci. USA* **88**, 1039-1043.

37. Dumont, J.N. (1972) *J. Morphol.* **136**, 153-180.

38. Smith, M., and Khorana, H.G. (1963) *in* Methods in Enzymology (Colowick, S.P., and Kaplan, N.O., Eds.), Vol. 6, pp659, Academic Press, London

39. Neuhard, J., Randerath, E., and Randerath, K. (1965) *Anal. Biochem.* **13**, 211-22130.

40. Dworkin, M.B., and Dawid, I.B. (1980) *Dev. Biol.* **76**, 435-448.

41. Kuribayasi, K., Hikata, M., Hiraoka, O., Miyamoto, C., and Furuiti, Y. (1988) *Nucletic Acids Reseach Symposium Serise* **19**, 61-64.

42. Lohka, M.J., and Maller, J.L. (1985) *J. Cell Biol.* **101**, 518-523.

43. Mans, R.J., and Novelli, G.D. (1961) *Arch. Biochem. Biophys.*

94, 48-58.

44. O'Farrel, P.H. (1975) *J. Biol. Chem.* **250**, 4007-4021.

45. Laemmli, U.K. (1970) *Nature* 227, 680-685.

46. Klenow, H. (1963) *Biochem. Biophys. Acta* 76, 354-365.

47. Shigeura, H.T., and Gordon, C.N. (1965) *J. Biol. Chem.* 240, 806-810.

49. Pennequin, P., Schouderet-Slatkine, S., Drury, K.C., and Baulieu, E.-E. (1975) *FEBS Letters* 51, 156-160.

50. Ballantine, J.E.M., Woodland, H.R., and Sturgess, E.A. (1979) *J. Embryol. Exp. Morphol.*, 51, 137-153.

51. Younglai, E.V., Godeau, F., and Baulieu, E.-E. (1981) *FEBS Letters* 127, 233-236.

52 6. Younglai, E.V., Godeau, F., Mulvihill, B., and Baulieu, E.-E.(1982) *Dev. Biol.* 91, 36-42.

53. Wasserman, W.J., Richter, J.D. and Smith, L.D. (1982) *Dev. Biol.* 89, 152-158.

54. Wilson, T., and Treisman, R. (1988) *Nature* 336, 396-399.

55. Green, L.L., and Dove, W.F. (1988) *J. Mol. Biol.* 200, 321-328.

56. Sagata, N., Daar, I., Oskarsson, M., Showalter, S.D., and Vande Woude, G.E. (1989) *Science* 245, 643-645.

57. Paris, J., and Richter, J.D. (1990) *Mol. Cell. Biol.* 10, 5634-5645.

Chapter III

Amplification of MPF Activity in *Xenopus laevis* Oocytes Occurs through a Protein Synthesis

Introduction

The studies on the temporal sequence of events in the cell cycle have reached the conclusion that the cell cycle consists of at least four functionally distinct phases, DNA synthesis occurs during S phase, mitosis during M phase, and each of these two phases is generally separated by periods of time during which neither DNA synthesis nor mitosis is occurring. In recent years considerable attention has been given in oocytes and eggs particularly those of *Xenopus laevis* .

Fully grown *Xenopus laevis* oocytes are physiologically arrested in the first meiotic prophase. When oocytes are exposed to progesterone, they resume meiotic maturation to complete undergoing breakdown of the nuclear envelope (germinal vesicle breakdown, GVBD), chromosome condensation, spindle formation, and extrusion of the first polar body [1,2]. In addition to these morphological changes, a transferable activity is known to appear in the cytoplasm that, when microinjected into immature oocytes, can induce them to engage in meiotic maturation in the absence of progesterone. This cytoplasmic activity has been called maturation-promoting factor or MPF [3-5].

Although MPF was first discovered in amphibian oocytes it plays the same role in mitosis as it does in meiosis. MPF actually oscillates in the mitotic division of cleaving embryos with the same periodicity as the cell cycle [6,7]. MPF activity exists in a variety of eukaryotic cells such as starfish oocytes and eggs [8], mammalian cultured cells [9] and yeast [10]. In

each case MPF activity, which was assayed by ability of the induction of *Xenopus* oocytes maturation, was detected only in M phase.

In recent years, the character of MPF and the mechanism of its activation come into sight. It was shown that the entity of MPF is the activated form of histone H1 kinase which is a complex of $p34^{cdc2}$ and a cyclin protein family [11-17] and that during meiotic maturation of *Xenopus* oocytes the cyclin B-$p34^{cdc2}$ complex is activated by dephosholylation of $p34^{cdc2}$ [18]. However, the physiological significance of this complex in the induction of M phase and the regulatory mechanism of its activity is unknown.

When oocytes are treated with progesterone in the presence of cycloheximide, neither morphological change [19] nor production of MPF [20] occurs. However, cytoplasm containing MPF can induce recipient oocytes to complete meiotic maturation even in the presence of cycloheximide [20]. These results have been interpreted to mean that progesterone induces oocytes to synthesize an initiator protein(s) that activates MPF and then MPF triggers meiotic events without a requirement of protein synthesis.

It was reported by Masui and Market [3] that MPF itself has an autocatalytic activity since injection of small amount of MPF triggers the production of a much larger amount of MPF by the recipient oocytes. This autocatalytic property of MPF is considered to be important for the rapid and sudden increase in MPF activity when cells enter into M-phase. It is important to

study the mechanism of the autocatalysis in MPF activity not only for understanding the mechanism of feedback control in cell cycle but also for understanding the mechanism of activation of MPF by hormone stimulation. It was reported by Wassrman and Masui [20] , Gerhardt et al. [7]. and Cyert and Kirshner [20] that amplification of MPF activity occurs even in the absence of any protein synthesis, while Druly and Schorderet-Slatekine [22] reported that the protein synthesis is required for the renewal of MPF in the serial transfer. However, the absolute value of MPF activity during serial transfer in the presence and absence of cycloheximide has not yet been reported.

