Cyclin A and B in Goldfish (*Carassius auratus*): Their Roles and Mechanisms of Synthesis during Hormone-Induced Oocyte Maturation

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SUMMARY

Oocyte maturation is a prerequisite for successful fertilization. This process in fish oocytes has been reported to be regulated by three major mediators: gonadotropin, maturation-inducing hormone (17α,20β-dihydroxy-4-pregnen-3-one, 17α,20β-DP, in fish) and maturation-promoting factor (MPF, a complex of cdc2 kinase and cyclin B). Cyclins have been reported to regulate the kinase activity of cdc2, leading to the activation and inactivation of MPF. The present study investigates the roles of cyclin A and B and the mechanisms of translational activation of maternal cyclin B mRNA during 17α,20β-DP-induced oocyte maturation in goldfish (*Carassius auratus*).

Western blot analysis was used to examine changes in the protein levels of cdc2 kinase and cyclin B during goldfish oocyte maturation induced *in vitro* with 17α,20β-DP. Immature oocytes contained a 35-kDa cdc2. In addition to this protein, a 34-kDa cdc2 was detected in mature oocytes. The purified MPF contained the 34-kDa cdc2, but not 35-kDa cdc2. Thus, it is concluded that the 34- and 35-kDa cdc2 proteins are active and inactive forms, respectively. The 34-kDa active cdc2 appeared in accordance with the onset of germinal vesicle breakdown (GVBD). Cyclin B was absent in immature oocyte extracts and appeared when oocytes underwent GVBD, coinciding with the appearance of the 34-kDa active cdc2. Precipitation experiments with $p13^{suc1}$ and anti-cyclin B antibody revealed that cyclin B that appeared during oocyte maturation formed a complex with cdc2, as soon as it appeared. It is most likely that the 35-kDa inactive cdc2 preexisting in immature oocytes forms a complex with *de novo* synthesized cyclin B at first, then is immediately converted into the 34-kDa active form, which triggers all changes that accompany oocyte maturation, such as GVBD, chromosome condensation and spindle formation. Introduction of *E. coli*-produced cyclin B into immature oocytes using microinjection induced oocyte maturation under condition of inhibited protein synthesis. These results strongly suggest that MPF activation in fish oocytes is induced by complex formation with preexisting cdc2 kinase and newly synthesized cyclin B during oocyte

maturation, a situation differing from that in *Xenopus* and starfish, in which the cdc2 kinase-cyclin B complex is already present in immature oocytes.

Both in *Xenopus* and fish, unfertilized mature oocytes are arrested at the second meiotic metaphase until fertilized. In contrast to *Xenopus*, an inhibition of protein synthesis in unfertilized mature goldfish oocytes with cycloheximide caused a 30-50 % decrease in the cdc2 kinase activity/cyclin B protein levels and an exit from meiotic metaphase-arrest. As compared with that occurring upon normal activation, the induced-decrease in the MPF activity was partial, and the cell cycle of the cycloheximide-treated oocytes was arrested again at the second meiotic anaphase. These results show that cdc2 kinase activity, cyclin B protein level, and cell cycle progression are closely linked. Furthermore, it is suggested that in addition to a difference in the mechanisms of MPF activatiion, the mechanisms of maintaining MPF activity in unfertilized mature goldfish oocytes differ from those in mature *Xenopus* oocytes.

Although cyclin A is thought to be involved in the regulation of both S and M phase in eukaryotic cell cycle, its exact role in the cell cycle, especially in the meiotic cycle (oocyte maturation) is uncertain. To investigate the role of cyclin A in oocyte maturation, a goldfish cyclin A cDNA was cloned and antibodies against its product were produced. Unlike goldfish cyclin B that is absent in immature oocytes, cyclin A was already present in immature oocytes and its protein level was not remarkably changed during oocyte maturation. These observations differ from those of *Xenopus* oocytes, showing an undetectable amount of cyclin A and a large amount of stockpiled cyclin B at the onset of oocyte maturation. Thus, the behavior of goldfish cyclin A resembles that of *Xenopus* cyclin B, whereas that of goldfish cyclin B resembles *Xenopus* cyclin A. In the goldfish oocyte system, cyclin A binds to cdc2, but not cdk2, and that it activates cdc2 both *in vivo* and *in vitro*, raising the possibility that cyclin A plays a role in oocyte maturation. Changes in cyclin A-cdc2 and cyclin B-cdc2 kinase activity during oocyte maturation were also examined. Cyclin B-cdc2 kinase activity increases according to the occurrence of GVBD. Although the timing of the activation of the cyclin B-cdc2 and cyclin A-cdc2 complexes is almost the same, the rapid increase in cyclin A-cdc2 kinase activity ocurrs

only after the completion of GVBD. It is possible that cyclin A-cdc2 kinase may play an important role in steps after GVBD; for example, the kinase may help the rapid activation of cyclin B-cdc2 kinase at meiosis I to II transition or play a part in the maintenance of high cyclin B-cdc2 kinase activity in mature unfertilized oocytes. It is concluded from these results that cyclin B-cdc2 kinase, but not cyclin A-cdc2 kinase, is important for oocyte maturation (especially GVBD).

In the preceding sections, it was demonstrated that in goldfish oocytes *de novo* synthesis of cyclin B protein is required for the activation of MPF (cyclin B-cdc2 complex) during oocyte maturation. The next series of experiments were designed to investigate the mechanism of 17α,20β-DP-induced cyclin B synthesis. It was found that 17α,20β-DP-induced oocyte maturation was inhibited by a protein synthesis inhibitor (cycloheximide), but not by an RNA synthesis inhibitor (actinomycin D). Northern blot analysis showed that cyclin B mRNA is present in both immature and mature oocytes with no significant difference between them. Taken together, these results suggest that the synthesis of cyclin B protein is regulated at the translational level. Since it has long been recognized that the translational activity of maternal mRNAs generally correlates with changes in polyadenylation, I examined the involvement of polyadenylation in cyclin B mRNA translation. Examination of the 3'UTR of goldfish cyclin B mRNA revealed that it posesses a conseved sequence AAUAAA with four copies of cytoplasmic polyadenylation element (CPE, consensus $U_{4-6}A_{1-2}U$) motifs which are the *cis*-acting sequence that specifies cytoplasmic polyadenylation. A PCR poly (A) test revealed that poly (A) elongation occurs in goldfish cyclin B mRNA during oocyte maturation. However, it is noteworthy that this poly (A) addition occurred in oocytes which underwent GVBD, that is, after the appearance of cyclin B protein in oocytes, probably after MPF activation. Thus, it is concluded that cyclin B mRNA polyadenylation is not the major mechanism which is responsible for the initiation of cyclin B mRNA translation in goldfish oocytes.

It has also been suggested that some RNA-binding proteins mediate translational process of stored maternal mRNAs. When poly $(A)^+$ RNA from goldfish immature

oocytes was mixed with the reticulocyte lysate system, cyclin B could be synthesized, suggesting the translational ability of goldfish maternal cyclin B mRNA. Furthrmore, the efficiency of cyclin B translation was much lower with extracts from immature oocytes than with those from mature oocytes. Thus, it is most likely that the initiation of cyclin B synthesis in goldfish oocytes is regulated by a translational inhibitory factor (RNA-binding protein). In this study, a cDNA clone encoding a goldfish Y box protein, which is known to sequester mRNA from translation in *Xenopus* oocytes, was isolated and partially characterized. The identification of the inhibitory factor and clarification of its role should elucidate the translational regulation of cyclin B synthesis during hormone-induced oocyte maturation in goldfish. Thus, the goldfish oocyte will provide a valuable model to gain better understanding of a basic mechanism for translational regulation of gene expression which has currently been accumulating as a primary regulatory mechanism in eukaryotic systems.

Chapter I.

Behavior of the Components of Maturation-Promoting Factor, cdc2 Kinase and Cyclin B, during Oocyte Maturation of Goldfish

INTRODUCTION

Under the influence of maturation-inducing hormone (MIH) secreted from follicle cells, oocyte maturation is finally induced by maturation-promoting factor (MPF), which triggers all the changes that accompany oocyte maturation, such as germinal vesicle breakdown (GVBD), chromosome condensation, and spindle formation (reviewed by Nagahama, 1987a,b; Nagahama and Yamashita 1989). MPF has been purified from mature oocytes of *Xenopus* (Lohka *et al.*, 1988), starfish (Labbé *et al.*,1989a,b), and carp (Yamashita *et al.*, 1992a), and consists of two components; one is a homolog of the $cdc2^+$ gene product of fission yeast, referred to as cdc2 kinase, and the other is cyclin B (see review by Nurse, 1990; Maller, 1991; Jacobs, 1992). The association of cdc2 kinase with cyclin B is necessary to turn on its protein kinase activity (Desai *et al.*,1992; Solomon *et al.*, 1990; Solomon *et al.*, 1992), and the destruction of cyclin B just before the onset of anaphase turns off the kinase activity (Luca and Ruderman, 1989; Murray *et al.*, 1989; Luca *et al.*, 1991).

In contrast to mammals and starfish, the activation of MPF in immature frog and fish oocytes requires *de novo* protein synthesis during the first few hours after MIH treatment (Ford, 1985; Maller, 1985; Goetz, 1983). The proteins that are synthesized at the initial phase of oocyte maturation and induce MPF activation are called initiators, but their chemical natures have not yet been understood. The finding that microinjection of cyclin mRNA can induce *Xenopus* oocyte maturation (Swenson *et al.*, 1986; Pines and Hunt, 1987) suggested that one of the initiators is cyclin. However, cyclin B is present in immature *Xenopus* oocytes as a component of inactive MPF (pre-MPF; Gerhart *et al.*, 1984; Cyert and Kirschner 1988; Gautier and Maller 1991; Kobayashi *et al.*; 1991) and that new cyclin synthesis is not required for inducing oocyte maturation (Minshull *et al.*, 1991). Therefore, proteins other than cyclin should be synthesized during *Xenopus* oocyte maturation. One of the most likely initiators is c-mos, since its synthesis is required and is sufficient for inducing *Xenopus* oocyte maturation (Sagata *et al.*, 1988; 1989; Yew *et al.*, 1992).

To date, detailed examinations of the proteins responsible for inducing oocyte maturation, such as cdc2 kinase, cyclin B, and c-mos during oocyte maturation, have been confined to *Xenopus*. Studies using other species could provide further evidence for the control mechanisms of MPF activation. In the present study, I first describe the behavior of the components of MPF, cdc2 kinase and cyclin B, during goldfish oocyte maturation by means of immunoblotting using monoclonal antibodies raised against the C-terminal region of goldfish cdc2 kinase and *Escherichia coli*-produced full length goldfish cyclin B. I also describe changes in cdc2 kinase activity, the cyclin B protein level and cell cycle progression, which were induced in unfertilized mature goldfish oocytes treated with the protein synthesis inhibitor, cycloheximide.

MATERIALS AND METHODS

Animals and Oocytes

Goldfish were purchased commercially and raised at 15°C until use. Full-grown immature oocytes were isolated from ovaries using a pipet, and induced to mature *in vitro* by incubating at room temperature in goldfish Ringer's solution (Kagawa *et al.*, 1984) containing 1 μg/ml 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP), a natural MIH in fish (Nagahama and Adachi, 1985). Maturational processes were assessed by immersing the oocytes in a clearing solution (5% formalin and 4% acetic acid in Ringer; Lessman and Kavumpurath, 1984), facilitating microscopic examination of the occurrence of GVBD.

Preparation of Oocyte Extracts

Oocyte extracts were obtained as described previously (Hirai *et al.*, 1992a). Briefly, oocytes were homogenized with a pestle (Pellet Pestle; Kontes) in 1 μl/oocyte of ice-cold extraction buffer (100 mM β-glycerophosphate, 20 mM HEPES, 15 mM $MgCl_2$, 5 mM EGTA, 100 μM p-amidinophenyl methanesulfonyl fluoride, 3 μg/ml leupeptin, pH 7.5).

The homogenate was centrifuged at 15,000 g for 10 min at 4°C, and the supernatant was frozen in liquid nitrogen and kept at -80°C until use.

Production of Monoclonal Antibodies against cdc2 Kinase and Cyclin B

A peptide (GFC3C, CPYFDDLDKSTLPASNLKI), which corresponds to the C-terminal sequence of goldfish cdc2 cDNA (Kajiura *et al.*, 1993) with an additional cysteine in the N-terminus, was synthesized using the solid-phase method (using the F-moc protocol) on an Applied Biosystems Model 431A peptide synthesizer. The peptide was purified by reverse-phase HPLC and coupled to bovine serum albumin and keyhole limpet hemocyanin through its N-terminal cysteine by N-(ε-maleimidocaproyloxy) succinimide (Dojin). The coupled peptides were injected into 6-week-old female BALB/c mice to obtain monoclonal antibodies, according to the procedures described previously Yamashita *et al.*, 1991). One clone (GFC3C-9) was used to detect cdc2 kinase by immunoblotting. The isotype of this antibody was IgG1 containing κ light chans. This antibody could not precipitate active cdc2 kinase binding to cyclin B.

Anti-cyclin B monoclonal antibodies were raised against *E. coli*-produced full-length goldfish cyclin B. Cyclin B proteins were produced as follows: A cDNA GFCYCB1-1, which encodes full length goldfish cyclin B and contains *Eco*RI adaptors (Amersham) at both ends of the insert (Hirai *et al.*, 1992a), was digested with *Bam*HI and ligated into the *Bam*HI site of the pET3b expression vector (Studier *et al.*, 1990). The predicted amino acid sequence of the new construct includes the first 12-amino acid sequence of the T7 gene 10 protein, MASMTGGQQMGR, followed by DPGTMGNLPK, which is derived from the adaptor and the 5' noncoding region of GFCYCB1-1, and full-length cyclin B. The protein expressed in *E. coli* BL21 (DE3) was purified by SDS-PAGE as described (Hirai *et al.*, 1992a) and injected into mice to produce monoclonal antibodies. Two clones (B111 and B112) were selected for this study. Both antibodies are thought to recognize the first 41 amino acids of goldfish cyclin B (Δ41 cyclin B; Hirai *et al.*, 1992a) did not react to these antibodies in immunoblotting. Immunoprecipitation was preformed using the B112 antibody (the isotype was IgG2a with a κ light chain) that precipitated the

formation of a complex between cyclin B and cdc2 kinase. B111 antibody was used for immunoblotting anti-cyclin B (B112) immunoprecipitates. Since the isotype of the B111 antibody was IgM, anti-IgM as the second antibody coud not reveal the IgG heavy chains of the B112 antibody used for immunoprecipitation, which migrated near cyclin B, and disturbed detailed observations (cf. Fig. 2B).

Precipitation with $p13^{suc1}$ and Anti-cyclin B Antibody

Precipitation of cdc2 kinase and cyclin B with $p13^{suc1}$-Sepharose and anti-cyclin B (B112) antibody, respectively, was performed as described previously (Yamashita *et al.*, 1991). Precipitates from 10 µl extracts derived from 10 oocytes were separated by SDS-PAGE and immunoblotted with monoclonal antibodies against cdc2 kinase and cyclin B, as described previously (Yamashita *et al.*, 1991).

Kinase Assay

The activity of cdc2 kinase was measured with a synthetic peptide (SP-peptide: KKAAKSPKKAKK), which is phosphorylated specifically by cdc2 and its related kinases (Yamashita *et al.*, 1992b). The sample was incubated for 20 min at 30°C in the presence of the following: 100 µM SP-peptide, 500 µM ATP, 1.5 µCi [γ-^{32}P]ATP, 1 mM EGTA, 10 mM $MgCl_2$, 4.5 mM β-mercaptoethanol, 20mM Tris-HCl (pH 8.0). The reaction was stopped with phosphoric acid, and the SP-peptide was adsorbed onto P81 phosphocellulose paper (Whatman). The paper was washed in phosphoric acid, and the radioactivity remaining on the paper was measured by Cherenkov counting.

Microinjection of Cyclin B protein into Goldfish Oocytes

Fully grown immature oocytes were injected with 20 nl of various concentrations of full-length cyclin B produced in *E. coli*. The injected oocytes were cultured in goldfish Ringer's for 8 hr in the absence or presence of cycloheximide (10 µg/ml) and then fixed in a clearing solution to examine the occurrence of GVBD.

Cytological Examination

After fixation of oocytes in Bouin's solution, the cytoplasm at the animal pole was skinned from the underlying yolk layer with a fine razor blade under a dissecting microscope. The cytoplasm was embedded in paraffin and the serial sections were stained with Delafield's hematoxylin and eosin to determine the cell cycle progression by means of chromosome and spindle morphology.

RESULTS

Characterization of Antibodies Against cdc2 Kinase and Cyclin B

The specificity of the antibodies was examined by immunoblotting highly purified carp MPF (Yamashita *et al.*, 1992a). The purified MPF contained four proteins, with apparent molecular masses of 33-, 34-, 46-, and 48-kDa (Fig. 1, lane 1). Both the 33- and 34-kDa proteins were recognized by anti-PSTAIR antibody (Fig. 1, lane 2), indicating that they are the cyclin-dependent kinases (Meyerson *et al.*, 1992). A monoclonal antibody against the C-terminal region of goldfish cdc2 kinase reacted with the 34-, but not the 33-kDa protein (Fig. 1 lane 3). The latter was recognized with monoclonal antibody against the C-terminal sequence of goldfish cdk2 kinase (Fig. 1 lane 4, see also Hirai *et al.*, 1992b). Thus, the anti-cdc2 kinase antibody does not react with cdk2 and seems to be specific for cdc2 kinase.

The 46- and 48-kDa proteins, which have been already identified as cyclin B using monoclonal antibodies against N-terminal truncated goldfish cyclin B (Yamashita *et al.*, 1992a), were also recognized by anti-full length goldfish cyclin B antibodies (Fig. 1, lane 5).

Absence of Cyclin B in Immature Goldfish Oocytes

It has been reported that in *Xenopus* (Gautier and Maler, 1991; Kobayashi *et al.*, 1991), starfish (Strausfeld *et al.*, 1991) and clam (Westendorf *et al.*, 1989), immature

oocytes contain sufficient cyclin B to induce oocyte maturation. In contrast, Hirai *et al.* (1992a) previously reported that immature goldfish oocytes contained no cyclin B detectable by immunoblotting. However, because of the high protein concentration, only a small amount of the sample (corresponding to the extract from 5/8 oocyte) could be analyzed previously (Hirai *et al.*, 1992a). The new method using two species of antibodies, in which cyclin B was first precipitated with one antibody (IgG) then immunoblotted with another (IgM), increased the detection sensitivity of cyclin B (see MATERIALS AND METHODS). I reexamined cyclin B in immature goldfish oocytes using this procedure. However, cyclin B was not detected in immature oocyte extracts, whereas it was in mature oocyte extracts (Fig. 2A, lane 1).

Cyclin B is stored in immature clam oocytes as an insoluble form, which is solubilized during oocyte maturation (Westendorf *et al.*, 1989). To investigate whether immature goldfish oocytes also contain insoluble cyclin B, I extracted immature oocytes by sonication in a buffer containing detergents or a high concentration of salt. However, cyclin B was not detected in the extracts (data no shown), suggesting that immature goldfish oocytes have no stores of cyclin B.