In the present study, we found that M-phase extract contain an activity to increase MPF activity even in the presence of cycloheximide. However, this activity disappeared by a serial transfer in the presence of cycloheximide. A large increase in the MPF activity requires protein synthesis. We report the existence of an active factor (amplification factor, AF) which induce an amplification of MPF activity through protein synthesis.

MATERIALS AND METHODS

Materials. Cycloheximide and progesterone were obtained from Nacalai Tesque (Kyoto).

Animals. Adult *Xenopus laevis* females were obtained from Hamamatsu Seibutsu Kyouzai (Shizuoka). The ovaries were removed surgically from animals that had been anaesthetized by immersion in ice-cold water into OR-2 medium [23] (82.5 m*M* NaCl, 2.5 m*M* KCl, 1.0 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 1.0 m*M* Na_2HPO_4, 5.0 m*M* Hepes at pH 7.8) and 10 m*M* glucose. The lobes of the ovaries were washed in OR-2 medium and dissected into clumps of 100 to 200 oocytes in OR-2. Then, oocytes were removed manually from ovaries, and kept in OR-2. Oocytes at stage VI [24], having a diameter of more than 1.2 mm, were selected for the study.

Maturation of Oocytes and Preparation of M-phase Extract. Maturation of oocytes were carried out under the influence of 10 μM progesterone in the OR-2 medium for 6 hrs. Oocyte maturation was also induced by injection of 50-80 nl extract. Oocyte showing GVBD was washed with the extraction buffer (buffer A: 80 m*M* 2-glycerophosphate, 5 m*M* NaF, 20 m*M* EGTA, 15m*M* $MgCl_2$, 1 m*M* DTT, 100 μM PMSF, 1 m*M* ATP, and 20 m*M* Hepes at pH 7.5 with NaOH). Oocytes were homogenized in the same buffer and centrifuged at 100,000 x g for 10 min, and the translucent layer between the cap of fat and the pellet of yolk was recovered. Cytosol containing active MPF was also prepared from *Xenopus* eggs using a procedure of Lohka and Maller [25]. For experiments on the serial transfers recipient oocytes were treated with 100 μM

cycloheximide 30 min prier to the injection. Injected oocytes were incubated for 2 hrs at 25° C. Then, the extract of oocytes was prepared as described above.

Assay of MPF Activity. To determine MPF activity, oocytes were injected with 50-80 nl each of sample in the presence of 50 μg/ml cycloheximide. Injected oocytes were incubated for 3 hours at 25° C, and the fraction that underwent GVBD was determined by scoring for 'white spot' formation in the pigment hemisphere. For each experiment the activities of various fractions were tested with oocytes from the same animal and more than 20 oocytes were injected for each sample.

MPF activity was quantified by stepwise dilution. The concentration that caused GVBD in just 50% of the oocytes was estimated with due consideration of the dependence of MPF activity on dilution. One unit of activity was arbitrarily defined as the amount of activity that causes GVBD in 50% of the oocytes injected with a volume of 50 nl of the sample.

Preparation of Cell Free Extract from Xenopus Oocyte. The lobes of the ovaries of *Xenopus* were washed in Barth's medium (88 m*M* NaCl, 1.0 m*M* KCl, 2.4 m*M* $NaHCO_3$, 0.82 m*M* $MgSO_4$, 0.33 m*M* $Ca(NO_3)_2$, 0.41 m*M* $CaCl_2$, 5 m*M* Hepes pH 7.6, 0.01 g/l streptomycin, 0.01 g/l penicillin) [26] and dissected into clumps of 100 to 200 oocytes in the medianum. The clumps of oocytes were gently agitated in the dissociation medium (0.6 U/ml dispase, 2 mg/ml collagenase in Barth's medium) for 2 h. This procedure results in complete dissociation of the oocytes from the ovarian envelope within 2 h. Then, oocytes were washed

extensively with Barth's medium. Oocytes having a diameter more than 0.6 mm were selected by sedimentation in 0.5 *M* sucrose in Barth's medium under gravity. The selected oocytes were wash with the extraction buffer (buffer B: 80 m*M* 2-glycerophosphate, 5 m*M* NaF, 20 m*M* EGTA, 15 m*M* $MgCl_2$, 20 m*M* HEPES (pH 7.5 with NaOH), 250 m*M* sucrose, 1 m*M* DTT, 1 m*M* ATP, 0.5 mg/ml trypsin inhibitor, 10 μg/ml leupeptin, 10 μg/ml chymostatin, 10 μg/ml pepstatin, 0.5 m*M* spermidine, 100 μg/ml cytochalasin B) and sedimented under 100 x g for 1 min at 4 °C. Excess buffer was removed and 1/10 volume of extraction buffer was added to the oocyte pellet. The oocytes were crushed by centifugation at 30,000 x g for 15 min at 4°C. The cytoplasmic layer was taken to the other tube. RNase inhibitor (250 U/ml) was added into the extract. One tenth volume of ATP regeneration mixture (150 mg/ml creatine phosphate, 2 mg/ml creatine phosphokinase, 10 m*M* $MgCl_2$, 100 m*M* EGTA at pH 7.5, 100 μg/ml cytochalasin B) was added to the cytoplasmic extract at the start of the incubation. For *in vitro* amplification assay, the oocyte extract was mixed with M-phase extract at various ratios and incubated at 25 °C for indicated periods. Then, aliquot of the sample was taken to assay of MPF activity or histone H1 kinase activity.

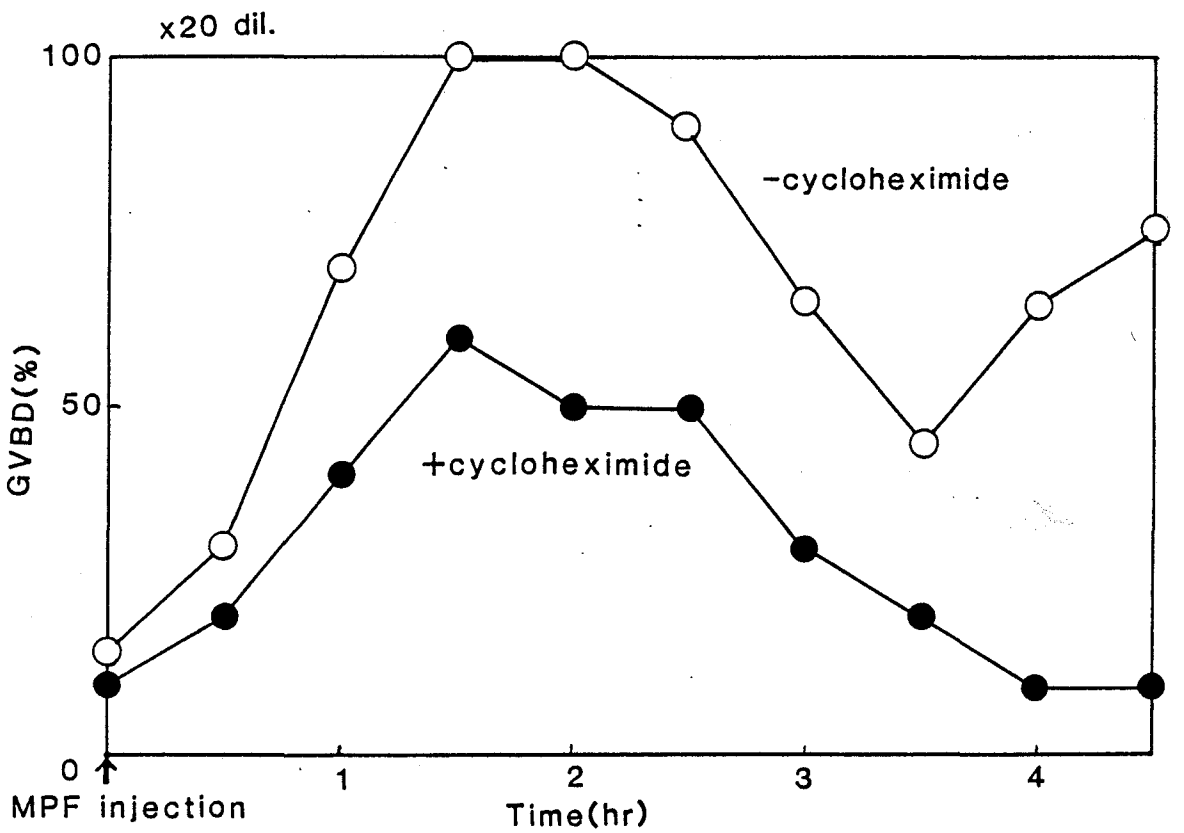
Assay of histone H1 kinase. Histone H1 kinase activity was measured as described by Felix *et al* [27], after diluting the sample with the extraction buffer at 100 μ*M* ATP.

Dependence on Protein Synthesis of the Amplification of MPF Activity in the Oocyte After Injection with M-Phase Extract.

We examined the involvement of protein synthesis in the process of amplification of MPF activity in *Xenopus*oocytes. M-phase extract was prepared from the oocytes that had undergone GVBD by treatment with progesterone 7 hrs. M-phase extract containing MPF activity was injected into oocytes in the presence or absence of 50 μg/ml cycloheximide. The injected oocytes were incubated for various period. MPF activity in extract of each sample of oocytes was measured by injection into the other oocytes in the presence of cycloheximide after the 20 fold dilution with the extraction buffer. After 3 h from the second injection, the fraction of recipient oocytes that underwent GVBD was scored (Fig. 1).

The first recipient oocytes underwent GVBD within 2 h after MPF injection even in the presence of cycloheximide, as reported previously [3,20]. If the first recipient oocytes were not treated with cycloheximide, MPF activity in their cytoplasm reached maximal level at 90-120 min, and the extract of these oocytes induced GVBD in all the second recipients. If the first recipient oocytes were treated with 50 μg/ml cycloheximide, MPF activity in their cytoplasm also reached at maximal level at 90-120 min. However, the cytoplasm from these oocytes induce GVBD only 50% of the second recipient oocytes (Fig.1). Therefore,

Fig. 1 Amplification of MPF activity in oocyte in the presence or absence of cycloheximide. Batches of 30 oocytes were injected with M-phase extract containing MPF activity in the presence (●) or absence (○) of 50 μg/ml cycloheximide. The injected oocytes were incubated for indicated period. The extracts of each batch of oocytes were injected into the other oocyte in the presence of cycloheximide after 20 fold dilution with the extraction buffer, and GVBD was scored after 3 h.



even in the presence of cycloheximide, MPF activity was amplified but in the presence of cycloheximide the extent of amplification in MPF activity was low.

Amplification of MPF Activity Disappeared by a Serial Transfer in the Presence of Cycloheximide

We confirmed the involvement of protein synthesis in the process of MPF amplification by a serial transfer of the recipient cytoplasm in the presence of cycloheximide (Table 1). The MPF activity of M-phase extract measured by injection to the other oocytes in the presence or absence of cycloheximide were 1,000 and 1,300 units/oocyte, respectively (Table 1, lane 2). M-phase extract (20 units of MPF activity) was injected into oocytes in the presence and absence of cycloheximide. After incubation for 2 hrs, MPF activity in cytosol of each recipient oocytes were measured by injection to the other oocyte (Table 1, lane 4 and 3, respectively). The injected oocyte in each treatment produced about 150 and 800 units, respectively, of MPF activity in their cytoplasm in the presence of cycloheximide. These results agree with those of Fig, 1, and showed that MPF activity is amplified even in the presence of cycloheximide but the extent of amplification is low in the presence of cycloheximide.