Extracts derived from 10 immature oocytes were analyzed as described, and the amount of cyclin B detected by immunoblotting was 50 pg. Therefore, even if stocks are present in immature oocytes, the cyclin B content in one immature oocyte should be less than 5 pg (the concentration in the oocyte is 10 ng/ml when the oocyte diameter is estimated to be 1 mm). On the other hand, the cyclin B level in one mature oocyte was 1 ng (the concentration is 2 μg/ml). Therefore, cyclin B should increase more than 200 times during oocyte maturation.

Cyclin B in mature goldfish oocytes migrated as two distinct bands on SDS-PAGE with apparent molecular mass of 46- and 48-kDa, as found in purified carp MPF (Fig. 1). The two bands were equally recognized by 5 species of monoclonal antibodies raised against *E. coli*-produced goldfish cyclin B (data not shown), indicating that they are the same molecule with different chemical modifications or are highly homologous proteins. Since the two bands were equally labeled with ^{32}P (Yamashita *et al.*, 1992a) and

phosphatase digestion did not promote conversion of the two bands into one (data not shown), it is unlikely that the two bands are differently phosphorylated forms of the same molecule. Although we can not exclude the possibility that the two bands have different functions (see also DISCUSSION), I do not distinguish between them in this study.

Appearance of 34-kDa Active cdc2 Kinase in Mature Oocytes

In a previous study using immunoblotting with anti-PSTAIR monoclonal antibody, Hirai *et al.* (1992a) were unable to find any remarkable changes in cdc2 kinase during oocyte maturation. However, it has been revealed that the anti-PSTAIR antibody also reacts to cdc2-related proteins including cdk2 kinase, which has the same electrophoretic mobility as cdc2 kinase (Hirai *et al.*, 1992b). Thus, it is plausible that changes in cdc2 kinase during oocyte maturation were masked behind the cdk2 kinase also visualized by the PSTAIR antibody. I investigated changes in cdc2 kinase more definitively during oocyte maturation, using the new monoclonal antibody specific to cdc2 kinase (Fig. 1). Immature oocytes contained a 35-kDa cdc2 kinase, whereas mature oocytes contained a 34-kDa cdc2 kinase, in addition to the 35-kDa form (Fig. 2B, lane 2). As described, the purified MPF contained the 34-, but not the 35-kDa cdc2 kinase, which was eliminated at the first step of the purification with a Q-Sepharose Fast-Flow anion exchange column (Fig. 1, see also Yamashita *et al.*, 1992a). In addition, anti-cyclin B immunoprecipitates from mature oocyte extracts, which contained only the 34-kDa cdc2 kinase (Fig. 2B, lane 1), had high kinase activity (data not shown), whereas anti-PSTAIR immunoprecipitates from mature oocyte extracts, which contained mainly the 35-kDa cdc2 kinase, had no kinase activity (Yamashita *et al*, 1991). Therefore, it is concluded that the 35- and 34-kDa cdc2 kinases are the inactive and active forms, respectively.

Association of Cyclin B with cdc2 Kinase

The finding that immature goldfish oocytes contain an inactive cdc2 kinase but no cyclin B (Fig. 2) shows that in goldfish the association of inactive cdc2 kinase and cyclin B might be a step regulating MPF activation, in contrast to *Xenopus* and starfish, in

which the cdc2 kinase-cyclin B complex is already present in immature oocytes (Gautier and Maller, 1991; Kobayashi *et al.*, 1991; Strausfeld *et al.*, 1991). I examined the complex formation of cdc2 kinase and cyclin B during goldfish oocyte maturation by precipitation with $p13^{suc1}$ and anti-cyclin B antibody. Cyclin B was found in the $p13^{suc1}$ precipitates from mature, but not from immature oocytes (Figs. 2A and B, lane 2), indicating the complex formation of cdc2 kinase and cyclin B in mature oocytes. Anti-cyclin B immunoprecipitates from mature oocyte extracts contained the 34-kDa active, but not the 35-kDa inactive form (Fig. 2B, lane 1). This result demonstrates that the cdc2 kinase which binds to cyclin B in mature oocytes is mainly the 34-kDa active form.

Changes in cdc2 Kinase and Cyclin B Protein Levels During Oocyte Maturation

To further investigate cdc2 kinase and cyclin B protein levels during oocyte maturation, oocyte extracts at various times after the addition of 17α,20β-DP were precipitated with either $p13^{suc1}$ or anti-cyclin B antibody, and immunoblotted with anti-cdc2 kinase and anti-cyclin B antibody. As described above, immature oocytes contained the 35-kDa inactive cdc2 kinase but no cyclin B, and mature oocytes contained both the 35-kDa inactive and the 34-kDa active cdc2 kinases, and cyclin B (Figs. 3A and C). The appearance of the 34-kDa active cdc2 kinase coincided with the appearance of cyclin B just before GVBD (Figs. 3A and C). Anti-cyclin B immunoblots of the $p13^{suc1}$ precipitates (Fig. 3B) and anti-cdc2 kinase immunoblots of anti-cyclin B immunoprecipites (Fig. 3D) showed that the binding of cdc2 kinase and cyclin B coincided the appearance of cyclin B and the 34-kDa active cdc2 kinase.

Hirai *et al.* (1992a) previously showed that the cyclin B which appeared during oocyte maturation was labeled with ^{35}S-methionine, demonstrating *de novo* synthesis during oocyte maturation. On the other hand, anti-cyclin B immunoprecipitates from mature oocyte extracts sometimes contained the 35-kDa inactive cdc2 kinase (Hirai *et al.*, 1992a), and the 35-kDa cdc2 kinase, as well as the 34-kDa form, can bind to cyclin B in a cell-free system (Yamashita *et al.*, unpublished). Therefore, it is most likely that the 35-

kDa inactive cdc2 kinase binds to *de novo* synthesized cyclin B at first, then is rapidly converted into the 34-kDa active form.

Induction of MPF Activation by Cyclin B Protein

These results indicate that the appearance of cyclin B is required and is sufficient for inducing oocyte maturation in goldfish. To confirm that the appearance of cyclin B is sufficient for inducing oocyte maturation, purified *E. coli*-produced full length goldfish cyclin B protein was injected into immature oocytes. Even under condition of inhibited protein synthesis, injected cyclin B induced oocyte maturation within 1hr after injection, in a dose-dependent manner (Fig. 4). Injection of 1 ng of cyclin B fully induced GVBD in the recipient oocytes. The concentration of cyclin B within the injected oocyte was estimated to be 2 μg/ml. This is about equal to the cyclin B concentration in mature oocytes, as described above. Introducing of cyclin B protein into immature oocyte extracts also induced MPF activation (data not shown, Katsu *et al.*, 1993). The threshold concentration of cyclin B for inducing the activation was around 2 μg/ml, being equivalent to that for inducing oocyte maturation by injection. These results demonstrated that the presence of 2 μg/ml cyclin B, corresponding to the concentration in mature oocytes, is sufficient for inducing oocyte maturation.

MPF Activity in Cycloheximide-Treated Mature Oocytes

Both in *Xenopus* and fish, mature oocytes are arrested at the second meiotic metaphase until fertilized (cf., Fig. 6A). When inseminated or artificially activated, the kinase activity maintained at a high level during arrest is precipitated, accompaning cyclin B destruction (Minshull *et al.*, 1989; Murray and Kirschner, 1989; Murray *et al.*, 1989; Hirai *et al.*, 1992a; Yamashita *et al.*, 1992b). Unfertilized mature *Xenopus* oocytes can maintain a high MPF level, even if protein synthesis is inhibited (Gerhart *et al.*, 1984). I examined whether unfertilized mature goldfish oocytes can resist protein synthesis inhibition. When protein synthesis was inhibited by 20 μg/ml cycloheximide, the kinase activity fell to 50%~70%, according to the batch, of the original activity within 30 min

after the exposure (Fig. 5A). The control mature oocytes without cycloheximide maintained high kinase activity level under the same culture conditions (data not shown). In accordance with the drop in kinase activity, the cyclin B protein level also decreased in the cycloheximide-treated oocytes, but did not disappear completely (Fig. 5B). After the sudden drop of the kinase activity and cyclin B protein level within 30 min after the treatment, the remaining activity and cyclin B were maintained even after a prolonged incubation of the oocytes with cycloheximide (Fig. 5B). When mature goldfish oocytes are activated, the kinase activity decreases to 1/10 of the initial value (Yamashita *et al.*, 1992b), and the cyclin B level drops below the detection limit of immunoblotting (Hirai *et al.*, 1992a). Thus, the decreases in the kinase activity and the cyclin B level induced by cycloheximide-treament is partial, as compared with that occurring upon normal activation. Cytological examination of the cycloheximide-treated mature oocytes showed that the cell cycle was liberated from the arrest at the second meiotic metaphase within 30 min and proceeded to the second meiotic anaphase, where it was arrested again (Figs. 6B and C). Therefore, the cell cycle progression is also partial in the cycloheximide-treated oocytes, as compared with normally activated mature oocytes, in which the cell cycle proceeds to interphase (Yamashita *et al.*, 1990). These results show that cdc2 kinase activity, cyclin B protein level, and cell cycle progression are closely linked. Furthermore, it is suggested that the mechanisms of maintaining MPF activity in unfertilized mature goldfish oocytes differ from those in mature *Xenopus* oocytes, in addition to a difference in the mechanisms of MPF activation.

DISCUSSION

MPF consists of cdc2 kinase and cyclin B (Dunphy *et al.*, 1988; Gautier *et al.*, 1988, 1990; Labbé *et al.*, 1989a,b; Yamashita *et al.*, 1992a). To understand how MPF is activated, I examined the time course of cdc2 kinase and cyclin B protein levels during goldfish oocyte maturation, using monoclonal antibodies specific to goldfish cdc2 kinase and cyclin B (Figs. 1-3). The following results were obtained. Cyclin B is absent in immature oocytes, and, probably by means of *de novo* protein synthesis, it appears just before GVBD. Immature oocytes contain only the 35-kDa inactive cdc2 kinase, but in accordance with the appearance of cyclin B, the 34-kDa active cdc2 kinase forming a complex with cyclin B (MPF) appears in maturing oocytes. These results indicate that cyclin B synthesized during oocyte maturation forms a complex with preexisting 35-kDa inactive cdc2 kinase and activates it, which is accompanied with a shift in the electrophoretic mobility of cdc2 kinase from 35- to 34-kDa. In this case, MPF activation should be controlled at the level of cyclin B accumulation. These mechanisms of MPF activation in fish apparently differ from those in *Xenopus* (Gautier and Maller, 1991; Kobayashi *et al.*, 1991) and starfish (Strausfeld *et al.*, 1991), in which cyclin B is present in immature oocytes and forms a complex with cdc2 kinase (pre-MPF).

The cdc2 kinase is activated by post-translational modifications including phosphorylation and dephosphorylation after binding to cyclin (see review by Jacobs, 1992). Therefore, it is plausible that cdc2 kinase undergoes chemical modifications in maturing fish oocytes after binding to cyclin B, although the complex formation of cyclin B and cdc2 kinase during maturation is a critical step for activating cdc2 kinase in these oocytes. Actually, this has shown that the activation of cdc2 kinase is accompanied with a shift in its electrophoretic mobility from 35- to 34-kDa (Figs. 2 and 3). Yamashita *et al.* (1992a) previously showed that the 34-kDa cdc2 kinase, but not the 35-kDa form, was labeled on threonine residue(s) when oocytes matured in the presence of ^{32}P. Thus, it is likely that threonine phosphorylation of cdc2 kinase, which may cause the electrophoretic mobility shift, is involved in the activation of cdc2 kinase. Indeed, recent studies using

mutant cdc2 kinases have demonstrated that in addition to dephosphorylation of Thr14 and Tyr15, phosphorylation of Thr161 is required for cdc2 activation (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Solomon *et al.*, 1992).

In contrast to *Xenopus* (Gautier and Maller 1991; Kobayashi *et al.*, 1991), goldfish cyclin B is not stockpiled in immature oocytes (this study), and it is abruptly synthesized during oocyte maturation (Hirai *et al.*, 1992a). Thus, cyclin B is still a potential initiator of goldfish oocyte maturation. If so, synthesis of cyclin B is required and is sufficient for inducing oocyte maturation in goldfish. In fact, Cyclin B is sufficent for inducing goldfish oocyte maturation, as demonstrated by inducing MPF activation by cyclin B proteins both *in vivo* (Fig. 4) and *in vitro* (Katsu *et al.*, 1993). To confirm the requirement of cyclin B during goldfish oocyte maturation, I investigated the effects of antisense oligonucleotides on 17α,20β-DP-induced oocyte maturation. So far, however, I have been unable to obtain an inhibitory effect that is significantly different between sense and antisense oligonucleotide injections. Therefore, it is premature to say that cyclin B is an initiator of goldfish oocyte maturation.

In unfertilized mature *Xenopus* oocytes, MPF activity is maintained at a high level, even if protein synthesis is inhibited by cycloheximide (Gerhart *et al.*, 1984). The stability of MPF activity depends on a cytostatic factor (CSF; Gerhart *et al.*, 1984; Newport and Kirschner 1984; Murray *et al.*, 1989). Since c-mos, a probable component of CSF, is not affected by protein synthesis inhibition (Watanabe *et al.*, 1989), this protein seems to be involved in stabilizing MPF in cycloheximide-treated mature oocytes. Unlike *Xenopus*, the cycloheximide-treated unfertilized mature goldfish oocytes caused a drop in MPF activity to 50~70% of its initial level (Fig. 5) and an exit from metaphase followed by arrest at anaphase (Fig. 6). Release from metaphase-arrest caused by protein synthesis inhibitors has been reported in the mouse (Clarke and Masui, 1983) and mollusk (*Patella*, Loon *et al.*, 1991). However, in those species, the cell cycle is not arrested at anaphase but it reaches interphase. The difference in the sensitivity of MPF to protein synthesis inhibitors indicates the existence of species specific mechanisms that maintain MPF activity in unfertilized mature oocytes. The mechanisms functioning in

Xenopus are independent of protein synthesis, whereas those in the mouse and mollusk are dependent. Mature goldfish oocytes may be equipped with both mechanisms. Since cyclin B continues to be synthesized in unfertilized mature goldfish oocytes (Hirai *et al.*, 1992a), the partial decrease in the cyclin B protein level found in cycloheximide-treated oocytes may be due to an interruption of the continuous supply of cyclin B. The presence of molecular mechanisms of maintaining MPF activity independent of protein synthesis is not certain, but c-mos may contribute to them, as it works in unfertilized mature *Xenopus* oocytes (Watanabe *et al.*, 1989).

It is notable that a 30~50% decrease in the kinase activity and cyclin B protein level induces cell cycle progression to some extent (from metaphase to anaphase). This finding indicates a close correlation between the kinase activity/cyclin B protein level and cell cycle progression. The mechanism that links the kinase activity and the cell cycle progression is not certain, but, as proposed for the cell cycle control in somatic cells (see review by Jacobs, 1992), one possibility is that plural cyclins are involved at each step of the cell cycle. For example, although I was unable to detect any difference between cyclin B that disappeared after the cycloheximide-treatment and cyclin B that remained even thereafter (Fig. 5), there may be at least two cyclin Bs, both of which are equally recognized by the anti-cyclin B antibodies used in this study. One is sensitive to protein synthesis inhibition and its destruction may be responsible for inducing an exit from metaphase, and the other is insensitive to the inhibitors and its destruction may induce further progression of the cell cycle towards interphase. Indeed, Hirai *et al.* (1992a) isolated two species of goldfish cyclin B cDNA between which, the homology is more than 95%. These cyclins may have different functions in initiating oocyte maturation and maintaining MPF activity, irrespective of their high homology. Further studies are required to understand the precise roles of each player involved in oocyte maturation, as well as to identify new players.

MPF, consisting of a cdc2 kinase-cyclin B complex, is a universal factor that promotes oocyte maturation (Kishimoto, 1988). As this study shows however, the mechanisms of MPF activation and its stabilization vary among species, in spite of the

involvement of the same players. Despite the impressive progress in recent years, there is still much to be learned about the control of oocyte maturation. Further studies using various species should provide comprehensive understanding of the control mechanisms of oocyte maturation.

FIGURES AND FIGURE LEGENDS

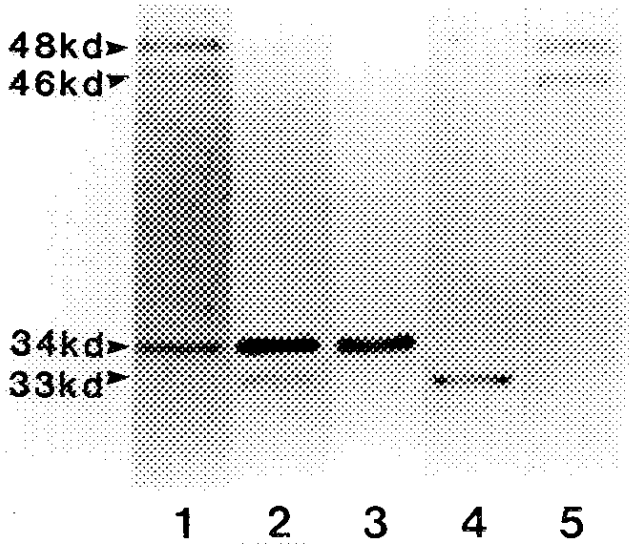

Fig. 1. SDS-PAGE analysis of highly purified MPF from carp eggs. Silver staining (lane 1) and immunoblotting with anti-PSTAIR (lane 2), anti-cdc2 (lane 3), anti-cdk2 (lane 4) and anti-cyclin B (lane 5) antibodies.

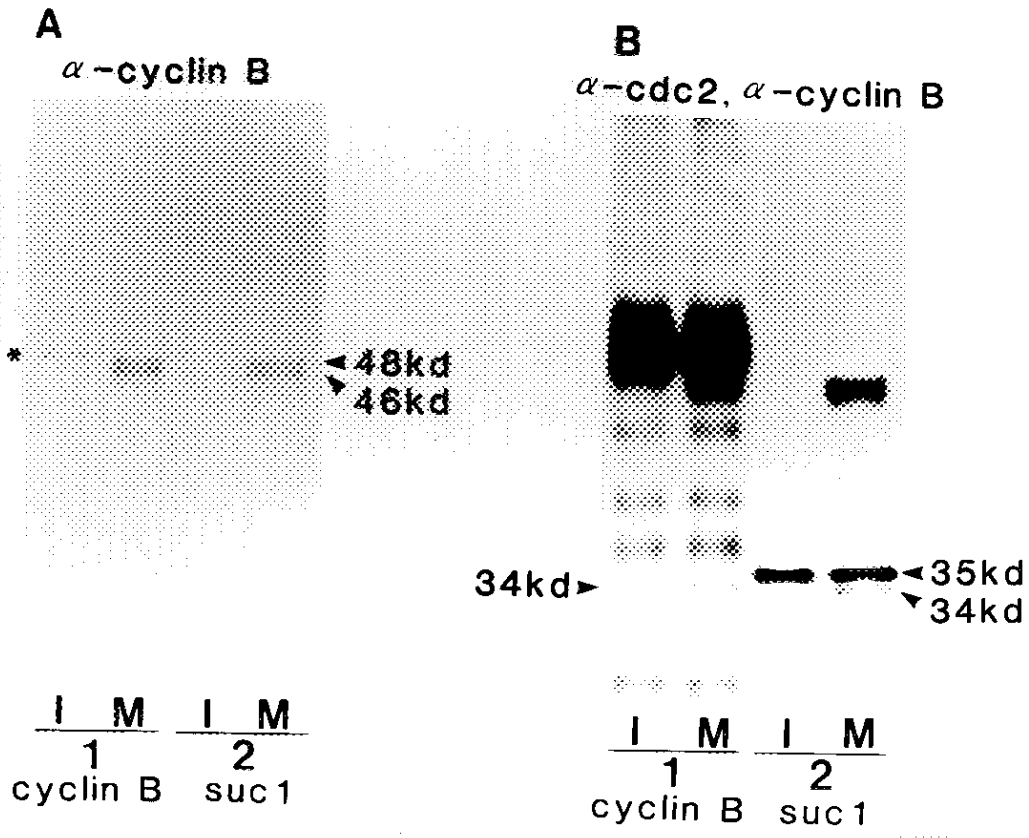

Fig. 2. Cyclin B and cdc2 kinase in goldfish immature (I) and mature (M) oocytes.

Anti-cyclin B (A) and anti-cdc2 antibody and anti-cyclin B (B) immunoblots of anti-cyclin B (lane 1) and $p13^{suc1}$ (lane 2) precipitates. The band with lower electrophoretic mobility than cyclin B, noted by the asterisk, is not cyclin B, but was recognized non-specifically with the second antibody.

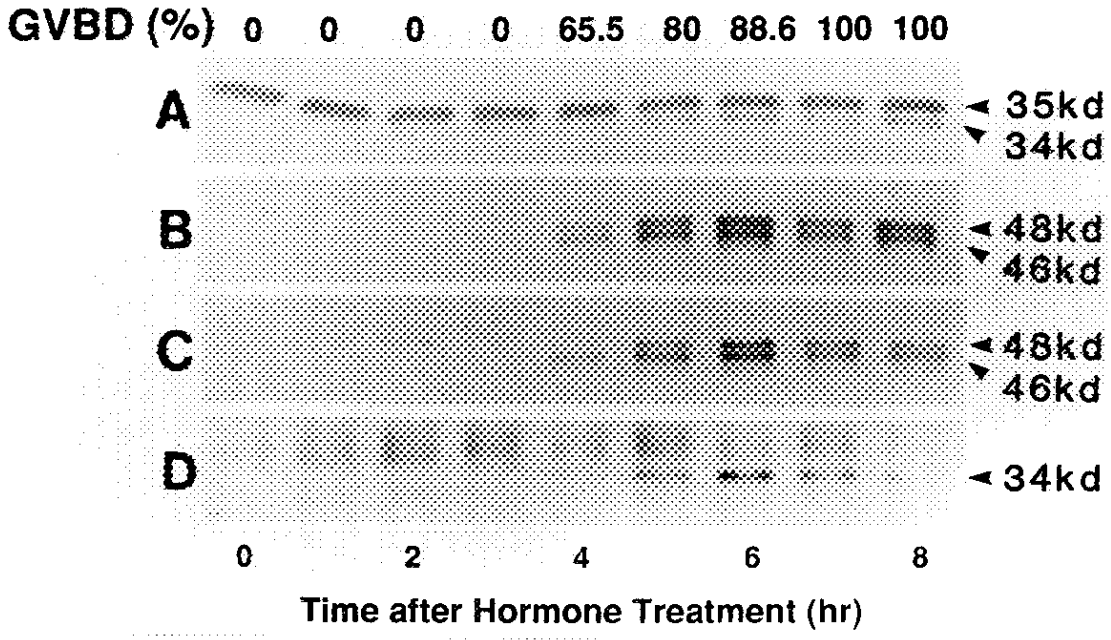

Fig. 3. Changes in cdc2 kinase (A and D) and cyclin B (B and C) protein levels during goldfish oocyte maturation induced by 17α, 20β-DP.

Anti-cdc2 kinase (A and D) and anti-cyclin B (B and C) immunoblots of $p13^{suc1}$ (A and B) and anti-cyclin B (C and D) precipitates.

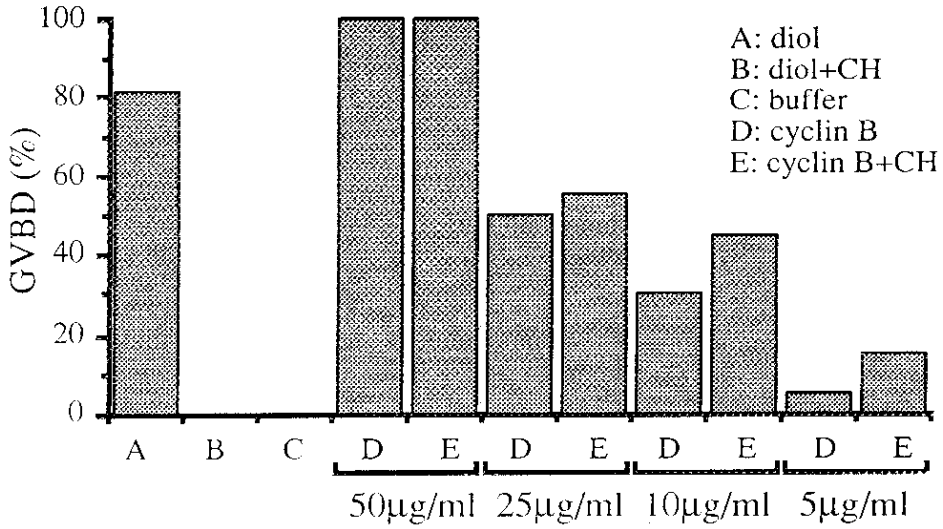

Fig. 4. Induction of oocyte maturation by *E. coli*-produced cyclin B protein in the absence of protein synthesis.

Oocytes were incubated for 8hr with 17α, 20β-DP or after microinjection of indicated amount of cyclin B protein, and were scored for GVBD. A, 17α,20β-DP (diol); B, 17α,20β-DP (diol)+cycloheximide (CH); C, buffer alone (1mM HEPES, pH7.0); D, cyclin B protein; E, cyclin B protein+cycloheximide (CH).

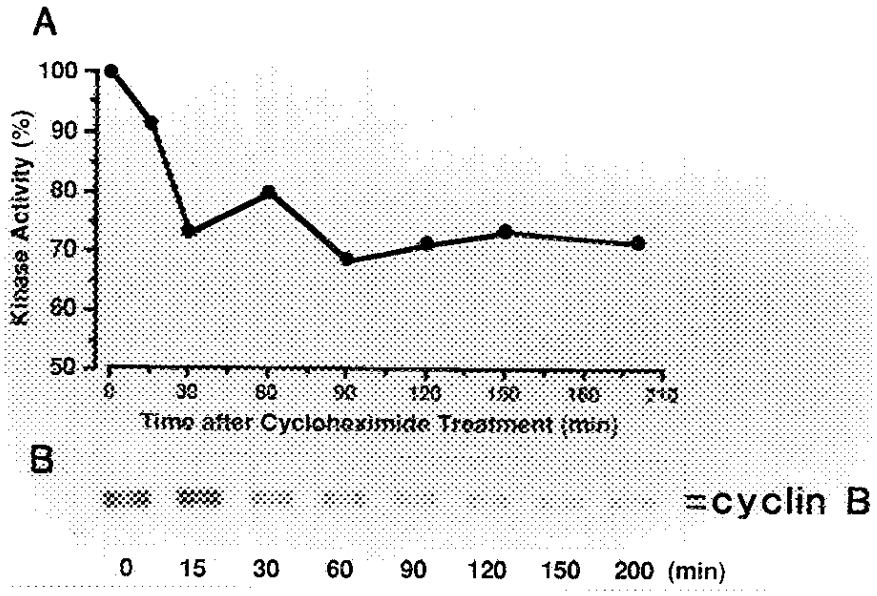

Fig. 5. Decrease in cdc2 kinase activity (A) and cyclin B protein levels (B) in mature oocytes treated with a protein synthesis inhibitor.

Oocytes matured *in vitro* were continuously treated with 20 μg/ml cycloheximide and the kinase activity and cyclin B protein levels were examined at the indicated times.

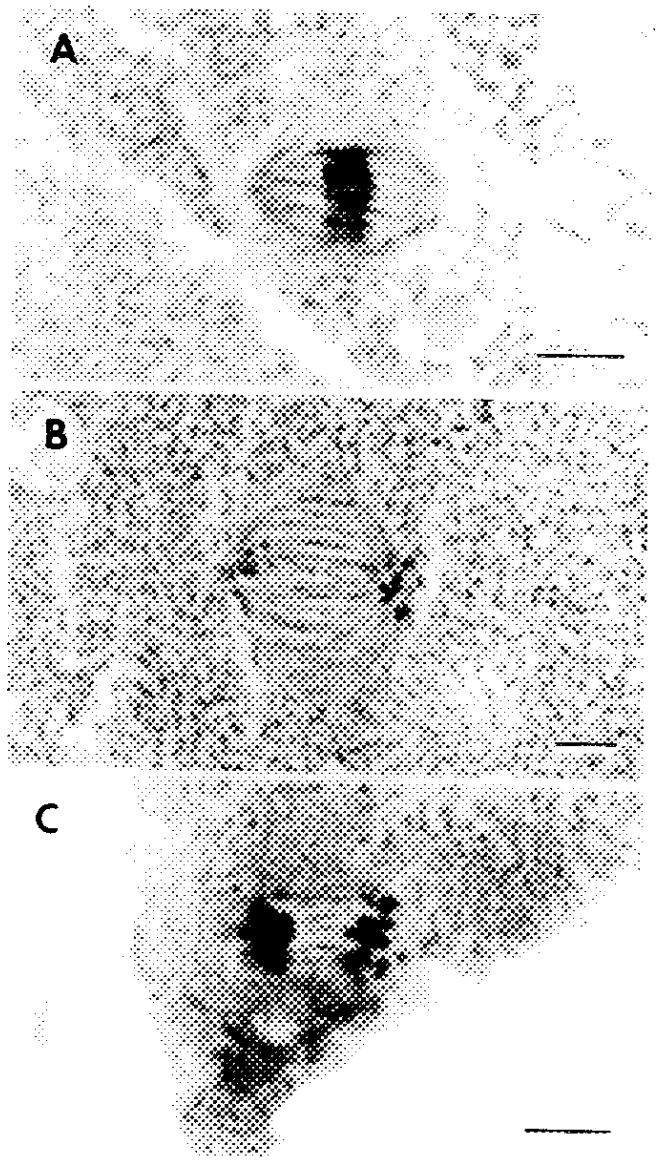

Fig. 6. Cytological examination of mature oocytes treated with a protein synthesis inhibitor.

The cell cycle arrested at metaphase II (A) was released and proceeded to anaphase (B and C). Bar=5 μm. A) untreated, 60 min B) cycloheximide, 20 min C) cycloheximide, 60 min

Chapter II.

Molecular Cloning and Immunological Analysis of Goldfish Cyclin A During Oocyte Maturation

INTRODUCTION

Cyclins are proteins that regulate progression through the eukaryotic cell cycle, and are classified into G1 cyclins or CLNs and mitotic cyclins or CLBs (reviewed by Hunter and Pines, 1991; Pines, 1993; Sherr, 1993). They associate, as regulatory subunits, with members of cyclin-dependent kinase (CDK) family, such as cdc2 and cdk2, and are thought to regulate distinct steps in the cell cycle (Girard *et al.*, 1991; Meyerson *et al.*, 1992; Dulic *et al.*, 1992; Koff *et al.*, 1992; Pagano *et al.*, 1992a; Heuvel and Harlow, 1993 and reviewed by Pines, 1993; Sherr, 1993).

Cyclin A was first discovered in clam embryos and was the first cyclin to be cloned and sequenced (Swenson *et al.*, 1986). The involvement of cyclin A in the G2-M transition has been demonstrated by the entry into metaphase of prophase-arrested *Xenopus* oocytes microinjected with cyclin A mRNA (Swenson *et al.*, 1986). In addition to the involvement in the G2-M transition, cyclin A forms an S phase-specific complex with cdk2, E2F (a cellular transcription factor) and p107 (an Rb-related protein), and plays a role in regulating S phase (Giordano *et al.*, 1989; Pines and Hunter, 1990; Pagano *et al.*, 1992b; Mudryj *et al.*, 1991; Faha *et al.*, 1992; Ewen *et al.*, 1992 Cao *et al.*, 1992; Devodo *et al.*, 1992; Shirodkar *et al.*, 1992 and see review by Nevins, 1992). Besides cyclin A, cyclin B complexed with cdc2 controls the G2-M transition in the cell cycle (see review by Nurse, 1990; Maller, 1991). The functional differences between cyclin A and cyclin B in the G2-M transition have not been clarified. Introduction of either cyclin A or B into interphase-arrested cell extracts induces entry into M phase (Murrey and Kirschner, 1989; Clarke *et al.*, 1992). In addition, both A- and B-type cyclin-cdk complexes have very similar substrate specificities *in vitro*; they phosphorylate histone H1 and H2B on identical residues (Draetta *et al.*, 1989; Minshull *et al.*, 1990; Parker *et al.*, 1991). Although there are many functional similarities, cyclins A and B do not seem to be redundant in the regulation of the cell cycle. For example, the histone H1 kinase activity of cyclin A-dependent kinase increases earlier than cyclin B-dependent kinase, although their activities patterns partially overlap (Minshull *et al.*, 1990; Pines

and Hunter, 1990; Dulic *et al.*, 1992). In mitotic cycles, cyclin A is degraded ahead of cyclin B in several organisms (Westendorf *et al.*, 1989; Minshull *et al.*, 1989).

The functional difference between cyclin A and B during oocyte maturation (meiotic cell cycle) is also uncertain. Maturation-promoting factor (MPF), a key regulator of oocyte maturation, consists of cdc2 kinase and cyclin B, but not of cyclin A (see review by Nurse, 1990; Maller, 1991; Jacobs, 1992). However, the microinjection of cyclin A, as well as cyclin B, into immature oocytes induces maturation (Swenson *et al.*, 1986; Pines and Hunt, 1987; Westendorf *et al.*, 1989). In *Xenopus* oocyte maturation, cyclin A is first detectable at about the time of germinal vesicle breakdown (GVBD) and its protein level decreases slightly when GVBD is completed and increases thereafter. However, *Xenopus* cyclin A binds neither to cdc2 nor cdk2 and it is not associated with histone H1 kinase activity until the egg is activated at fertilization (Kobayashi *et al.*, 1991). Therefore, it is completely uncertain whether or not cyclin A plays any roles in oocyte maturation.

I first describe the molecular cloning of goldfish cyclin A cDNA and immunological characterization of its product. This study also determine the association of cyclin A with cdc2 and cdk2 proteins, and changes in the kinase activity of the cyclin A-cdc2 complex during oocyte maturation. Furthermore, I examined whether cyclin A mRNA can induce GVBD when microinjected into goldfish immature oocytes. The results suggest the role of cyclin A-cdc2 kinase in the later events of oocyte maturation, such as the transition between meiosis I and II and the maintenance of high MPF activity in unfertilized eggs.

MATERIALS AND METHODS

cDNA Cloning of Goldfish Cyclin A

I used two cDNA libraries for isolating goldfish cyclin A cDNA; one is a λgt10 library constructed from ovulated goldfish eggs (Hirai *et al.*, 1992a), another is a λZAPII library

constructed from a full-grown goldfish ovary. The λgt10 library was screened under relaxed hybridization conditions with a full-length *Xenopus* cyclin A cDNA probe (Minshull *et al.*, 1990). Hybridization was performed at 45°C for 16 hr in a buffer containing 5x SSC (1x SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5x Denhardt's regent (1 mg/ml each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS), and 200 μg/ml herring sperm DNA. Membranes were washed in 5x SSC at the same temperature. Fifty clones were isolated, but even the longest clone seemed to be truncated, lacking several amino acids at the N-terminal. Therefore, we screened the λZAPII cDNA library with an oligonucleotide probe corresponding to the 5'-terminal sequence of the truncated cyclin A (Fig. 1), to obtain a clone containing the complete cyclin A coding sequence. Hybridization was carried out at 65°C for 14 hr in the same buffer as described above. Bluescript plasmids containing the cDNA inserts were rescued from the ZAPII phages according to the instructions of the manufacturer (Strategene) and sequenced by dideoxy chair termination sequencing methods (BcaBEST sequencing kit, TAKARA) using double-stranded plasmid DNA as templates.

Animals and Oocytes

Goldfish were purchased commercially and raised at 15°C until use. Immature oocytes were isolated from ovaries using a pipet, and induced to mature *in vitro* by incubating at room temperature in goldfish Ringer's solution (Kagawa *et al.*, 1984) containing 1 μg/ml 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP), a natural maturation-inducing hormone in fish (Nagahama and Adachi, 1985). Maturational processes were assessed by immersing the oocytes in a clearing solution (5% formalin and 4% acetic acid in Ringer; Lessman and Kavumpurath, 1984), which facilitates microscopic examination of the occurrence of GVBD.

Xenopus laevis was obtained from a dealer in Hamamatsu, and maintained in laboratory aquaria at 20°C. Oocytes were obtained by surgically removing a portion of an

ovary and then removing the follicular layers of full-grown oocytes with forceps. All operations on oocytes were conducted in modified Barth's medium (Laskey *et al.*, 1977).

Preparation of Goldfish Oocyte Extracts

Oocyte extracts were obtained as described previously (Hirai *et al.*, 1992a). Briefly, oocytes were homogenized with a pestle (Pellet Pestle; Kontes) in 1 µl/oocyte of ice-cold extraction buffer (100 mM β-glycerophosphate, 20 mM HEPES, 15 mM $MgCl_2$, 5 mM EGTA, 100 µM p-amidinophenyl methanesulfonyl fluoride, 3 µg/ml leupeptin, pH 7.5). The homogenate was centrifuged at 15,000 g for 10 min at 4°C, and the supernatant was used for kinase assay, immunoblotting and immunoprecipitation, as described below.

Bulk extracts were prepared from immature and mature oocytes by crushing them in extraction buffer followed by ultracentrifugation (100,000 g, 1 hr, 4°C), as described previously (Yamashita *et al.*, 1992a). A portion of the extracts was mixed with SDS sample buffer immediately after the extraction and the rest was frozen in liquid nitrogen and stored at -80°C until use in the cell-free system described below.

Production of Goldfish Cyclin A Proteins

Cyclin A truncated by 13 amino acids at the N-terminal, was produced from a cDNA clone (Fig. 1). The cDNA was digested with *Bam*HI and ligated into the *Bam*HI site of the pET3a (Studier *et al.*, 1990). The predicted amino acid sequence of the construct is as follows: MASMTGGQQMGR (the first 12-amino-acid sequence of the T7 gene 10 protein), GSGYHG (a sequence derived from the adapter) (Amersham), and the goldfish cyclin A sequence starting from the 14th amino acid (Fig. 1).