The extract from the oocytes injected in the presence of cycloheximide (The second extract, Table 1, *2) was injected into the next oocyte in the presence or absence of cycloheximide after the 5-fold dilution. After incubation for 2 hrs, MPF activity in

Table 1 Amplification of MPF activity disappeared by a serial transfer in the presence of cycloheximide. MPF-activity (GVBD inducing activity) of the extract prepared from the immature oocytes (lane 1) and oocyte at M-phase induced by progesterone (lane 2) were measued by injecting into the other oocytes in the presence (left column) or absence (right) of cycloheximide. M-phase extract was injected into the next oocyte in the presence (lane 4) or absence (lane 3) of cycloheximide. After 2 hour incubation, the extracts of these oocytes was prepared and the MPF activity was measured by injection in the presence or absence of cycloheximide. The extract from the oocytes injected the in the presence of cycloheximide (Table 1 *2) was injected again into the next oocyte in the presence (lane 6) or absence (lane 5) of cycloheximide. After 2 hour incubation, the extracts of these oocytes was prepared, and the MPF activity was measured in the presence or absence of cycloheximide.

	Acceptor	
Donor	+Cycloheximide	-Cycloheximide
control	< 20	< 20
15 μM progesterone *1	1000	1300
MPF (20 unit*1) injected in the absence of cycloheximide	800	1000
MPF (20 unit*1) *2 injected in the presence of cycloheximide	150	300
2nd MPF (20 unit*2) injected in the absence of cycloheximide	600	1000
2nd MPF (20 unit*2) injected in the presence of cycloheximide	< 20	150

1 unit = activity which induce 50% GVBD

cytosol of each recipient oocytes were measured by injection of the extract to the other oocyte (Table 1. lane 5 and 6). By the serial injection in the presence of cycloheximide MPF activity in the cytoplasm of third oocyte was less than 20 units, but in the absence of cycloheximide the MPF activity increased to 600 units/oocyte.

Thus, in the case of serial transfer in the continuous presence of cycloheximide, MPF activity was increased 7.5-fold in the first recipient (Table 1, lane 4). But in the second recipient, amplification of MPF activity was no more observed (Table 1, lane 6). Even in the absence of amplification of MPF oocytes showed GVBD. In parallel, when serial transfer was performed in the absence of cycloheximide. 50-fold amplification of MPF activity was observed even in the second recipient (Table 1. lane 5).

Dependence on Protein Synthesis of the Amplification of MPF Activity in Cell Free System.

To characterize the process of MPF amplification in more detail, we examined the MPF amplification in the cell-free system. In the present paper, oocytes were prepared by dispase treatment (see "MATERIAL and METHOD". Isolated oocytes were gently lysed in a minimal amount of buffer and centrifuged at 30,000 x g to remove yolk platelet. Then the extract was then incubated at room temperature with an ATP-regeneration system in the presence or absence of 50 μg/ml cycloheximide and with one-tenth volume

Table 2 Amplification of MPF activity in cell free system was promoted by protein synthesis. Cytosolic extract of oocytes was mixed with one-tenth volume of M-phase extract containing MPF activity and incubated at room temperature with an ATP-regeneration system in the presence ( ) or absence ( ) of 50 μg/ml cycloheximide. After 1 hour incubation, an aliquot was withdrawn from the reaction mixture and injected into the other oocytes to assay for MPF activity. After 2 h, the percentage of oocytes that had undergone M-phase was scored.

Amplification of MPF Activity in Cell Free System

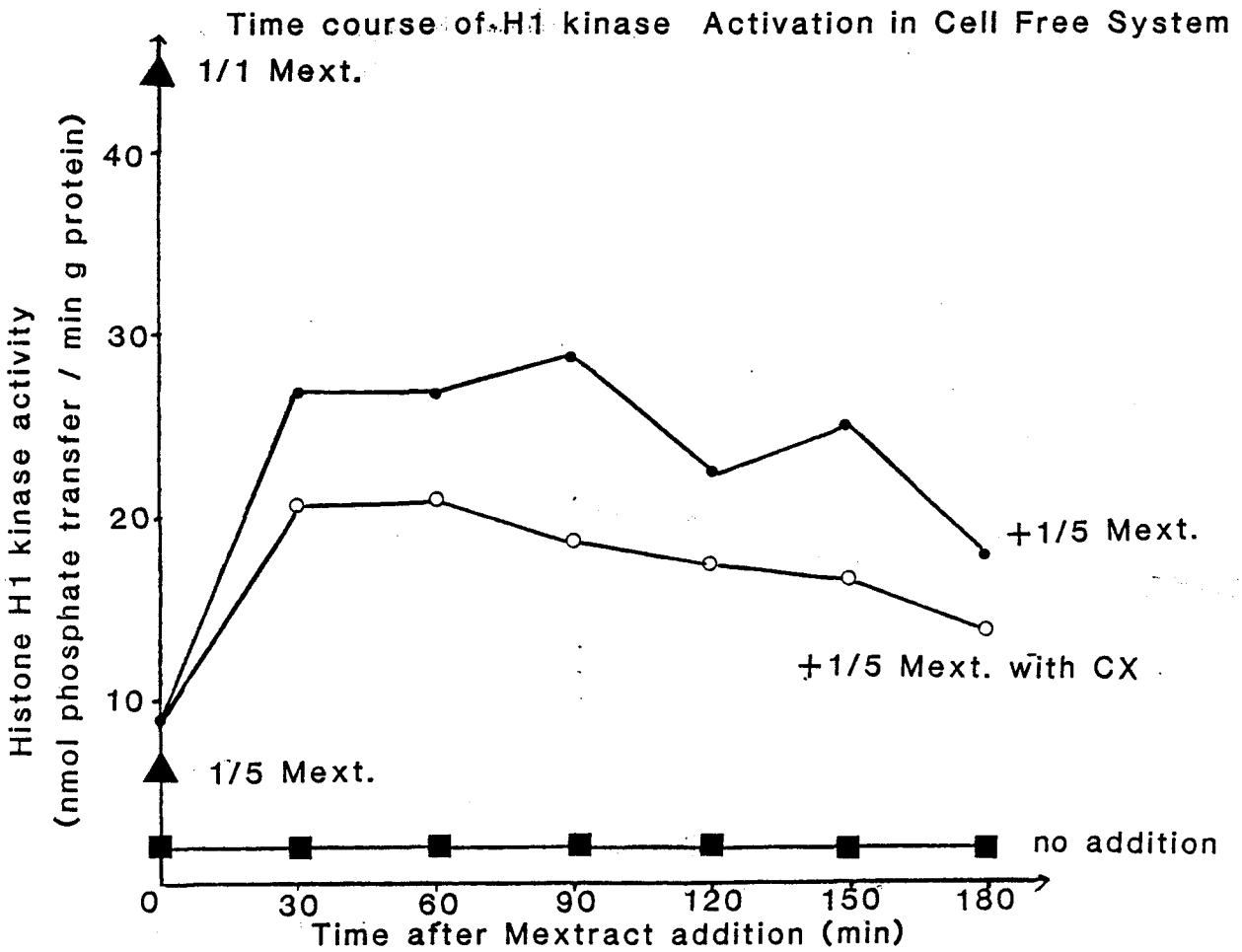
donor extract	GVBD in CX
oocyte extract	7/36 19%
Mextract	26/33 79%
oocyte ext.+1/10 Mext. (1 h incubation)	23/34 68%
oocyte ext.+1/10 Mext.+CX (1 h incubation)	11/36 31%