A full-length cyclin A protein was constructed as follows: The cDNA clone encoding the full-length cyclin A (Fig. 1) was amplified by PCR using the oligonucleotide 5'-CGCCATATGGCTTCCCGTGGCTTCGCTCC-3' (5' primer introducing a *Nde*I site) and 5'-CGCGATCCAAAACATCAAGGGAGAGGC-3' (3' primer introducing a *Eco*RV site). The PCR fragment was digested with *Nde*I and *Eco*RV, then subcloned

into pET3a that was cut with *Nde*I and *Eco*RV. The construct expresses the full-length goldfish cyclin A without any additional amino acid residues on its N- and C-terminals.

Both the truncated and full-length goldfish cyclin A proteins were expressed in *Escherichia coli* BL21 (DE3), and the produced cyclin A proteins were electrophoretically purified with a Prep-Cell (Bio-Rad).

Goldfish cyclin A fused with protein A (PA-cyclin A) was constructed by amplifying the full-length cDNA clone by PCR with the oligonucleotide; 5'-CGCGAATTCGATGGCTTCCCGTGGCTTCG-3' (5' primer introducing a *Eco*RI site) and 5'-CGCGAATTCCAAAACATCAAGGGAGAGGC-3' (3' primer introducing a *Eco*RI site). The PCR fragment was digested with *Eco*RI and ligated into *Eco*RI-digested protein A-pET3b vector (a gift from Tim Hunt). The predicted sequence of the fusion site between PA and cyclin A is DDPGNS (sub C-terminal region of PA) followed by the first methionine of goldfish cyclin A. PA-cyclin A protein was translated in the TNT T7 coupled reticulocyte lysate system (Promega).

Antibodies

Anti-goldfish cyclin A monoclonal antibody was raised against the N-terminal truncated proteins, according to the procedure described previously (Yamashita *et al.*, 1991). The monoclonal antibody does not react with goldfish cyclin B. This antibody, however, was unable to precipitate cyclin A from oocyte extracts (data not shown). Therefore, immunoprecipitation was performed using an anti-cyclin A polyclonal antibody (antiserum) raised against the full-length cyclin A protein. Anti-cdc2 antibody, anti-cdk2 antibody and anti-cyclin B antibody were described elsewhere (Kajiura *et al.*, 1993; Hirai *et al.*, 1992b; Katsu *et al.*, 1993).

Immunoprecipitation

Oocyte extracts (50 µl) were mixed with 20 µl of protein A-Sepharose beads (Pharmacia) and incubated for 2 hr at 4°C. After centrifugation at 3,000 g for 1 min, the supernatant was mixed with 1 µl of either ascites fluid containing anti-cyclin B

monoclonal antibody or anti-cyclin A serum. After incubation for 5 hr at 4°C, 20 µl of protein A-Sepharose beads was added and incubated overnight at 4°C under continuous agitation. After washing in extraction buffer containing 0.2% Tween 20, the beads were divided into two; one portion was used to measure kinase activity associated with the beads and the other was mixed with 2x SDS sample buffer and boiled for 2 min, then proteins associated with the beads were analyzed by immunoblotting. Anti-cyclin B immunoblotting of anti-cyclin A immunoprecipitates has revealed that cyclin B is not coprecipitated with anti-cyclin A antibody. Similarly, cyclin A was not precipitated with anti-cyclin B antibody.

Kinase Assay

The kinase activity of immunocomplexes was measured using a synthetic peptide (SP-peptide: KKAAKSPKKAKK), which is phosphorylated specifically by cdc2 and its related kinases (Yamashita *et al.*, 1992b). Samples were incubated for 20 min at 30°C in the presence of the following: 100 µM SP-peptide, 500 µM ATP, 1.5 µCi [γ-^{32}P]ATP, 1 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 20 mM Tris-HCl (pH 8.0). The reaction was stopped with phosphoric acid, and the SP-peptide was adsorbed onto P81 phosphocellulose paper (Whatman). After washing in phosphoric acid the radioactivity remaining on the paper was counted by liquid scintillation.

Activation of Cdc2 Kinase by Cyclin A in a Cell-Free System

Immature goldfish oocyte extracts (100,000 g supernatant) were thawed and centrifuged at 15,000 g for 10 min at 4°C. The supernatant (75 µl) was mixed with 25 µl of *in vitro* translated PA-cyclin A protein. The mixture was incubated with 1 mM ATP at 25°C. One hour later, 30 µl IgG-Sepharose beads (Pharmacia) were added, and the incubation was continued for 2 hr at 4°C. After washing with extraction buffer containing 0.2% Tween 20, the kinase activity was measured and proteins were analyzed by immunoblotting.

In Vitro RNA Synthesis

To synthesize cyclin mRNA *in vitro*, the pBluescript vector containing the cyclin A cDNA and the pET3b vector containing cyclin B cDNA (Hirai *et al.*, 1992) were digested with either *Eco*RV (for sense mRNA) or *Sma*I (for antisense mRNA), and capped mRNA was synthesized by T3 RNA polymerase (for sense mRNA) or T7 RNA polymerase (for antisense cyclin A mRNA and sense cyclin B mRNA) using a mCAP mRNA capping kit (Stratagene).

Assay for Meiosis-Reinitiation Activity

In vitro synthesized mRNA (1 mg/ml), was microinjected into immature oocytes (50 nl for *Xenopus* and 20 nl goldfish). In *Xenopus*, GVBD was detected by a white spot appearing at the animal pole within 6 hr after the injection. The presence or absence of the germinal vesicle was verified by dissection of the oocytes after fixation with 10% trichloroacetic acid. In goldfish, the injected oocytes were incubated in goldfish Ringer's solution for 8 hr and then fixed in a clearing solution to examine the occurrence of GVBD.

RESULTS

Molecular Cloning of Goldfish Cyclin A cDNA

I screened a goldfish egg cDNA library with *Xenopus* cyclin A cDNA. Of $1x10^5$ plaques screened, 50 were positive and the longest clone was sequenced. This clone had an insert of 1486 bp (Fig. 1), and the predicted amino acid sequence exhibited significant homology with A-type cyclins of other species, although the entire coding region was not covered. To isolate clones including the entire coding region, I screened another goldfish cDNA library constructed from full-grown ovary with the 5'- fragment of the truncated cDNA (Fig. 1, underlined). Twenty-seven positive plaques were isolated from 50,000, and the longest clone was sequenced. Although the longest cDNA was about 350 bases shorter than the corresponding mRNA that was estimated at 1.9 kb by Northern blotting

(data not shown), the sequence contained an open reading frame of 391 amino acids, starting with an AUG codon in accordance with Kozak's rules (Kozak, 1986). The sequence contained a stop codon in the same reading frame upstream of this AUG, suggesting that this is the authentic translation starting point. The predicted molecular weight and pI of the cDNA product were 43,594 and 5.1, respectively.

The deduced amino acid sequence of the isolated cDNA resembled cyclin A, rather than cyclin B (Fig. 2). When the cyclin box were compared, the identity with *Xenopus* cyclin A was 85% (Minshull *et al.*, 1990) and that with human cyclin A, 81% (Wang *et al.*, 1990). The identity was 49% with goldfish cyclin B1 (Hirai *et al.*, 1992a) and 50% with *Xenopus* cyclin B1 (Minshull *et al.*, 1989). Therefore, I referred to the isolated clone as goldfish cyclin A cDNA.

Cyclin A Protein Is Present in Both Immature and Mature Goldfish Oocytes

To examine the behavior of cyclin A protein in goldfish oocytes, I raised monoclonal antibody against bacterially-produced goldfish cyclin A. This antibody does not react with *E. coli*-produced goldfish cyclin B or anti-cyclin B reactive 46-48 kDa proteins in highly purified carp MPF (data not shown; see Yamashita *et al.*, 1992a), indicating that the anti-cyclin A antibodies do not react with cyclin B. This monoclonal antibody recognized a single 47 kDa band in both immature prophase- and mature metaphase II-arrested oocytes by immunoblotting (Fig. 3A). The 47 kDa band disappeared when the eggs were activated by insemination (data not shown). Based on the disappearance upon egg activation and the approximate correspondence between the molecular weight of the anti-cyclin A reactive protein and that of goldfish cyclin A deduced from its cDNA, I concluded that the 47 kDa protein found in both immature and mature oocytes is goldfish cyclin A.

I then examined changes in cyclin A protein levels during goldfish oocyte maturation induced *in vitro* by 17α,20β-DP (Fig. 3B), and found that there were no remarkable changes. In this experiment however, I used the 100,000 g supernatant of crushed oocytes for immunoblotting. Since cyclin A has been reported to be associated with

chromatin (Maldonado-Codina and Glover, 1992), I cannot exclude the possibility that cyclin A was precipitated by the ultracentrifugation.

Anti-Cyclin A Immunoprecipitates from Mature Goldfish Oocyte Extracts Contain Active Cdc2 Kinase

Immunoblotting revealed the presence of cyclin A in both immature and mature goldfish oocytes. I then examined whether cyclin A confers kinase activity in goldfish oocytes. Anti-cyclin A immunoprecipitates from mature goldfish oocyte extracts had kinase activity, but those from immature oocyte extracts did not (Fig. 4A). I further examined whether goldfish cyclin A was interacted with cdc2 and/or cdk2 during oocyte maturation. The anti-cyclin A immunoprecipitates from mature oocyte extracts contained an anti-cdc2 reactive 34 kDa protein, but those from immature oocyte extracts did not (Fig. 4B). Anti-cdk2 reactive proteins were not detected in either immunoprecipitate (data not shown). I showed that the anti-cdc2 reactive 34 kDa protein is active cdc2 (Katsu *et al.*, 1993; Kajiura *et al.*, 1993). Therefore, these results suggest that the kinase activity associated with anti-cyclin A immunoprecipitates from mature oocyte extracts is responsible for cdc2.

Activation of Cdc2 Kinase by Cyclin A in a Cell-Free System

Immunological studies showed that cyclin A binds to cdc2 and probably activates it during goldfish oocyte maturation. To confirm this, experiments were designed to examine whether recombinant cyclin A can bind to and activate cdc2 in a cell-free system. I used PA-cyclin A produced in the reticulocyte lysate system and immature goldfish oocyte extracts, in which all cdc2 is inactive and monomeric (Kajiura *et al.*, 1993). PA-cyclin A was added to the extracts, and after an appropriate incubation, it was recovered with IgG-Sepharose beads. The kinase activity associated with the beads was apparently higher than that in controls (Fig. 5A). Protein A alone did not confer kinase activity (data not shown). The catalytic partner of PA-cyclin A was examined by immunoblotting the proteins associated with the IgG-Sepharose beads with anti-cdc2 and anti-cdk2

antibodies. The IgG-Sepharose beads contained a 34 kDa active cdc2, but not cdk2 (Fig. 5B). These results indicate that cyclin A binds to and activates cdc2 in a cell-free system, as it does *in vivo* (Fig. 4B).

Changes in Cyclin A-Cdc2 and Cyclin B-Cdc2 Kinase Activities During Oocyte Maturation

I demonstrated that goldfish cyclin A binds to and activates cdc2. To determine the changes in cyclin A-cdc2 kinase activity during oocyte maturation, I prepared oocyte extracts at various stages of oocyte maturation induced by 17α,20β-DP, immunoprecipitated them with anti-cyclin A serum and measured the kinase activity associated with the anti-cyclin A immunoprecipitates (Fig. 6). Cyclin A-cdc2 kinase activity was undetectable in immature oocytes, and appeared at about the time of GVBD. The level increased slightly until GVBD was completed, then increased drastically after completion of the first meiotic division.

Next, I compared cyclin A- and cyclin B-cdc2 kinase activities, by measuring the kinase activity in anti-cyclin B immunoprecipitates. Cyclin B-cdc2 kinase activity appeared just before GVBD and increased gradually during oocyte maturation (Fig. 6), in agreement with the previous observation (Katsu *et al.*, 1993). On the other hand, the level of cyclin A-cdc2 kinase activity was about 10-times lower than that of cyclin B-cdc2 and its activation occurred more abruptly at the later phase of oocyte maturation (Fig. 6).

Synthetic Cyclin A mRNA can not Induce GVBD of Goldfish Oocytes

Goldfish cyclin A mRNA was transcribed *in vitro*. When microinjected into *Xenopus* immature oocytes, the goldfish synthetic sense cyclin A mRNA caused GVBD, but the antisense mRNA did not (Table 1). The results are consistent with those of previous studies showing that cyclin A drives frog oocytes into meiosis (Swenson *et al.*, 1986; Pines and Hunt, 1987; Westendorf *et al.*, 1989).

I then examined whether goldfish cyclin A mRNA can induce GVBD in goldfish oocytes. Cyclin A mRNA did not cause GVBD in goldfish oocytes, whereas cyclin B

mRNA did cause GVBD (Table 1). Cyclin A mRNA microinjection increased H1 kinase activity in oocyte extracts (Fig. 7). These results suggest that in contrast to cyclin B-cdc2 kinase, cyclin A-cdc2 kinase can not induce GVBD in goldfish oocytes, in spite of the activation of the kinase.

DISCUSSION

To study the behavior of cyclin A during goldfish oocyte maturation, I cloned goldfish cyclin A cDNA and produced antibodies against its product. I found that goldfish cyclin A protein is present in both immature and mature oocytes, and remains at a steady level during oocyte maturation; cyclin A binds to cdc2, but not to cdk2, and activates it during oocyte maturation; and that the kinase activity of the cyclin A-cdc2 complex starts to increase just before GVBD like cyclin B-cdc2, but it increases only slightly until GVBD is completed. A rapid increase occurs after the completion of GVBD.

I showed that goldfish cyclin B is absent in immature oocytes and that it appears in concert with the beginning of GVBD (Hirai *et al.*, 1992a; Katsu *et al.*, 1993). This situation differs from that of *Xenopus* (Gautier and Maller, 1991; Kobayashi *et al.*, 1991) and starfish (Strausfeld *et al.*, 1991), in which cyclin B is already present in immature oocytes and forms a complex with cdc2 kinase. In contrast to goldfish cyclin B, this study demonstrated that goldfish cyclin A is stockpiled in immature oocytes, and that its content is not remarkably changed during maturation. In *Xenopus*, cyclin A is undetectable at the outset of oocyte maturation, in contrast to the large amounts of stockpiled cyclin B in immature oocytes (Kobayashi *et al.*, 1991). These results showed that the behavior of goldfish cyclin A resembles that of *Xenopus* cyclin B, whereas that of goldfish cyclin B resembles *Xenopus* cyclin A. According to its homology and its absence in purified MPF, it is certain that the anti-cyclin A reactive 47 kDa protein is not cyclin B but cyclin A. Therefore, this study showed that the behavior of cyclins A and B differ completely between goldfish and *Xenopus*, suggesting the presence of different mechanisms of cyclin A and B activation that are species specific. Indeed, I revealed that the molecular mechanisms of cyclin B activation during oocyte maturation differs among species (Katsu *et al.*, 1993; Yamashita *et al.*, submitted).

A previous study of *Xenopus* cyclin A during oocyte maturation found that $p13^{suc1}$-precipitates and anti-cyclin A immunoprecipitates from mature *Xenopus* oocyte extracts

do not have cyclin A or histone H1 kinase activity, respectively (Kobayashi *et al.*, 1991). This suggests the absence of complex formation of cyclin A with cdc2 and/or cdk2 during *Xenopus* oocyte maturation, implying that cyclin A does not play any roles at least in oocyte maturation. Contrary to this finding, I have shown that, in the goldfish oocyte system, cyclin A binds to cdc2, but not to cdk2, and that it activates cdc2 both *in vivo* and *in vitro*, raising the possibility that cyclin A plays a role in oocyte maturation at least in goldfish. The question is the role that cyclin A-cdc2 kinase plays during oocyte maturation. MPF, a final inducer of oocyte maturation (see review by Nagahama, 1987a), consists of cdc2 kinase and cyclin B, and cyclin A is not a component (Draetta *et al.*, 1989; Dunphy *et al.*, 1988; Gautier *et al.*, 1988, 1990; Labbé *et al.*, 1989a,b; Yamashita *et al.*, 1992a,b). Cyclin B-cdc2 kinase activity increases according to the occurrence of GVBD. Although the point where cyclin B-cdc2 and cyclin A-cdc2 begin to be activated is almost the same, the rapid increase of cyclin A-cdc2 kinase activity ocurrs only after the completion of GVBD. Furthermore, microinjection of cyclin A mRNA into goldfish immature oocytes does not induce GVBD. Collectively, these results suggest that cyclin A-cdc2 kinase is not required for inducing GVBD, which is under the control of cyclin B-cdc2 activity. It is possible that cyclin A-cdc2 may play an important role in steps after GVBD. For example, cyclin A-cdc2 kinase may help the rapid activation of cyclin B-cdc2 kinase at meiosis I to II transition. The cyclin A-cdc2 kinase may play a part in the maintenance of high cyclin B-cdc2 kinase activity in mature unfertilized oocytes by inactivating cdc2-specific tyrosine kinases, such as wee1 and mik1 (Devault *et al.*, 1992). Further investigations are required to understand the role of cyclin A-cdc2 kinase and the connection between cyclin A- and B-cdc2 kinase during oocyte maturation.

The present findings that goldfish cyclin A binds to and activates cdc2 in both mature oocytes and a cell-free system derived from immature oocytes raise the question of why cyclin A-cdc2 kinase is not activated in immature oocytes regardless of the presence of cyclin A. Kajiura *et al.* (1993) have shown in a previous study that all cdc2 proteins in immature goldfish oocytes are monomeric. Thus, it is most likely that cyclin A is

prevented from interacting with the immature oocyte cdc2 proteins. Although cyclin A is found in the soluble fraction (100,000 g supernatant), it should be in a 'masked' form in immature oocytes. It is plausible that the hormone changes the chemical state of cyclin A and allows it to interact with cdc2. This situation is similar to that of cyclin B during clam oocyte maturation, except that cyclin B in immature clam oocytes is stocked as the insoluble form (Westendorf *et al.*, 1989). Unfortunately, the unavailability of an anti-cdc2 antibody that immunoprecipitates cdc2 proteins does not allow us to determine the timing of the association of cyclin A with cdc2 kinase nor the relationship between this association and the increase in the kinase activity. I also examined anti-cyclin A immunoblots of suc1 precipitates. However, I found that in goldfish oocytes cyclin A protein binds to suc1 protein directly, so that I could not identify the timing of cyclin A-cdc2 complex formation. The molecular mechanisms of the chemical modifications of cyclin A during oocyte maturation remain to be resolved.

FIGURES AND FIGURE LEGENDS

Table 1

EFFECT OF MICROINJECTION OF GOLDFISH CYCLIN mRNA INTO *XENOPUS* AND GOLDFISH OOCYTES

Materials injected	% GVBD in *Xenopus* oocytes	% GVBD in Goldfish oocytes
Synthetic RNA		
sense cyclin A mRNA	100(21)[1]	0(28)
antisense cyclin A mRNA	8(25)	—[2]
sense cyclin B mRNA	—	81(21)
Control experiments		
oocytes treated with P[3]	91.7(24)	—
oocytes treated with 17,20-DP[4]	—	84(25)
nontreated oocytes	0(24)	0(24)

1. Number of oocytes used.
2. Not examined.
3. Progesterone (10 μg/ml)
4. 17α, 20β-Dihydroxy-4-pregnen-3-one (1 μg/ml)

```
                                                    TATTGGTTCTTTAAGAGTTTAACGCAGCAAAAGCAGATAAG  -41

ATGGCTTCCCGTGGCTTCGCTCCTTTGTCCGGTCGTCAGGAGAACATCATGGTCCTGGGAAGAGCGGATGGCCTG   75
M  A  S  R  G  F  A  P  L  S  G  R  Q  E  N  I  M  V  L  G  R  A  D  G  L     25

CACGCTCTCAAGCCTGGCCAGAGGGTCGTGCTCGGTGTTCTGACCGAGAATGATCAGCATAATCGAGTATTTGGA  150
H  A  L  K  P  G  Q  R  V  V  L  G  V  L  T  E  N  D  Q  H  N  R  V  F  G     50

CAGGTTTCGTCCAAATATGTGCCAGCCCTTCGTGATGCATCGACCCTTGACGTCAGCACATCGAGTGCTACACTG  225
Q  V  S  S  K  Y  V  P  A  L  R  D  A  S  T  L  D  V  S  T  S  S  A  T  L     75

GGTGTTCACGTGGTCGAGCCAGTCATTGCGCAGGCAACTAAACCGACCTCATTCTTACTGCCCTCAGAGCTTCTG  300
G  V  H  V  V  E  P  V  I  A  Q  A  T  K  P  T  S  F  L  L  P  S  E  L  L    100

CTAGTAGATGATGTGGTCCAAGATCTTGGTTCAGGGTCTTGCATGGATTCTTCCATGCAGTCATTGCCGGAGGAG  375
L  V  D  D  V  V  Q  D  L  G  S  S  S  C  M  D  S  S  M  Q  S  L  P  E  E    125

GCTGCTTATGAGGACATCCTGTGTGTTCCAGAATATGCGGAGGACATCCACAGATACCTGCGTGAATGTGAAGTT  450
A  A  Y  E  D  I  L  C  V  P  E  Y  A  E  D  I  H  R  Y  L  R  E  C  E  V    150

AAATACAGGCCGAAGCCTGGTTACATGAGAAAGCAGCCTGACATAACCAACTGTATGAGGGTCATCCTTGTTGAC  525
K  Y  R  P  K  P  G  Y  M  R  K  Q  P  D  I  T  N  C  M  R  V  I  L  V  D    175

TGGCTGGTTGAGGTTGGTGAGGAATACAAACTGTGCTCGGAGACCCTTTTCTTGGCTGTCAATTACCTGGACCGC  600
W  L  V  E  V  G  E  E  Y  K  L  C  S  E  T  L  F  L  A  V  N  Y  L  D  R    200

TTCCTTTCGTGCATGTCTGTCCTGAGAGGAAAATTGCAGCTTGTGGGAACAGCTGCTGTACTCCTGGCTGCGAAA  675
F  L  S  C  M  S  V  L  R  G  K  L  Q  L  V  G  T  A  A  V  L  L  A  A  K    225

TATGAGGAGGTGTATCCTCCGGAAGTGGATGAGTTTGTGTACATCACGGATGACACCTACACAAAGAAACAGCTG  750
Y  E  E  V  Y  P  P  E  V  D  E  F  V  Y  I  T  D  D  T  Y  T  K  K  Q  L    250

CTTCGGATGGAGCAGCACCTGCTCCGTGTGCTGGCTTTTGACATGACCGCTCCCACGGTTCACCAGTTTTTGATG  825
L  R  M  E  Q  H  L  L  R  V  L  A  F  D  M  T  A  P  T  V  H  Q  F  L  M    275

CAGTACACATTGGAGGGGCATATCTGTGCCAGGACTGTAAACCTTGCTTTGTATCTTTCAGAGTTGAGCCTGCTT  900
Q  Y  T  L  E  G  H  I  C  A  R  T  V  N  L  A  L  Y  L  S  E  L  S  L  L    300

GAGGTGGATCCCTTTGTGCAGTATCTACCTTCAAAGACTGCTGCAGCTGCATATTGTCTGGCCAACTACACTCTA  975
E  V  D  P  F  V  Q  Y  L  P  S  K  T  A  A  A  A  Y  C  L  A  N  Y  T  L    325

AATGGGGTTTTGTGGCCTGAGAACCTGTATGCCTTCACTGGTTACTCACTGGCCGTGATCATCCCATGTCTGATG 1050
N  G  V  L  W  P  E  N  L  Y  A  F  T  G  Y  S  L  A  V  I  I  P  C  L  M    350

GAGCTTCATAAACTTCATCTAGGGGCTGCAGGTCGCCCCCAACAGGCTATCCAGGAGAAATACAAGGGCTCAAAA 1125
E  L  H  K  L  H  L  G  A  A  G  R  P  Q  Q  A  I  Q  E  K  Y  K  G  S  K    375

TATTGTGGCGTGTCCCTGCTTGAGCCTGTGGAGTCTCTGCCTCTCCCTTGATGTTTTGGTGACCACATTGGTTTG 1200
Y  C  G  V  S  L  L  E  P  V  E  S  L  P  L  P  *                            391

AGGCCAACAAATGAACTGCAGCGCTTAAGTCTATTGTAAATATTGATTGAACTTTTGTTTTTAAATGTGGCTTTT 1275
AATCTTTTTAAAACGTGATTTTTATCATAGGTTGCTTAAGACTAATCAACAAGTGACTTGTTTCTGTAAATCCAA 1350
CACGCAGTTGGTCTGAAGGCTGTTCTCCCCTCTTTGTATATGACATGAACAAATGTACCACTTTATCTACTCTCC 1425
TTAGGGTCTCTTTCTCTGGTAACCATGTATTTTACTTGTATAGTAAGTGCAATATCATGTACTTCAAACTGAATT 1500
TCAATAAACGAAACAAGTTGCAAAA                                                    1525
```

Fig. 1. Nucleotide and deduced amino acid sequence of goldfish cyclin A.

The underlined sequence was used as a probe for isolating clones that contain the entire coding region. The first nucleotide of the truncated cDNA clone isolated initially is shown by #.

```
A consensus   ## ###############  ### ## ## ##### ####
GFcycA    169 MRVILVDWLVEVGEEYKLCSETLFLAVNYLDRFLSCMSVL
XLcycA    196 MRTILVDWLVEVGEEYKLHTETLYLAMNYLDRFLSCMSVL
HScycA    210 MRAILVDWLVEVGEEYKLQNETLHLAVNYIDRFLSSMSVL
GFcycB    167 MRAILIDWLVQVQIKFKLLQETMYMTVAVIDRFLQDHPVP
XLcycB1   167 MRAILIDWLVQVQMKFRLLQETMFMTVGIIDRFLQFHPVP
XLcycB2   162 MRAILVDWLVQVHSRFQLLQETLYMGVAIMDRFLQVQPVS
AB consensus ** ** **** *     *  **        ****    *

A consensus   ########### ### # ## ### # ########## ##
GFcycA    209 RGKLQLVGTAAVLLAAKYEEVYPPEVDEFVYITDDTYTKK
XLcycA    236 RGKLQLVGTAAILLASKYEEIYPPDVDEFVYITDDTYSKK
HScycA    250 RGKLQLVGTAAMLLASKFEEIYPPEVAEFVYITDDTYTKK
GFcycB    207 KKQLQLVGVTAMFIASKYEEVYPPEIADFAFVTDRAYTTG
XLcycB1   207 KNQLQLVGVTAMFLAAKYEEMYPPEIGDFTFVTDHTYTKA
XLcycB2   202 RSKLQLVGVTSLLIASKYEEMYTPEVADFVYITDNAYTAS
AB consensus    *****       * * ** * *    *   **  *

A consensus   # ####   # ## ##   ### ###
GFcycA    249 QLLRMEQHLLRVLAFDMTAPTVHQFL
XLcycA    276 QLLRMEHVLLKVLAFDLTVPTVNQFL
HScycA    290 QVLRMEHLVLKVLTFDLAAPTVNQFL
GFcycB    247 QIRDMEMKILRVLDFSFGKPLPLQFL
XLcycB1   247 QIRDMEMKILRVLKFAIGRPLPLHFL
XLcycB2   242 QIREMEMIILRLLNFDLGRPLPLHFL
AB consensus *   **   *  * *    *    **
```

Fig. 2. Comparison of amino acid sequence of cyclin.

A comparison of goldfish cyclin A (GFcycA) with cyclin A homologs from *Xenopus* (XLcycA) (Minshull *et al.*, 1990) and human (HScycA) (Wang *et al.*, 1990) and with cyclin B homologs from goldfish (GFcycB) (Hirai *et al.*, 1992a) and *Xenopus* (XLcycB1 and XLcycB2) (Minshull *et al.*, 1989). The most conserved region is shown. Residues identical in A-type cyclins (GFcycA, XLcycA and HScycA) are marked # above the sequence of GFcycA. Residues conserved in all A-type and B-type cyclins are marked with asterisks below the sequence of XLcycB2.

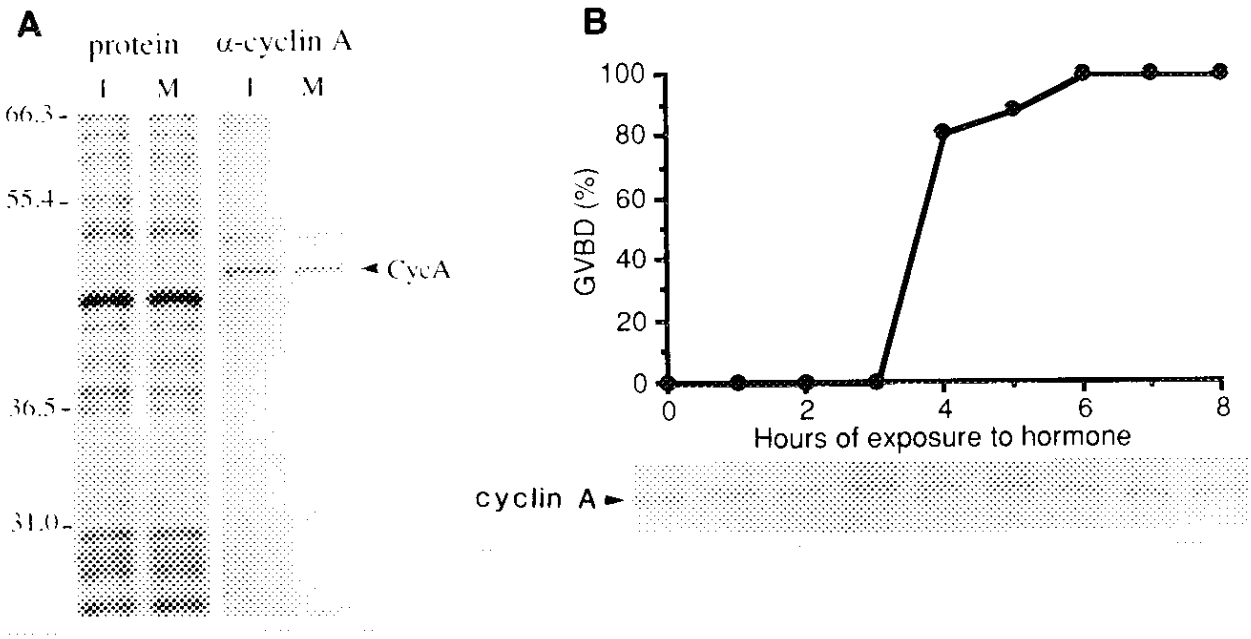

Fig. 3. Immunoblots of goldfish oocyte extracts with an anti-cyclin A monoclonal antibody.

Oocyte extracts (5 μl) were separated by 12.5% SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue for total proteins. Identical samples were blotted and probed with the anti-cyclin A antibody. (A) Cyclin A (CycA), 47 kDa in immature (I) and mature (M) oocyte extracts. The positions of protein molecular mass markers (in kD) are indicated. (B) Anti-cyclin A immunoblots of goldfish oocyte extracts during maturation. The percentage of GVBD in the same experiment is shown above the blots. The 47 kDa cyclin A protein is not remarkably changed during oocyte maturation.

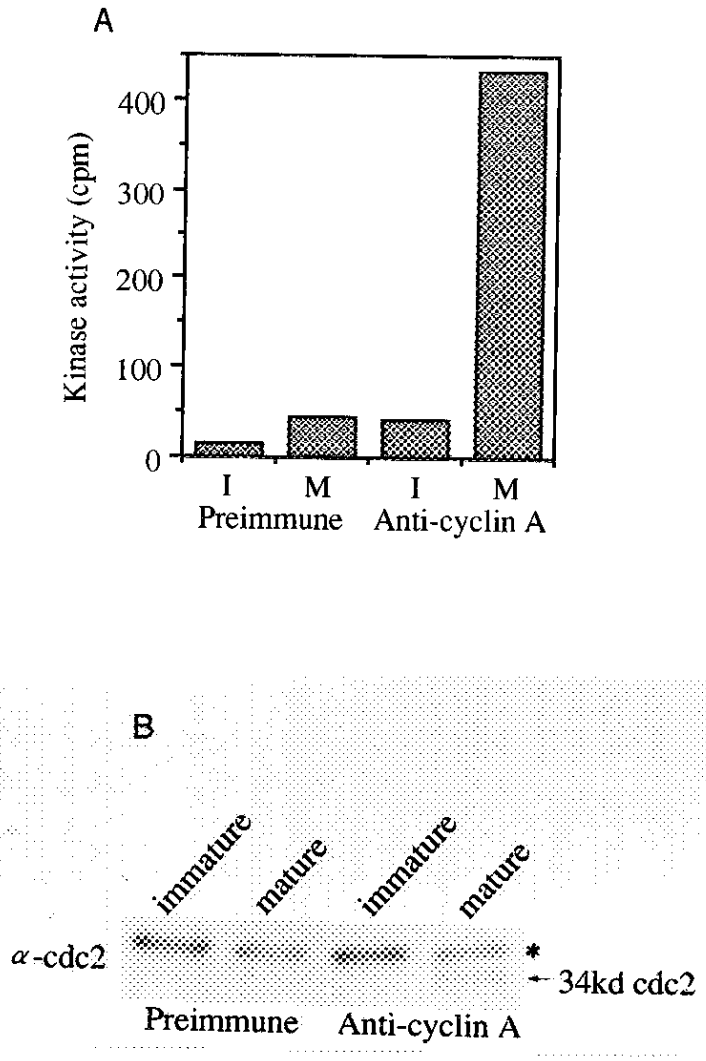

Fig. 4. Binding of cyclin A and cdc2 and its activation in mature oocytes.

(A) Kinase activity of immunoprecipitates from immature (I) and mature (M) oocyte extracts with anti-cyclin A or preimmune serum. (B) Anti-cdc2 immunoblots of immunoprecipitates from immature and mature oocyte extracts with anti-cyclin A antiserum or preimmune serum. The band noted by an asterisk is 35 kDa inactive cdc2, which is nonspecifically precipitated with protein A-Sepharose beads.

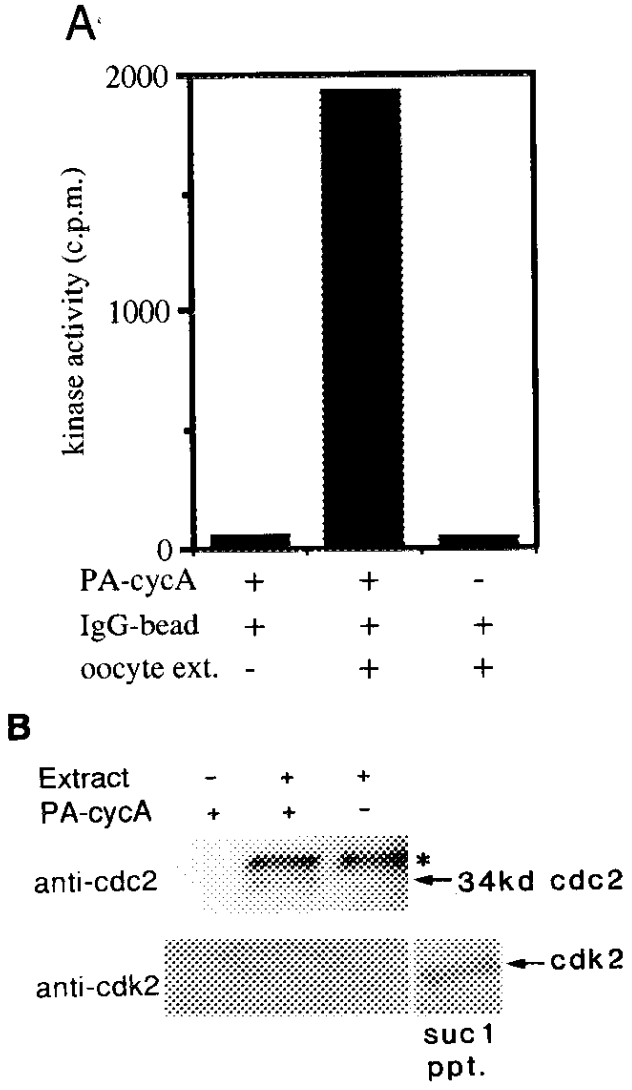

Fig. 5. The binding of recombinant PA-cyclin A to cdc2 and its activation in a cell-free system.

(A) Kinase activity of PA-cyclin A protein harvested from immature oocyte extracts with IgG-Sepharose. (B) Immunoblots of the same sample as A, probed with anti-cdc2 antibody or anti-cdk2 antibody. Inactive 35 kDa cdc2 noted by the asterisk was nonspecifically precipitated with IgG-Sepharose. Anti-cdk2 immunoblot of $p13^{suc1}$ precipitates from the same oocyte extract revealed cdk2 in the original extracts.

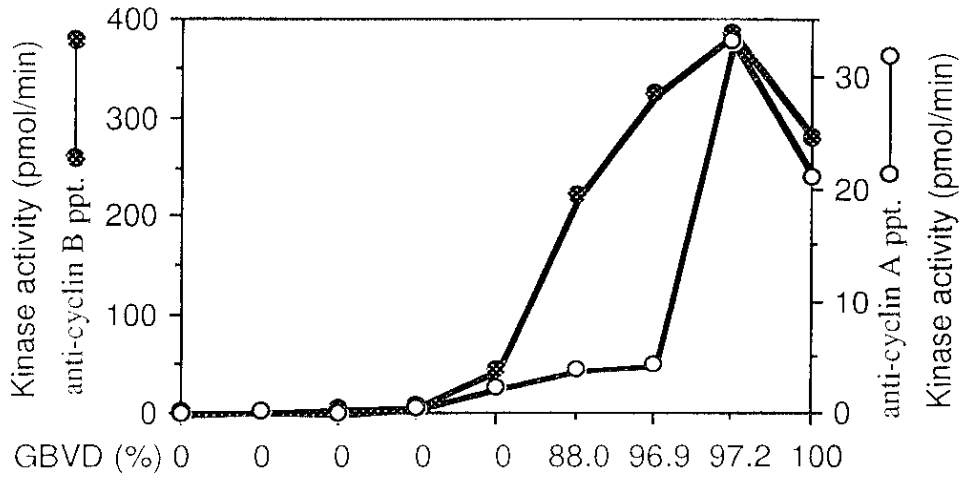

Fig. 6. Cyclin A- and B-cdc2 activities during goldfish oocyte maturation.