of M-phase extract containing MPF activity. After 1 hr incubation, aliquot was withdrawn from the reaction mixture and injected into *Xenopus* oocytes to assay the MPF activity. After 2 h, the percentage of oocytes that had undergone GVBD was scored (Table 2). When cycloheximide was not added to the mixture of cell-free extract and M-phase extract. the mixture induced GVBD in 68% of the recipients. However, when 50 μg/ml cycloheximide was added to the mixture, it induced GVBD only 31% of the recipient oocytes (Table 2) Therefore, as those of in vivo amplification (Fig.1, Table 1), the treatment of extract with cycloheximide inhibits partially the amplification of MPF activity.

It was shown that the entity of MPF is activated form of histone H1 kinase which is a complex of $p34^{cdc2}$ and a cyclin protein family [11-17]. Then, we examined whether histone H1 kinase was amplified in the cell free system. We mixed 0.8 volume of oocyte extract with 0.2 volume of M-phase extract containing MPF activity in the presence or absence of 50μg/ml cycloheximide. The mixed extract was incubated for various period and an aliquot was withdrawn to measure H1 kinase activity (Fig. 2).

The oocyte extract have 2 nmol/min/g H1 kinase activity through out the incubation upto 180 min. M-phase extract contained MPF activity of 45 nmol/min/g. At the beginning of incubation, the mixture has H1 kinase of 9 nmol/min/g. If the oocyte extract was not treated with cycloheximide, H1 kinase activity in the mixture reached the maximal level of 29

Fig. 2 Amplification of H1 kinase activity in cell free system. Oocyte extract was prepared as described in the text. One volume of oocyte extract was mixed with 0.25 volume of M-phase extract, which prepared from the egg, in the presence (○) or absence (●) of 50 μg/ml cycloheximide. The mixed extract was incubated for indicated period, and an aliquot was withdrawn to measure H1 kinase activity. H1 kinase activity in the oocyte extract alone was shown by (▲).



nmol/min/g at 30-90 min. Thus, there is about 3.2-fold activation in H1 kinase activity in the extract system.

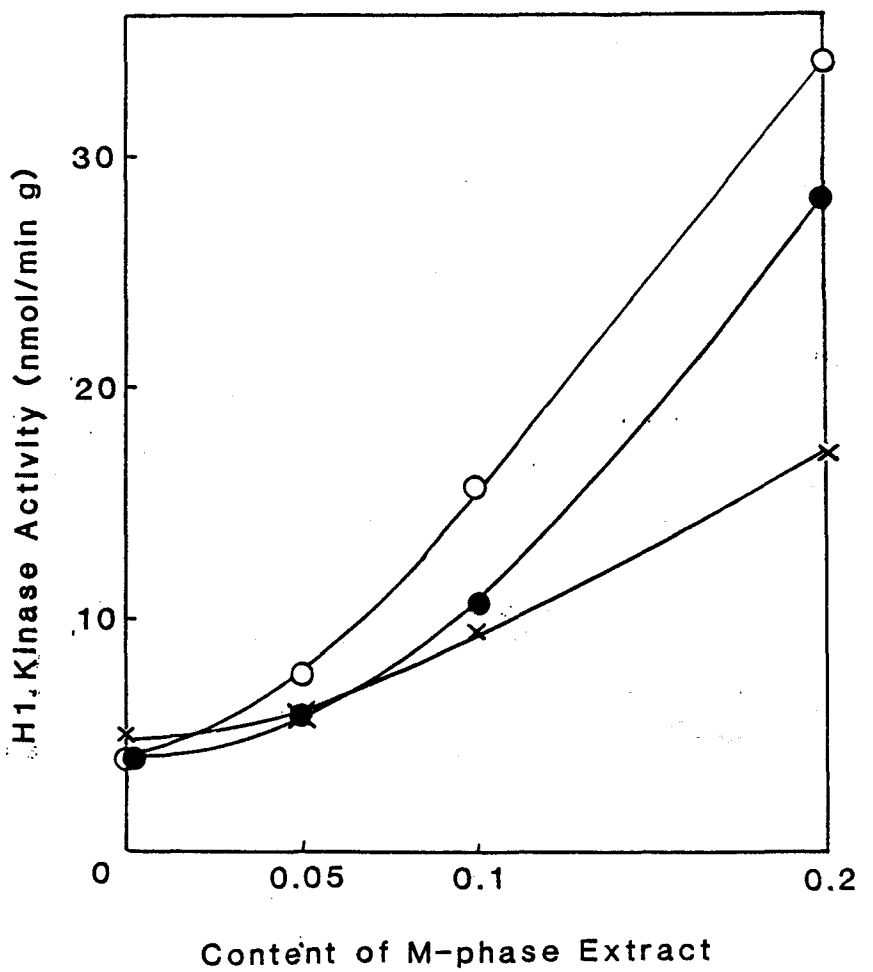
If the oocyte extract was treated with 50 μg/ml cycloheximide, H1 kinase activity in the mixture reached maximal level of 20 nmol/min/g at 30-60 min after the mixing (2.3-fold activation). Therefore, the treatment of oocyte extract with cycloheximide inhibits 28 % of the amplification of H1 kinase activity in the extract. Thus, the result on *in vitro* system is closely related to that on in vivo amplification of MPF activity, as shown in Fig. 1.