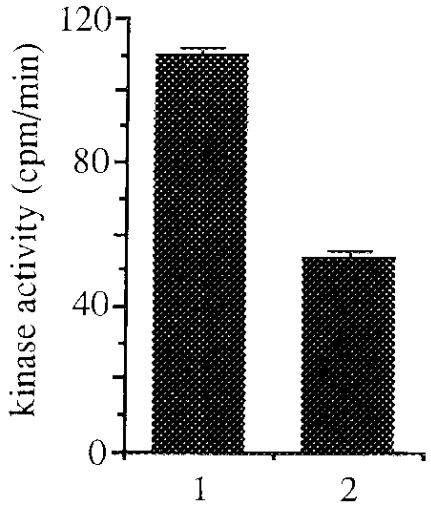

Fig. 7. Kinase activity of synthetic cyclin A mRNA injected oocytes. Ten oocytes were incubated in goldfish Ringer's solution after injection of synthetic cyclin A mRNA. After 8 hr, oocytes were washed with extraction buffer and homogenized with a pestle in 10 μl of ice-cold extraction buffer. The homogenate was centrifuged at 15,000 g for 10 min at 4℃, and the supernatant was used for kinase assay as described in Materials and Methods. The kinase activity of cyclin A mRNA-injected oocytes (lane 1) and non-injected control oocytes (lane 2) was measured. Mean±standard deviation (n=2)

Chapter III.

Translational Control of Maternal Cyclin B mRNA during Goldfish Oocyte Maturation

INTRODUCTION

MPF has been purified from mature oocytes of *Xenopus* (Lohka *et al.*, 1988), starfish (Labb *et al.*, 1898a,b), and carp (Yamashita *et al.*, 1992) and consists of two components; one is a homolog of the $cdc2^+$ gene product of fission yeast, referred to as cdc2 kinase, and the other is cyclin B (reviewed by Nurse, 1990; Maller, 1991; Jacobs, 1992). The association of cdc2 kinase with cyclin B is necessary to turn on its protein kinase activity (Desai *et al.*, 1992). The mechanisms of MPF activation differ from species to species, in spite of the involvement of the same molecules. For example, in immature oocytes of *Xenopus* (Gautier *et al.*, 1991; Kobayashi *et al.*, 1992) and starfish (Strausfeld *et al.*, 1991), inactive cdc2 is already in a complex with cyclin B as pre-MPF; in clam oocytes, cdc2 forms a complex with cyclin B that insolubilized in process of oocyte maturation (Westendorf *et al.*, 1989); in goldfish, there is no detectable cyclin B in immature oocytes and cyclin B is synthesized *de novo* during oocyte maturation (Hirai *et al.*, 1992; see also chapter I in this thesis). In goldfish, therefore, the synthesis of cyclin B during oocyte maturation appears to have a crucial role in activating cdc2 and in inducing oocyte maturation.

Patterns of translational control in the oocyte maturation and early embryo are diverse. Maternal mRNAs that regulate key events in the embryo often are repressed during oogenesis and activated either during meiotic maturation or shortly after fertilization. It is not yet known how many different biochemical mechanisms are responsible for the timing of translational control. Generally regulation of maternal mRNA translation is separated into two control mechanisms: the control of translational repression and the control of translational activation. The majority of the maternal mRNA that will be untilized during early development is sequestered during oogenesis into storage ribonucleoprotein particles (mRNPs)(Richter, 1991). Assembly of these storage mRNPs is believed to prevent the recruitment of mRNA to ribosomes for translation until the correct devevopmental time; thus, the maternal mRNA is described as being masked from the translational apparatus (Kandror and Stepanov, 1988). In *Xenopus* oocytes,

two major proteins, mRNP3 and mRNP4, are associated with maternal mRNA in the storage mRNPs of mature oocytes (Darnbroygh and Ford, 1981) and these proteins have been directly implicated in the repression of mRNA translation in the oocyte (Richter and Smith, 1983, 1984). Recently, the deduced amino acid sequences of mRNP3 and mRNP4 have been obtained (Murray *et al.*, 1992) and these proteins are identical to the protein FRGY2, recently reported (Tafuri and Wolffe, 1990) as a DNA-binding transcription factor specific for the DNA "Y box" motif located in the promoter region of certain genes. However, the molecular mechanisms for repression system by FRGY2 and for releasing the template for translation have not been defined.

Regulation of the length of poly (A) on specific maternal mRNA appears to be a common means of translational activation in oocytes and early embryos. The extent of polyadenylation of mRNAs is correlated with its translation (MacGrew *et al.*, 1989; Vassalli *et al.*, 1989; Paris and Richter, 1990; Sheets *et al.*, 1994; Fox *et al.*, 1989). These general correlations are not absolute, however, with some maternal mRNAs, poly (A) tail length either does not change, or is even reduced, during translational activation (Ruderman *et al.*, 1979).

As an initial step to understand the regulation of cyclin B synthesis during 17α,20β-DP-induced oocyte maturation in goldfish, I first examined the effects of protein and RNA synthesis inhibitors on 17α,20β-DP-induced cyclin B synthesis and GVBD. A reticulocyte lysate system was also used to determine (1) whether goldfish maternal cyclin B mRNA has the ability to be translated *in vitro*, and (2) whether factors which inhibit cyclin B mRNA translation are present in cell extracts from immature and mature oocytes. In addition, a PCR poly (A) test was used to examine the polyadenylation state of cyclin B mRNA during oocyte maturation. Finally, a cDNA clone encoding goldfish Y box protein (RNA-binding protein) was isolated from a goldfish oocyte cDNA library, and its expression was examined by Northern blot analysis.

MATERIALS AND METHODS

Animals and Oocytes

Goldfish were purchased commercially and raised at 15°C until use. Full-grown immature oocytes were isolated from ovaries using a pipet, and induced to mature *in vitro* by incubating at room temperature in goldfish Ringer's solution (Kagawa *et al.*, 1984) containing 1 μg/ml 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP), a natural MIH in fish (Nagahama and Adachi, 1985). Maturational processes were assessed by immersing the oocytes in a clearing solution (5% formalin and 4% acetic acid in Ringer; Lessman and Kavumpurath, 1984), facilitating microscopic examination of the occurrence of GVBD.

Immunoblot

Oocyte extracts were obtained as described previously (Hirai *et al.*, 1992a). Proteins were electrophoresed in SDS/12.5% polyacrylamide gels, transferred to Immobilon membranes (Millipore), blocked with 10% skim milk, and probed with anti-cdc2 (Kajiura *et al.*, 1993) and anti-cyclin B (Katsu *et al.*, 1993) momoclonal antibodies. The blots were incubated with anti-mouse IgG peroxidase conjugate (ZYMED). The antigen-antibody complexes were visualized by chemiluminescence (ECL detection system; Amersham).

Northern Blot

Total RNA was isolated by the method of Chomczynski and Sacchi (1987) using ISOGEN (Nippon GENE). Poly $(A)^+$ RNA was purified from total RNA of immature and mature oocytes by using oligo(dT)-Latex beads (Oligotex^{TM}-dT30, TaKaRa) according to the manufacturer's procedure. RNA samples were treated with formamide and formaldehyde, and run on Mops-buffered agarose gels as described by Maniatis *et al.* (1982). The RNA was transferred onto Hybond-N+ (Amersham) after electrophoresis. Probes were labeled using the Random Primer PLUS Extension Labeling System

(NEN). Hybridizations were performed at 42°C overnight, and the blot was washed at high stringency (60°C, 0.1 x SSC: 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0), and analyzed by a BAS 2000 Bio-Image Analyzer (Fuji)

In Vitro Translation Assay

Poly (A)$^+$ RNAs from immature and mature oocytes were translated at a final concentration of 400 µg/ml in the nuclease-treated reticulocyte lysate (Promega). Typically, 3 µl of RNA was added to 17.5 µl of reticulocyte lysate mix with 0.5 mCi/ml ^{35}S methionine and incubated for 1 hr at 30°C. A portion of the translational products was mixed with SDS sample buffer immediately and the rest was immunoprecipitated with anti-cyclin B antibody. Samples were processed for analysis on SDS-polyacrylamide gels. For translational inhibition assay, synthetic goldfish cyclin B mRNA was used. To synthesize mRNA *in vitro*, the pET3b vector containing cyclin B cDNA (Katsu *et al.*, 1993) was digested with *Eco*RV, and capped mRNA was synthesized by T7 RNA polymerase for cyclin B mRNA using a mCAP mRNA capping kit (Stratagene). Synthetic mRNAs were incubated with oocyte extracts on ice for 20 min, followed by 1 hr of incubation at 30°C in reticulocyte lysate containing 0.5 mCi/ml ^{35}S methinine. The translational products were then mixed with SDS sample buffer and analyzed on SDS-polyacrylamide gels followed by autoradiography.

PCR Poly (A) Test

PCR poly (A) test was performed as described previously (Sallés *et al.*, 1992). RNA from oocytes was isolated and reverse transcribed with 20 units of SuperScript RNase H-reverse transcriptase (BRL) using 200 ng of the oligo (dT) primer/adapter (5'-GCGAGCTCCGCGGCCGCGTTTTTTTTTTTT-3'). The CG-rich adapter region allows increased annealing temperature in the subsequent PCR. The synthesized first-strand cDNAs were then amplified with adapter oligo (dT) primer as 3' primer and goldfish cyclin B specific 5' primer (5'-AAATGTTGTGAGAGTCAATGAGGG-3'). The amplification procedure was 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C for 30

cycles. Final products were electrophoresed on a 4% acrylamide-6M urea gel and then electrotransfered onto Hybond-N+ (Amersham). The filter was Southern blot using labeled goldfish cyclin B cDNA as a detection probe.

cDNA Cloning of Goldfish Y Box Protein

To screen a goldfish Y box protein cDNA, I used a λZAPII cDNA library constructed from an early vitellogenesis goldfish ovary. The λZAPII cDNA library was screened with a redundant oligonucleotide (5' AACAGRAATGACACCAARGAAGAY-GTGTTTGT 3', R:A or G, Y:C or T). Hybrydization was performed at 37°C for 16 hr in a buffer containing 5x SSC (1x SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5x Denhardt's regent (1 mg/ml each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS), and 200 μg/ml herring sperm DNA. Membranes were washed in 5x SSC at the same temperature. Fourteen clones were isolated and the longest clone was sequenced by dideoxy chair termination sequenceing methods (BcaBEST sequencing kit, TAKARA) using a double-stranded plasmid DNA as a template.

RESULTS

Cyclin B Protein Synthesis is Regulated at the Translational Level, but not at the Transcriptional Level

In Chapter I, it was shown that in goldfish cyclin B protein synthesis is necessary for 17α,20β-DP-induced oocyte maturation. To further study the mechanism of cyclin B protein synthesis by goldfish oocytes, the first series of experiments were carried out to examine the effects of inhibitors of protein synthesis (cycloheximide) and RNA synthesis (actinomycin D) on 17α,20β-DP-induced cyclin B synthesis and oocyte maturation. Immature oocytes were induced to mature *in vitro* by incubating in goldfish Ringer's solution containing 1 μg/ml 17α,20β-DP in the presence or absence of cycloheximide (10

μg/ml) or actinomycin D (100 μg/ml) for 8 hr. The occurrence of GVBD was used as an indication of oocyte maturation and was visualized in intact follicles through a dissecting microscope. Oocyte extracts were obtained for immunoblot analysis. Cycloheximide-treated oocytes failed to undergo maturation. In this group, neither cyclin B nor 34-kDa active cdc2 could be detected (Fig. 1, lane 3). In contrast, actinomycin D-treated oocytes underwent maturation in a fashion similar to that of 17α,20β-DP-treated oocytes without treatment of the inhibitors. In these groups, both cyclin B and 34-kDa active cdc2 were observed (Fig. 1, lane 4). Similar results were obtained when actinomycin D was microinjected into oocytes (data not shown).

The next experiment was designed to examine whether cyclin B mRNA is present in immature oocytes. Total RNA from immature and mature oocytes was extracted and fractionated on Mops-agarose gels, followed by transfer to nitrocellulose filters before hybridization to the goldfish cyclin B cDNA (Fig. 2). Northern blot analysis showed a single distinct signal at about 1.9 kb (Fig. 2A). In addition, poly $(A)^+$ RNA from immature and mature oocytes were analyzed by Northern blot using goldfish cyclin B and actin cDNAs as probes (Fig. 2B). A single distinct signal for cyclin B and actin mRNAs was found in both immature and mature oocytes, showing no marked difference in the amount of the mRNAs between two oocyte groups.

Goldfish Maternal Cyclin B mRNA Possesses the Translational Ability

Northern blot analysis using poly $(A)^+$ RNA clearly showed that goldfish immature oocytes contain cyclin B mRNA and this mRNA has poly (A) tails (see above). The next series of experiments were performed to examine whether maternal cyclin B mRNA is translationally active. Poly $(A)^+$ RNA isolated from immature and mature oocytes was used for template RNA in the reticulocyte lysate system. Translational products were analyzed by SDS-PAGE and autoradiography. Figure 3A shows protein products visualized by ^{35}S methionine incorporation. The patterns of products are essentially identical, except for two protein bands (about 50-kDa and 40-kDa, marked in Fig. 3A) which appear to be specific for immature and mature oocytes, respectively. Poly $(A)^+$

RNA from immature oocytes was immunoprecipitated with the anti-goldfish cyclin B antibody and analyzed by SDS-PAGE and autoradiography. The immunoprecipitates contained a distinct band corresponding to goldfish cyclin B protein (Fig. 3B). Cyclin B protein was also present in poly $(A)^+$ RNA translation products from mature oocytes (data not shown).

Inhibition of Cyclin B Translation by Goldfish Oocyte Extracts

As shown above, when poly $(A)^+$ RNA from goldfish immature oocytes was mixed with nuclease-treated rabbit reticulocyte lysate, cyclin B protein could be synthesized (Fig. 3). Thus, it is possible that cyclin B translation is inhibited in immature oocytes. To test this possibility, *in vitro* synthesized cyclin B mRNA was mixed with immature and mature oocyte extracts, and then rabbit reticulocyte lysate was added. As shown in Fig. 4, incubation of synthetic cyclin B mRNA with immature oocyte extracts resulted in approximately 2-fold lower translation than with mature oocyte extracts (Fig. 4).

3' End of Goldfish Cyclin B mRNA

It has been reported that changes in the translational activity of specific mRNAs are correlated with changes in the length of their poly (A) tails. In general, mRNAs that receive poly (A) become translationally active, whereas those that lose poly (A) become translationally quiescent (see reviews by Bachvarova, 1992; Wormington, 1993; Richter, 1993). The 3'-untranslated regions (3' UTR) of specific maternal mRNAs have been implicated in a wide variety of processes, including translational control, mRNA degradation, and intracellular localization of mRNAs. Sequences in the 3' UTR also determine whether or not an mRNA receives poly (A) during frog and clam oocyte maturation; maturation-specific polyadenylation depends on the presence of two sequences: the usual nuclear polyadenylation signal AAUAAA and a nearby U-rich cytoplasmic polyadenylation elements (CPE, consensus $U_{4\text{-}6}A_{1\text{-}2}U$), whereas mRNAs that lack such a sequence lose their tails instead (Fox *et al.*, 1989; Paris and Richter, 1990; Salles *et al.*, 1992).

A comparison of the 3' noncoding region of goldfish cyclin B mRNA with those of several other transcripts that undergo poly (A) elongation during early development reveals one conserved region, the hexanucleotide AAUAAA. Further examination of the distal portion of the 3' noncoding region also reveals that it contains four copies of U_{4-6}AA/U motifs, which are very similar in sequence to the U-rich cytoplasmic polyadenylation elements identified in frog mRNA (Fig. 5). Thus, it was confirmed that the 3'UTR of goldfish cyclin B possesses the cytoplasmic polyadenylation elements.

Polyadenylation of Cyclin B mRNA during Goldfish Oocyte Maturation

A PCR-based assay recently developed by Salles *et al.* (1992) was used to determine whether goldfish cyclin B mRNA is regulated by polyadenylation upon oocyte maturation. This assay is an application of anchored PCR and can rapidly determined the polyadenylation state of an mRNA. RNA was isolated from either immature or mature oocytes and reverse transcribed with an oligo (dT) primer/adapter, 5'-GCGAGCTCCGGCGGCCGCGT(12). In theory, the oligo (dT) primer/adapter can anneal anywhere on the poly (A) tail of the mRNA during the reverse transcription. If the specific mRNA is further polyadenylated upon meiotic maturation, an increase in the size of the cDNAs should occur, resulting from the greater number of priming sites for the oligo (dT) primer/adapter and a second sense primer (in this case goldfish cyclin B specific 5' primer) that recognizes the specific clone (Fig. 6A). The adapter anchors the oligo (dT) primer preventing internal priming in the poly (A) region, which would gradually shorten the PCR products. Increased heterogeneity can be visualized as a slower electrophoretic mobility of some of the PCR fragments. If the mRNA was not adenylated further, there should be no change in mobility of the amplified products.

Using the PCR poly (A) test, I first examined the poly (A) status of the cyclin B mRNA in immature and mature oocytes. The cDNAs from immature and mature oocytes were electrophoresed and transferred to membranes. Figure 6B showed that PCR products derived from the transcripts of mature oocytes were more heterogenous and longer than those mRNAs from immature oocytes. The poly (A) tail length of cyclin B

mRNA in mature oocytes as manifested by this assay is about 100 nt, indicating that cyclin B mRNA was polyadenylated during oocyte maturation. The same approach was also used to determine the poly (A) status of cyclin B mRNA during 17α,20β-DP-induced oocyte maturation. At various times after the addition of 17α,20β-DP, RNA was extracted, followed by the PCR poly (A) test. As shown in Fig. 6C, there was a significant change in the poly (A) length of cyclin B mRNA during oocyte maturation, but this change could be detected only after GVBD, that is, after cyclin B protein becomes detectable (Fig. 6C, also Fig. 4 in Chapter I).

Cloning of Goldfish Y Protein and its Expression

The *Xenopus* Y box proteins were originally identified as an oocyte-specific transcription factor that associates with the Y box, a regulatory element prevalent in genes selectively active in oocytes (Tafuri and Wolffe, 1990, 1993) and association of the Y box proteins with maternal mRNA offers a potential general repression mechanism for translation. To examine whether Y box proteins play an important role in repression of cyclin B mRNA translation in goldfish oocytes, I identified a goldfish Y box protein cDNA clone from a goldfish immature ovary cDNA library. From the most conserved region of *Xenopus* Y box proteins (FRGY1, 2: Tafuri and Wolffe, 1990) and a mouse Y box protein (MSY1: Tafuri *et al.*, 1993), a 8-fold redundant oligonucleotide was designed (Fig. 7A). Approximately 250,000 clones were screened and 14 potential positives isolated. The longest of these clones (1.5 kb) was partially sequenced and the peptide domains conserved among all Y box proteins (Wolffe *et al.*, 1992) were determined (Fig. 7B). Although a comparative analysis of the nucleic acid sequence and amino acid sequence showed that the isolated clone is one of the Y box protein family, this study could not determine whether this clone belongs to either the FRGY1 group or FRGY2 group. The next experiment was designed to determine the size of a goldfish Y box protein (GFYP) mRNA, RNA was extracted from oocytes, resolved on denaturing gels, blotted and probed with the GFYP cDNA (Fig. 8). Two signals of 1.7 kb and 3.3 kb were detected in total RNA with no significant changes during oocyte maturation (Fig.

8A). However, it was found that in poly $(A)^+$ RNA, the 1.7 kb signal was much stronger than that of 3.3 kb (Fig. 8B).

DISCUSSION

It has previously been shown that in goldfish cyclin B protein synthesis is required for 17α,20β-DP (maturation-inducing hormone of goldfish)-induced oocyte maturation (Hirai *et al.*, 1992; Katsu *et al.*, 1993; Chapter I in this thesis). The results of the present study demonstrate that (1) 17α,20β-DP-induced cyclin B protein synthesis is inhibited by cycloheximide, but not by actinomycin D, and (2) immature oocytes contain cyclin B mRNA which shows no significant change in its amount during oocyte maturation. Thus, this study confirms our previous findings and further suggests that 17α,20β-DP-induced expression of cyclin B protein is regulated at the translational level.

Oocytes synthesize and accumulate a large pool of maternal mRNAs. Some of these mRNAs are masked until their activation at subsequent developmental stages. This translational control is the principal mechanism for regulating gene activity during early development, as maternal mRNAs direct all protein synthesis before the onset of zygotic transcription. It has long been recognized that the stage-specific translational control of maternal mRNA is determined by their differential polyadenylation and deadenylation. The translational activity of maternal mRNAs generally correlates with changes in polyadenylation (reviews by Jackson and Standart, 1990; Bachvarove, 1992; Wickens, 1990). In general, mRNAs that receive poly (A) become translationally active, whereas those that lose poly (A) become translationally quescent. Furthermore, at least in certain mRNAs, the changes in poly (A) length cause the change in translational activity; in fact, the elongation of poly (A) tail is necessary and sufficient to trigger translation (Fox *et al.*, 1992; Sheets *et al.*, 1994; Vassalli *et al.*, 1989; McGrew *et al.*, 1989; Simon *et al.*, 1992). It has also been reported that the *cis*-acting sequences that specify cytoplasmic polyadenylation have been determined in several mRNAs from frog and mouse oocytes. These sequences include the usual cytoplasmic polyadenylation element (CPE, consensus $U_{4\text{-}6}A_{1\text{-}2}U$) (McGrew *et al.*, 1989; Fox *et al.*, 1989, 1992; Vassali *et al.*, 1989; McGrew and Richter, 1990; Paris and Richter, 1990; Fox and Wickens, 1990; Huarate *et al.*, 1992). Examination of the 3'UTR of goldfish cyclin B mRNA revealed that it contains

four copies of CPE motifs. This observation raises the possibility that goldfish cyclin B mRNA can be polyadenylated during oocyte maturation. Finally, the PCR poly (A) test revealed that cyclin B mRNA receives poly (A) during oocyte maturation. However, it is noteworthy that this poly (A) addition occurs after GVBD, that is, after the appearance of cyclin B protein, probably after MPF activation. In this regard, it is of great interest to note a recently proposed hypothesis that polyadenylation can be activated in oocyte extracts by $p34^{cdc2}$ kinase (Paris *et al.* 1991). In fact, a CPE-binding protein isolated from *Xenopus* oocytes was reported to contain a single putative cdc2 kinase phosphorylation site and is phosphorylated during oocyte maturation (Hake and Richter, 1994). Thus, it is unlikely that in goldfish 17α,20β-DP-induced cyclin B translation is initiated by poly (A) addition. It is possible that addition of poly (A) which occurs after GVBD, probably induced by activated MPF, may play a role in increasing translational rate during the later stages of oocyte maturation.

Another mechanim for translational control of maternal mRNA is that the proteins bound to maternal mRNA act as translational inhibitors which are released or inactivated at the appropriate stage in development, leading to translation of the previously dormant mRNA. I first demonstrated that cyclin B mRNA isolated from goldfish immature oocytes can be translated in the reticulocyte system. It was also shown in this study that the efficiency of cyclin B translation is much lower with extracts from immature oocytes than with those from mature oocytes. These results suggest that the initiation of cyclin B synthesis is controlled by a translational inhibitory factor, so called the RNA-binding proteins. In *Xenopus*, Y box proteins (FRGY2, mRNP3) which bind maternal mRNAs within 6S mRNP storage particles have been shown to regulate translation in developing oocytes and embryos. In this study, I was also able to isolate a goldfish cDNA clone, which encodes the Y box protein. Thus, it is possible that in immature oocytes cyclin B mRNA is packaged into ribonucleoprotein particles (RNPs) that render the mRNA nontranslatable and may be masked by this RNA binding protein. Activation of the signal transduction system by maturation-inducing hormone treatment may induce inactivation of the binding protein (probably by its phosphorylation or dephosphorylation), leading to

the initiation of translation (Cummings and Sommerville, 1988). I am now attempting to produce recombinant GFTPs which will be used to investigate the role of the GFYP in the translational regulation of cyclin B mRNA in goldfish oocytes.

In conclusion, these results, together with our earlier observations, suggest the following sequence for the translational control of cyclin B mRNA during hormone-induced oocyte maturation in goldfish. Cyclin B mRNA is stored, stable and untranslated in the cytoplasm of fully grown immature oocytes. 17α,20β-DP stimulation releases or inactivates the inhibitor of cyclin B mRNA translation, leading to cyclin B protein synthesis. As soon as cyclin B is synthesized, cyclin B binds to pre-existing cdc2, forming active MPF. Active MPF may then phosphorylate the *trans*-acting factor (CPE-binding protein), leading to addition of poly (A) to cyclin B mRNA. In turn, this adenylation may increase the rate of translation. The identification of the inhibitory factor and clarification of its roles should greatly increase our knowledge of not only the translational regulation of cyclin B synthesis but also the hormonal regulation of oocyte maturation in general. Thus, the goldfish oocyte should provide a valuable model to gain better understanding of a basic mechanism for translational activation of maternal mRNA which occurs during meiotic maturation and/or early embryogenesis.

FIGURES AND FIGURE LEGENDS

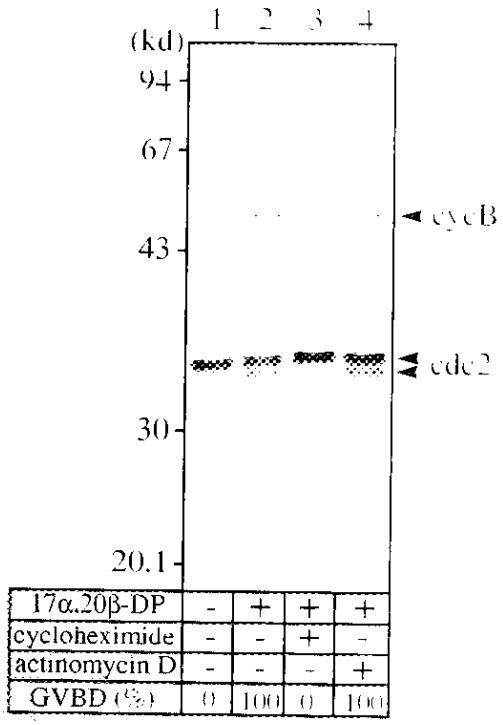

Fig. 1. Effects of cycloheximide and actinomycin D on expression of cyclin B protein 17α, 20β-DP. Goldfish oocytes were treated with 17α, 20β-DP (1 μg/ml) and a protein (cycloheximide, 10 μg/ml) or an RNA (actinomycin D, 100 μg/ml) synthesis inhibitor, and then oocyte extracts were obtained. lane 1, control; lane 2, 17α, 20β-DP treatment alone; lane 3, 17α, 20β-DP plus cycloheximide; lane 4, 17α, 20β-DP plus actinomycin D. Each extract was analyzed by SDS-PAGE and processed for Western blotting with anti-cyclin B and anti-cdc2 antibodies.

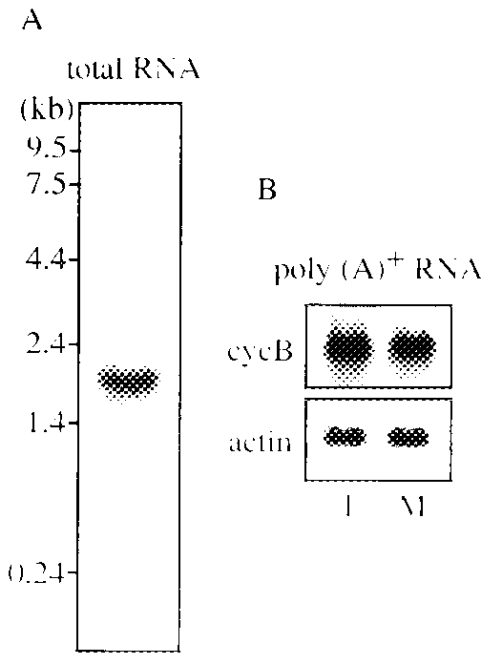

Fig. 2. Northern blot analysis of cyclin B mRNA in immature and mature oocyte of goldfish. (A) Total RNA (20 μg) extracted from immature oocytes was separated by electrophoresis on a Mopes-agarose gel. The positions of size standards are indicated in kb. (B) Four μg poly $(A)^+$ RNA from immature (I) and mature (M) oocytes were separeted as described above. Goldfish cyclin B cDNA (uper lane) and goldfish actin cDNA (lower lane) were used as detection probes.

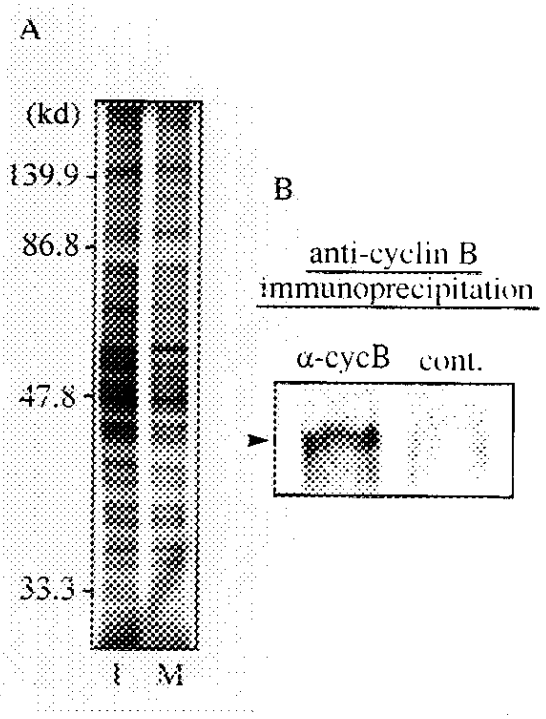

Fig. 3. ***In vitro*** **translation of goldfish maternal mRNAs.** (A) Poly $(A)^+$ RNAs of immature (I) or mature oocytes (M) were translated using the nuclease-treated rabbit reticulocyte lysate *in vitro*. The translation products were analyzed by SDS-PAGE and autoradiography. The size of molecular weight markers are indicated in kd. (B) The translation products from poly $(A)^+$ RNA of immature oocytes were immunoprecipitated with an anti-cyclin B antibody (α-cycB) or a rabbit anti-mouse IgG (cont.). The immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

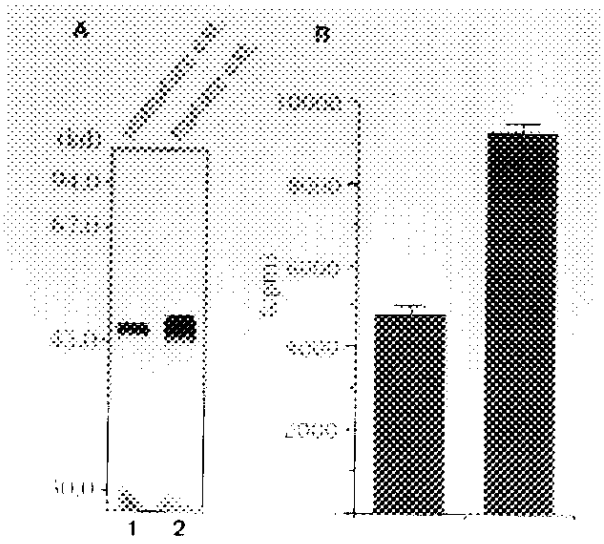

Fig. 4. Inhibition of cyclin B mRNA translation by goldfish oocyte extracts. (A) Extracts from immature oocytes (lane 1) or mature oocytes (lane 2) were mixed with cyclin B mRNA, followed by translation *in vitro* in the nuclease-treated rabbit reticulocyte lysate. The translational products were analyzed by SDS-PAGE and autoradiography. (B) The cyclin B signals in (A) were quantified by a Fuji BAS2000 phosphorimager.

UGUGGGAUUGUUAAAGAGACUUGAGCACUGUGUGCUGUUUUGUAAAAA

CUGUAAUUUAUGCAUUGAUUUUACUGUUGCAAUUUUUUAUUGUUUGAGG

UUUUUACAGAUUUUUUUUUCACCUGUACUGCCAUGUCCUGAAUUAAGG

UGAUAAGUGUUUAUGCAACCCUGUACAUUCUGAUUUCA**AAUAAA**CA

UUUUACCAUUUUUAAGGGTG(A)n

Fig. 5. The 3' untranslated regions of goldfish cyclin B mRNA. Shown are the terminal 209 nucleotides of the 3' UTR of goldfish cyclin B mRNA (Hirai *et al.*, 1992). The polyadenylation sequence AAUAAA is in bold letters, and the U-rich sequences referred to in the text are underlined.

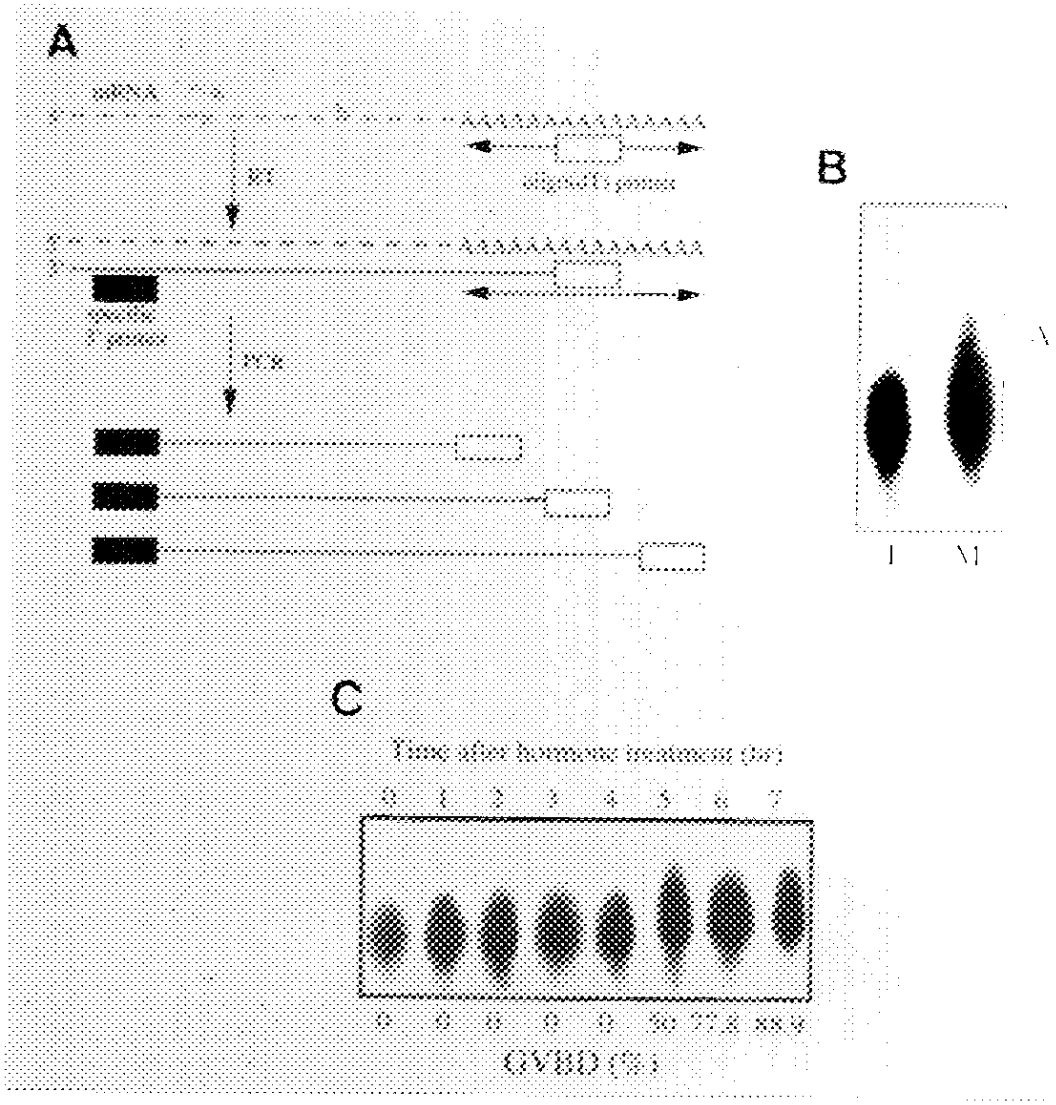

Fig. 6. Polyadenylation of goldfish cyclin B mRNA during oocyte maturation. (A) Shematic representation of the PCR poly (A) test. If an mRNA has long poly (A) tail, the products from anchored PCR amplification using a specific primer and adapter oligo (dT) primer will be various lengths. (B) Autoradiograph of the PCR poly (A) test applied to mRNAs from immature (I) and mature (M) oocytes using a cyclin B specific 5' primer. PCR products were then analyzed by PAGE and Southern blot. (C) Changes in polyadenylation state of cyclin B mRNA during 17α, 20β-DP-induced oocyte maturation in goldfish. The percentage of GVBD in the same experiment is shown below the blots.

A

```
   N   R   N   D   T   K   E   D   V   F   V      amino acid
5' AAC AG(G/A) AAT GAC ACC AA(G/A) GAA GA(T/C) GTG TTT GT 3'  oligonucleotide

5' AAC AGA AAT GAT ACT AAA GAA GAT GTG TTT GT 3'  cDNA
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B

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                ################### ##############
MSY 1:59  GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKN
FRGY1:39  GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKN
FRGY2:44  GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKN
mRNP3:44  GTVKWFNVRNGYGFINRNDSKEDVFVHQTAIKKN
GFYP  :23  GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKN

                #### ######## #######  ## ########
MSY 1:93  NPRKYLRSVGDGETVEFDVVEGEKGAEAANVTGP
FRGY1:73  NPRKYLRSVGDGETVEFDVVEGEKGAEAANVTGP
FRGY2:78  NPRKFLRSVGDGETVEFDVVEGEKGAEAANVTGP
mRNP3:78  NPRKFLRSVGDGETVEFDVVEGEKGAEAANVTGP
GFYP  :57  NPRKFLRSVGDGEVVEFDVVEAAKGSEAANVTGP
```

Fig. 7. Goldfish Y-box protein amino acid. (A) The sequences of the redundant oligonucleotide probe and the corresponding region in the goldfish cDNA clone for Y-box protein. The encoded amino acids are given in one-letter code. (B) Amino acid sequence comparison of the Y-box proteins. A comparison of goldfish Y-box protein (GFYP) with those proteins from mouse (MSY1, Tafuri *et al.*, 1993), and *Xenopus* (FRGY1 and FRGY2, Tafuri and Woffe, 1990), (mRNP3, Murray *et al.*, 1992). The most conserved region is shown. Residuces identical in all Y-box proteins are marked #.