We examined the dependence of initial ratios of M-phase extract to the oocyte extract on the amplification of H1 kinase activity (Fig. 3). We mixed the oocyte extract with the M-phase cytoplasm containing MPF activity at various ratio and in the presence or absence of 50μg/ml cycloheximide. The mixed extract was incubated for 90 min and an aliquot was withdrawn to measure the H1 kinase activity. The cycloheximide sensitive amplification was obvious when the ratio of M-phase extract to oocyte extract was more than 0.1. At every ratio of mixing, H1 kinase activation in the cycloheximide treated extract was lower than that in non treated extract. These result also showed that amplification of MPF is promoted by protein synthesis.

Primary characterization of Amplification Factor.

The extract of the oocytes which were injected at the second time in the continuous presence of cycloheximide had an activity which

Fig. 3 Dose dependence of M-phase extract on H1 kinase activation in the cell free system. Oocyte extract was mixed with M-phase extract containing MPF activity at various ratio in the presence ( ) or absence ( ) of 50 μg/ml cycloheximide. The mixed extract was incubated for 0 min ( ) or 90 min (,) and an aliquot was withdrawn to measure the H1 kinase activity.



could induce GVBD only when protein synthesis was available (Table 1, lane 6). We call this active factor as Amplification Factor, AF. To investigate the molecular nature of this factor, we characterized AF in the extract of the oocytes which were injected at the second time in the continuous presence of cycloheximide.

We estimated the molecular mass of AF activity by gel filtration method. The extract containing AF activity was loaded on a column of Sepharose 4B. AF activity was eluted with a mobility that corresponded to an apparent molecular mass of about 2,000 kDa (Fig. 4). We examined fractionation of the extract containing AF activity into ammonium sulfate. The majority of AF activity in the extract was recovered in the protein precipitated by 15-45% ammonium sulfate (Fig. 5).

Binding examination of AF activity to various resins was shown together in Table 3. AF activity was adsorbed by DEAE-cellulose column at 1/3 concentration of the extraction buffer (80 m*M* 2-glycerophosphate, 20 m*M* EGTA, 15 m*M* $MgCl_2$, and 20 m*M* Hepes at pH 7.5), and was eluted by the extraction buffer. AF was adsorbed by CM-cellulose column at 1/3 concentration of extraction buffer and eluted with the extraction buffer + 0.1 *M*KCl. It should be noted that MPF and CMF (Chapter I) were not adsorbed by CM-cellulose column. AF was trapped by phospho-cellulose column at 1/3 concentration of extraction buffer and eluted by the extraction buffer. AF was also adsorbed by phenyl-sepharose column at 1/3 extraction buffer + 10% ammonium sulfate, but was not eluted even by water. These primary characterization

Fig. 4 Estimation of molecular mass of AF activity.

The extract of the oocytes which were injected at the second time in the continuous presence of cycloheximide was loaded on a column of Sepharose 4B. An aliquot of each fraction was injected into the oocytes in the absence of cycloheximide. After 2 h, the percentage of oocytes that had undergone M-phase was scored. Ferritin (460 kDa), hemoglobin (65 kDa) and blue-dextran (∞) were eluted at the positions indicated by arrows.

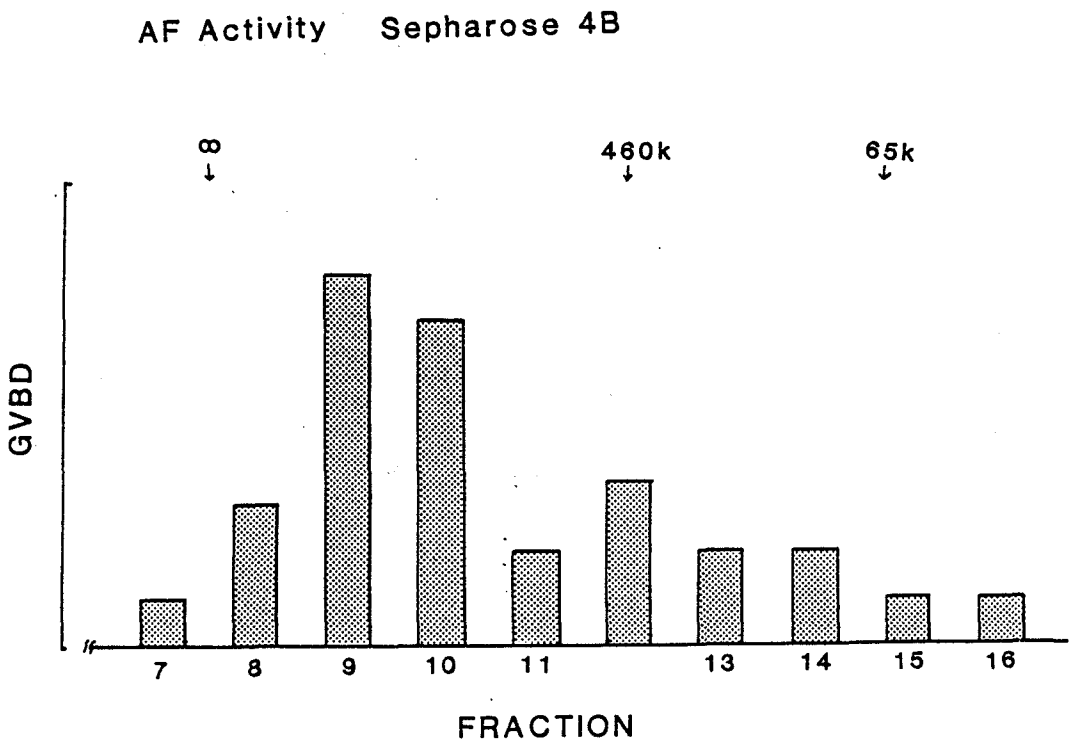


Fig. 5 Ammonium sulfate fractionation of the extract containing AF activity. The extract of the oocytes which were injected at the second time in the continuous presence of cycloheximide was fractionated by ammonium sulfate precipitation. The pellet of each fraction was dialyzed against extraction buffer for 12 h and then injected into the oocytes in the presence (open bar) or absence (shadowed bar) of cycloheximide. After 2 h, the percentage of oocytes that had undergone M-phase was scored.

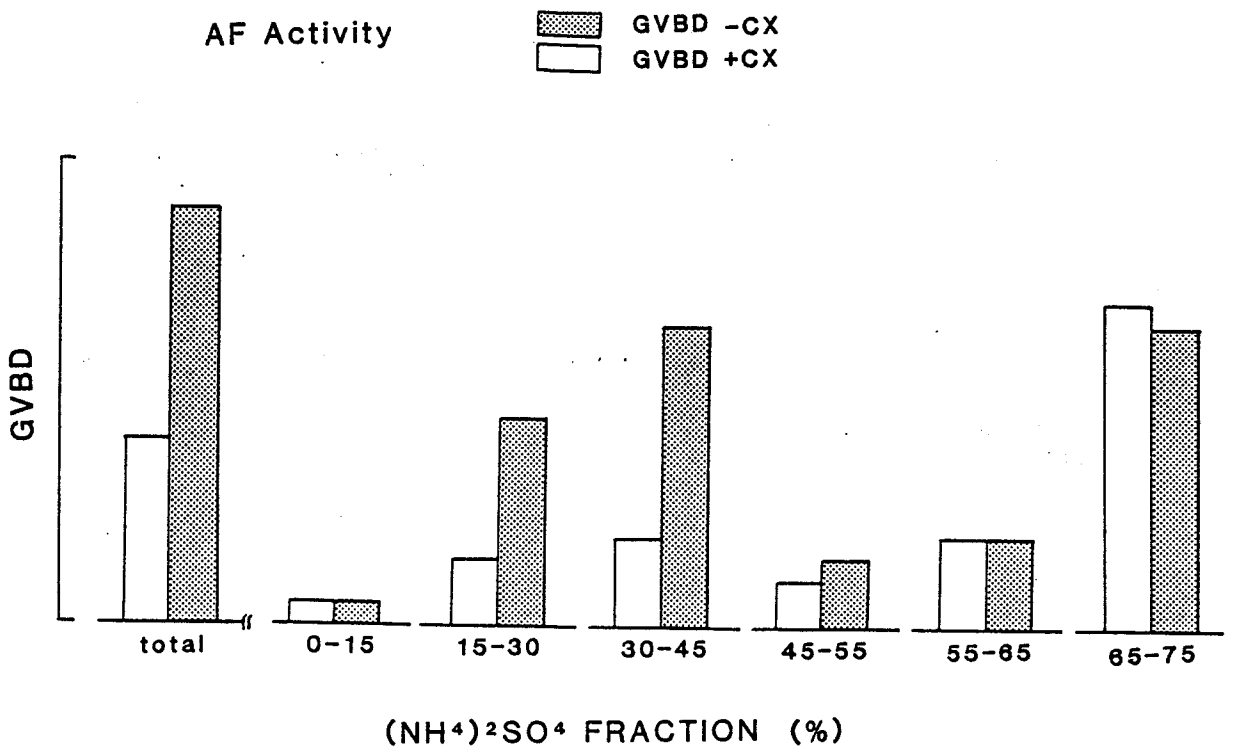


Table 3 Binding of AF activity to various resins.

The extract of the oocytes which were injected at the second time in the continuous presence of cycloheximide was applied to the column of various resin at the indicated condition. The fraction eluted by the indicated condition was injected into oocytes to assay AF activity. The quantity of the symbol '+' means the rough estimation of AF activity in the fraction.

Resin	Condition of Apply	Elution		
DEAE-cellulose	1/3 E.B.	E.B.	E.B.+0.1M KCl	E.B.+0.5M KCl
	−	++	+	−
CM-cellulose	1/3 E.B.	E.B.	E.B.+0.1M KCl	E.B.+0.5M KCl
	−	−	+++	−
Phospho-cellulose	1/3 E.B.	E.B.	E.B.+0.1M KCl	E.B.+0.5M KCl
	−	++	+	−
Phenyl-Sephalose	1/3 E.B.+10%(NH_4SO_4)		E.B.	Water
	−		−	−

E.B. (80 mM 2-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$ 20 mM HEPES pH 7.5)

of AF activity will contribute the purification of AF and the study of the mechanism of amplification of MPF activity.

DISCUSSION

Onset of M-phase is the start of irreversible process toward new generation of the cell. There must be an elaborate control mechanism to guarantee that various changes in M phase should take place irreversibly and contemporary. A sudden increase of MPF activity occurs surely in cytoplasm in the onset of M phase. In addition, injection of a small amount of MPF activity triggers the production of a much larger amount of MPF activity by the recipient oocyte. This result indicates that a positive feedback control exists in the regulating mechanism of MPF activity. However, the mechanism of the amplification of MPF activity is largely unknown. Then, we examined the involvement of protein synthesis in the process of MPF amplification.

In this study, we established the involvement of protein synthesis in the process of MPF amplification. However, M-phase extract contains an activity to increase MPF activity even in the absence of protein synthesis (Fig. 1 and Table 1). This result agrees with the results of Wasserman and Masui [20], Gerhart *et al* [7] and Cyert and Kirshner [21]. However, this activity disappeared by a serial transfer in the presence of cycloheximide (Table 1). Therefore, present result indicates that MPF cannot amplify by itself but that M-phase extract contains an factor which activates MPF and this factor is diluted by a serial transfer. Thus, there exist an cascade in the MPF activation.

Recently, the entity of MPF was highly purified and shown to be activated form of histone H1 kinase which is a complex of

$p34^{cdc2}$ and a cyclin protein family [11-17]. However, amplification of MPF activity (or histone H1 kinase activity) without protein synthesis by purified $p34^{cdc2}$ complex has not yet reported. We suggest that amplification of MPF activity by M-phase extract without translation is an effect the of activator of MPF in the extract. A large increase in the MPF activity was observed only when protein synthesis was available (Fig. 1 and Table 1). This result agrees with the result of Drury and Schorderet-Slatkine [22].

In this paper we found that the oocyte injected the second times in the presence of cycloheximide contained an active factor which induce GVBD only when protein synthesis was available, and we named this factor as amplification factor (AF). Primary characterization of AF activity indicated that AF and have different molecular entities (Fig. 2,3) from CMF (chapter I).

Cyert and Kirshner [21] reported the increase of MPF activity in a cell free system. However, protein synthesis was not involved in the activation of MPF in their system. We described a new method of preparation for *in vitro* system which can amplify MPF activity (Fig. 2,3). In this system, amplification of MPF activity have same dependency on protein synthesis as in the oocyte. By use of this *in vitro* system, the study on the mechanism of amplification of MPF by AF may proceed.

When MPF was injected into oocytes, AF was activated even in the presence of cycloheximide (Table 1). Once active MPF was produced in the cytoplasm, it induce self-activation through protein synthesis by activating AF (Fig. 4). These positive

feedback control of MPF activity guarantee the drastic activation of MPF activity and irreversible transition to M phase at G_2-M border.

REFERENCES

1. Schuetz, A.W. (1974) *Biol. Reprod.* **10**, 150-178.
2. El-Etr, M., chorderet-Slatkine, S., and Baulieu, E. (1979) *Science* **205**, 1397-1399.
3. Masui, Y. and Markert, C.L. (1971) *J. Exp. Zool.* **177**, 129-146.
4. Smith L.D. and Ecker R.E. (1971) *Dev. Biol.* **25**, 232-247.
5. Schorderet-Slatkine, S. (1972) *Cell Differenciation* **1**, 178-189.
6. Wasserman, W.J., and Smith, L.D. (1978) *J. Cell Biol.* **78**, R15-R22.
7. Gerhart, J., Wu, M., and Kirshner, M. (1984) *J. Cell Biol.* **98**, 1247-1255.
8. Sunkara, P.S., Wright, D.A., and Rao, P.N. (1979) *J. Supramol. Struct.* **11**, 195-198
9. Kishimoto, T., Kuriyama, R., Kondo, H., and Kanatani, H. (1982) *Exp. Cell Res.* **137**, 121-126
10. Weintraub, H., Buscaglia, M., Ferrez, M., Weiller, S., Boult, A., Fabrie, F., and Baulieu, E.E. (1984) *Gen. Comp. Endocrinol.* **53**, 461-469.
11. Lohka, M.J., Hayes, M.K., and Maller, J.L. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3009-3019.
12. Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J. (1988) *Cell* **54**, 433-439.
13. Arion, D., Meijer, L., Brizuela, L., and Beach, D. (1988) *Cell* **55**, 371-378.
14. Labbe, J.C., Picard, A., Peaucellier, G., Cavadore, J.C.,

Nurse, P., and Doree, M. (1989) *Cell* **57**, 253-263.

15. Meijer, L., Arion, D., Golsteyn, R., Pines, J., Brizuela, L., Hunt, T., and Beach, D. (1989) *EMBO J.* **8**, 2275-2282.
16. Dunphy, W.G. and Newport, J.W. (1988) *Cell* **55**, 925-928.
17. Murray, A.W. and Kirschner, M.W. (1989) *Science* **246**, 614-621.
18. Jessus, C., Rime, H., Haccard, O., Lint, J.V., Goris, J., Merlevede, W., and Ozon, R. (1991) Development **111**, 813-820.
19. Merrian, R.W. (1972) *J. Exp. Zool.* **180**, 421-426.
20. Wasserman, W.J. and Masui, Y. (1975) *Exp. Cell Res.* **91**, 381-388.
21. Cyert, M.S., and Kirshner, M.W. (1988) *Cell* **53**, 185-195.
22. Drury, K.C., Schorderet-Slatkine, S. (1974) *Cell* **4**, 264-274.
23. Wallace, R.A., Jared, D.W., Dumont, J.N., and Sega, M.W. (1973) *J. Exp. Zool.* **184**, 321-334.
24. Dumont, J.N. (1972) *J. Morphol.* **136**, 153-180.
25. Lohka, M.J. and Maller, J.L. (1985) *J. Cell Biol.* **101**, 518-523.
26. Gurdon, J.B. and Wickens, M.P. (1983) in "*Methods in Enzymology* (eds by Wu, R., Grossman, L., and Moldave, K.) vol. **101**, pp. 370-386
27. Felix, M-A., Pines, J., Hunt, T., and Karsenti, E. (1989) *EMBO J.* **8**, 3059-3069

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