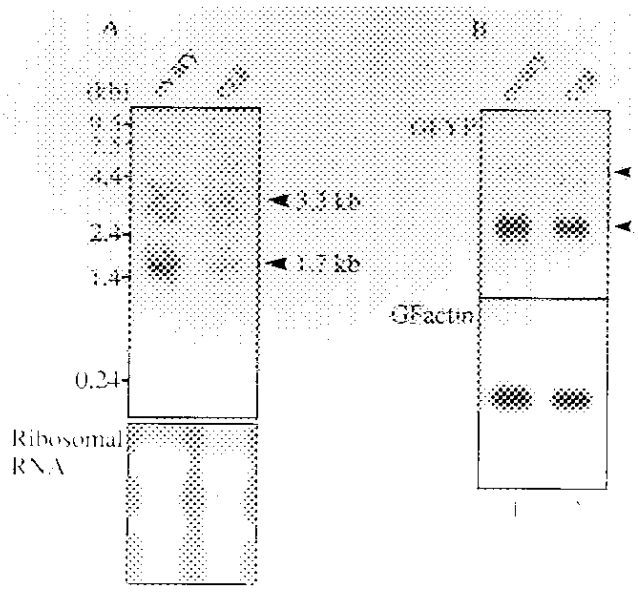

Fig. 8. Expression of goldfish Y-box protein mRNA in goldfish oocytes. Total RNA (20 μg) (A) or poly (A)$^+$ RNA (5μg) (B) from oocytes as indicated was electrophoresed on 1.2 % MOPS agarose gel. After transfer to nitrocellulose, the filter was hybridized to labelled GFYP cDNA insert, wash, and autoradiographed. Goldfish actin cDNA was used as a control.

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Behavior of the Components of Maturation-Promoting Factor, cdc2 Kinase and Cyclin B, during Oocyte Maturation of Goldfish

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We examined the changes that occurred in the two components of maturation-promoting factor (MPF), cdc2 kinase and cyclin B, during oocyte maturation in goldfish, using monoclonal antibodies against the C-terminal sequence of goldfish cdc2 kinase and *Escherichia coli*-produced full-length goldfish cyclin B. Immature oocytes contained a 35-kDa inactive cdc2 kinase. In addition to the 35-kDa form, a 34-kDa active cdc2 kinase was detected in oocytes undergoing germinal vesicle breakdown (GVBD). Cyclin B was absent in immature oocytes and appeared just before GVBD, coinciding exactly with the appearance of the 34-kDa active cdc2 kinase. Precipitation with $p13^{suc1}$ beads and anti-cyclin B antibody revealed that cyclin B formed a complex with cdc2 kinase as soon as it appeared. MPF activation was induced by 1 ng cyclin B after introduction into immature oocytes or oocyte extracts. This corresponds to the amount of cyclin B found in mature oocytes (the concentration in the oocyte is 2 μg/ml). These results suggest that MPF activation in fish oocytes is induced by complex formation with preexisting cdc2 kinase and newly synthesized cyclin B during oocyte maturation, a situation differing from that in *Xenopus* and starfish, in which the cdc2 kinase–cyclin B complex is already present in immature oocytes. Unlike that in *Xenopus*, an inhibition of protein synthesis in unfertilized mature goldfish oocytes caused a decrease in the cdc2 kinase activity/cyclin B protein level and led to a progression from meiotic metaphase to meiotic anaphase. This result indicates that the mechanisms of maintaining MPF activity in mature goldfish oocytes differ from those in *Xenopus*.

INTRODUCTION

Under the influence of maturation-inducing hormone (MIH) secreted from follicle cells, oocyte maturation is finally induced by maturation-promoting factor (MPF), which triggers all the changes that accompany oocyte maturation, such as germinal vesicle breakdown (GVBD), chromosome condensation, and spindle formation (reviewed by Nagahama, 1987a,b; Nagahama and Yamashita 1989). MPF has been purified from mature oocytes of *Xenopus* (Lohka *et al.*, 1988), starfish (Labbé *et al.*, 1989a,b), and carp (Yamashita *et al.*, 1992a) and consists of two components; one is a homolog of the $cdc2^+$ gene product of fission yeast, referred to as cdc2 kinase, and the other is cyclin B (see review by Nurse, 1990; Maller, 1991; Jacobs, 1992). The association of cdc2 kinase with cyclin B is necessary to turn on its protein kinase activity (Desai *et al.*, 1992; Solomon *et al.*, 1990, 1992), and the destruction of cyclin B just before the onset of anaphase turns off the kinase activity (Luca and Ruderman, 1989; Murray *et al.*, 1989; Luca *et al.*, 1991).

In contrast to that in mammals and starfish, the activation of MPF in immature frog and fish oocytes requires *de novo* protein synthesis during the first few hours after MIH treatment (Ford, 1985; Maller, 1985; Goetz, 1983). The proteins that are synthesized at the initial phase of oocyte maturation and induce MPF activation are called initiators, but their chemical natures are not yet understood. The finding that microinjection of cyclin mRNA can induce *Xenopus* oocyte maturation (Swenson *et al.*, 1986; Pines and Hunt, 1987) suggested that one of the initiators is cyclin. However, cyclin B is present in immature *Xenopus* oocytes as a component of inactive MPF (pre-MPF; Gerhart *et al.*, 1984; Cyert and Kirschner, 1988; Gautier and Maller, 1991; Kobayashi *et al.*, 1991) and new cyclin synthesis is not required for inducing oocyte maturation (Minshull *et al.*, 1991). Therefore, proteins other than cyclin should be synthesized during *Xenopus* oocyte maturation. One of the most likely initiators is c-mos, since its synthesis is required and is sufficient for inducing *Xenopus* oocyte maturation (Sagata *et al.*, 1988; 1989; Yew *et al.*, 1992).

To date, detailed examinations of the proteins responsible for inducing oocyte maturation, such as cdc2 kinase, cyclin B, and c-mos during oocyte maturation, have been confined to *Xenopus*. Studies using other species may provide further evidence for the control mecha-

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nisms of MPF activation. In this report, we describe the behavior of the components of MPF, cdc2 kinase and cyclin B, during goldfish oocyte maturation by means of immunoblotting with monoclonal antibodies raised against the C-terminal region of goldfish cdc2 kinase and *Escherichia coli*-produced full-length goldfish cyclin B. We demonstrate that although inactive cdc2 kinase is present, cyclin B is absent in immature oocytes and it appears just before GVBD, in accordance with the appearance of active cdc2 kinase binding to cyclin B. This result indicates that MPF activation is regulated by cyclin B accumulation and its association with the preexisting cdc2 kinase, in contrast to the situation in *Xenopus*, in which MPF activation is controlled by post-translational modifications (phosphorylation and dephosphorylation) of cdc2 kinase and cyclin B (Gautier and Maller, 1991; Kobayashi *et al.*, 1991). We also describe changes in cdc2 kinase activity, the cyclin B protein level, and cell cycle progression, which were induced in unfertilized mature goldfish oocytes treated with the protein synthesis inhibitor, cycloheximide. The induced changes are remarkably different from those reported for other species, suggesting that species-specific mechanisms are responsible for stabilizing MPF activity in unfertilized mature oocytes.

MATERIALS AND METHODS

Animals and Oocytes

Goldfish were purchased commercially and raised at 15°C until use. Full-grown immature oocytes were isolated from ovaries using a pipet and induced to mature *in vitro* by incubation at room temperature in goldfish Ringer's solution (Kagawa *et al.*, 1984) containing 1 μg/ml 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP), a natural MIH in fish (Nagahama and Adachi, 1985). Maturational processes were assessed by immersing the oocytes in a clearing solution (5% formalin and 4% acetic acid in Ringer's; Lessman and Kavumpurath, 1984), facilitating microscopic examination of the occurrence of GVBD.

Preparation of Oocyte Extracts

Oocyte extracts were obtained as described previously (Hirai *et al.*, 1992a). Briefly, oocytes were homogenized with a pestle (Pellet Pestle; Kontes) in 1 μl/oocyte of ice-cold extraction buffer (100 m*M* β-glycerophosphate, 20 m*M* Hepes, 15 m*M* $MgCl_2$, 5 m*M* EGTA, 100 μ*M* *p*-amidinophenylmethanesulfonyl fluoride, 3 μg/ml leupeptin, pH 7.5). The homogenate was centrifuged at 15,000g for 10 min at 4°C, and the supernatant was frozen in liquid nitrogen and kept at −80°C until use.

Production of Monoclonal Antibodies against cdc2 Kinase and Cyclin B

A peptide (GFC3C, CPYFDDLDKSTLPASNLKI), which corresponds to the C-terminal sequence of goldfish cdc2 cDNA (Kajiura *et al.*, unpublished) with an additional cysteine in the N-terminus, was synthesized using the solid-phase method (using the F-moc protocol) on an Applied Biosystems Model 431A peptide synthesizer. The peptide was purified by reverse-phase HPLC and coupled to bovine serum albumin and keyhole limpet hemocyanin through its N-terminal cysteine by *N*-(ε-maleimidocaproyloxy) succinimide (Dojin). The coupled peptides were injected into 6-week-old female BALB/c mice to obtain monoclonal antibodies, according to the procedures described previously (Yamashita *et al.*, 1991). One clone (GFC3C-9) was used to detect cdc2 kinase by immunoblotting. The isotype of this antibody was IgG1 containing κ light chains. This antibody could not precipitate active cdc2 kinase binding to cyclin B.

Anti-cyclin B monoclonal antibodies were raised against *E. coli*-produced full-length goldfish cyclin B. Cyclin B proteins were produced as follows: A cDNA GFCYCB1-1, which encodes full-length goldfish cyclin B and contains *Eco*RI adaptors (Amersham) at both ends of the insert (Hirai *et al.*, 1992a), was digested with *Bam*HI and ligated into the *Bam*HI site of the pET3b expression vector (Studier *et al.*, 1990). The predicted amino acid sequence of the new construct includes the first 12-amino-acid sequence of the T7 gene 10 protein, MASMTGGQQMGR, followed by DPGTMGNLPK, which is derived from the adaptor and the 5′ noncoding region of GFCYCB1-1, and full-length cyclin B. The protein expressed in *E. coli* BL21(DE3) was purified by SDS–PAGE as described (Hirai *et al.*, 1992a) and injected into mice to produce monoclonal antibodies. Two clones (B111 and B112) were selected for this study. Both antibodies are thought to recognize the first 41 amino acids of goldfish cyclin B, since N-terminal truncated cyclin B (Δ41 cyclin B; Hirai *et al.*, 1992a) did not react to these antibodies in immunoblotting. Immunoprecipitation was performed using the B112 antibody (the isotype was IgG2a with a κ light chain) that precipitated the formation of a complex between cyclin B and cdc2 kinase. B111 antibody was used for immunoblotting anti-cyclin B (B112) immunoprecipitates. Since the isotype of the B111 antibody was IgM, anti-IgM as the second antibody could not reveal the IgG heavy chains of the B112 antibody used for immunoprecipitation, which migrated near cyclin B, and disturbed detailed observations (cf. Fig. 2B).

Precipitation with $p13^{suc1}$ and Anti-cyclin B Antibody

Precipitation of cdc2 kinase and cyclin B with $p13^{suc1}$–Sepharose and anti-cyclin B (B112) antibody, re-

spectively, was performed as described previously (Yamashita *et al.*, 1991). Precipitates from 10-μl extracts derived from 10 oocytes were separated by SDS-PAGE and immunoblotted with monoclonal antibodies against cdc2 kinase and cyclin B, as described previously (Yamashita *et al.*, 1991).

Kinase Assay

The activity of cdc2 kinase was measured with a synthetic peptide (SP-peptide, KKAAKSPKKAKK), which is phosphorylated specifically by cdc2 and its related kinases (Yamashita *et al.*, 1992b). The sample was incubated for 2 min at 30°C in the presence of the following: 100 μM SP-peptide, 500 μM ATP, 1.5 μCi [γ-^{32}P]ATP, 1 mM EGTA, 10 mM $MgCl_2$, 4.5 mM β-mercaptoethanol, 20 mM Tris-HCl (pH 8.0). The reaction was stopped with phosphoric acid, and the SP-peptide was adsorbed onto P81 phosphocellulose paper (Whatman). The paper was washed in phosphoric acid, and the radioactivity remaining on the paper was measured by Cherenkov counting.

Microinjection of Cyclin B Protein into Goldfish Oocytes

Fully grown immature oocytes were injected with 20 nl of various concentrations of full-length cyclin B produced in *E. coli*. The injected oocytes were cultured in goldfish Ringer's for 8 hr in the absence or presence of cycloheximide (10 μg/ml) and then fixed in a clearing solution to examine the occurrence of GVBD.

Activation of cdc2 Kinase in a Cell-Free System

Extracts from immature goldfish oocytes were incubated with various concentrations of *E. coli*-produced full-length goldfish cyclin B for 30 min at 25°C. After incubation, the kinase activity was measured as described above.

Cytological Examination

After fixation of oocytes in Bouin's solution, the cytoplasm at the animal pole was skinned from the underlying yolk layer with a fine razor blade under a dissecting microscope. The cytoplasm was embedded in paraffin and the serial sections were stained with Delafield's hematoxylin and eosin to determine the cell cycle progression by means of chromosome and spindle morphology.

RESULTS

Characterization of Antibodies against cdc2 Kinase and Cyclin B

The specificity of the antibodies was examined by immunoblotting highly purified carp MPF (Yamashita *et al.*, 1992a). The purified MPF contained four proteins, with apparent molecular masses of 33, 34, 46, and 48 kDa (Fig. 1, lane 1). Both the 33- and the 34-kDa proteins were recognized by anti-PSTAIR antibody (Fig. 1, lane 2), indicating that they are the cyclin-dependent kinases (Meyerson *et al.*, 1992). A monoclonal antibody against the C-terminal region of goldfish cdc2 kinase reacted with the 34- but not the 33-kDa protein (Fig. 1, lane 3). The latter was recognized with a monoclonal antibody against the C-terminal sequence of goldfish cdk2 kinase (Fig. 1, lane 4; see also Hirai *et al.*, 1992b). Thus, the anti-cdc2 kinase antibody does not react with cdk2 and seems to be specific for cdc2 kinase.

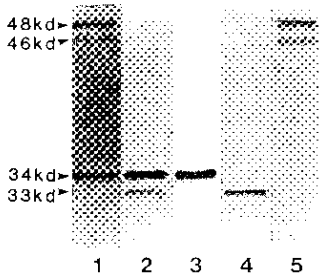

FIG. 1. SDS-PAGE analysis of highly purified MPF from carp eggs. Silver staining (lane 1) and immunoblotting with anti-PSTAIR (lane 2), anti-cdc2 (lane 3), anti-cdk2 (lane 4), and anti-cyclin B (lane 5) antibodies.

The 46- and 48-kDa proteins, which we have already identified as cyclin B using monoclonal antibodies against N-terminal truncated goldfish cyclin B (Yamashita *et al.*, 1992a), were also recognized by anti-full-length goldfish cyclin B antibodies (Fig. 1, lane 5).

Absence of Cyclin B in Immature Goldfish Oocytes

It has been reported that in *Xenopus* (Gautier and Maller, 1991; Kobayashi *et al.*, 1991), starfish (Strausfeld *et al.*, 1991), and clam (Westendorf *et al.*, 1989), immature oocytes contain sufficient cyclin B to induce oocyte maturation. In contrast, we previously reported that immature goldfish oocytes contained no cyclin B detectable by immunoblotting (Hirai *et al.*, 1992a). However, because of the high protein concentration, only a small amount of the sample (corresponding to the extract from 5/8 oocyte) could be analyzed (Hirai *et al.*, 1992a). The new method using two species of antibodies, in which cyclin B was first precipitated with one antibody (IgG) and then immunoblotted with another (IgM), increased the detection sensitivity of cyclin B (see Materials and Methods). We reexamined cyclin B in immature goldfish oocytes using this procedure. However, cyclin B was not detected in immature oocyte extracts,

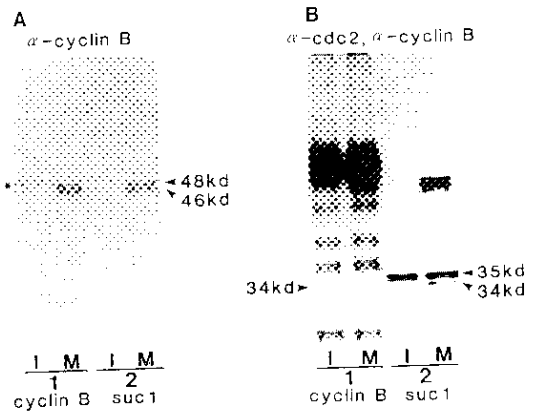

FIG. 2. Cyclin B and cdc2 kinase in goldfish immature (I) and mature (M) oocytes. Anti-cyclin B (A) and anti-cdc2 plus anti-cyclin B (B) immunoblots of anti-cyclin B (lane 1) and $p13^{suc1}$ (lane 2) precipitates. The band with an electrophoretic mobility lower than that of cyclin B, noted by the asterisk, is not cyclin B, but was recognized nonspecifically with the second antibody.

whereas it was in mature oocyte extracts (Fig. 2A, lane 1).

Cyclin B is stored in immature clam oocytes in an insoluble form, which is solubilized during oocyte maturation (Westendorf *et al.*, 1989). To investigate whether immature goldfish oocytes also contain insoluble cyclin B, we extracted immature oocytes by sonication in a buffer containing detergents or a high concentration of salt. However, cyclin B was not detected in the extracts (data not shown), suggesting that immature goldfish oocytes have no stores of cyclin B.

Extracts derived from 10 immature oocytes were analyzed as described, and the amount of cyclin B detected by immunoblotting was 50 pg. Therefore, even if stocks are present in immature oocytes, the cyclin B content in one immature oocyte should be less than 5 pg (the concentration in the oocyte is 10 ng/ml when the oocyte diameter is estimated to be 1 mm). On the other hand, the cyclin B level in one mature oocyte was 1 ng (the concentration is 2 μg/ml). Therefore, cyclin B should increase more than 200 times during oocyte maturation.

Cyclin B in mature goldfish oocytes migrated as two distinct bands on SDS-PAGE with apparent molecular masses of 46- and 48-kDa, as found in purified carp MPF (Fig. 1). The two bands were equally recognized by five species of monoclonal antibodies raised against *E. coli*-produced goldfish cyclin B (data not shown), indicating that they are the same molecule with different chemical modifications or are highly homologous proteins. Since the two bands were equally labeled with ^{32}P (Yamashita *et al.*, 1992a) and phosphatase digestion did not promote conversion of the two bands into one (data not shown), it is unlikely that the two bands are differently phosphorylated forms of the same molecule. Although we cannot exclude the possibility that the two bands have different functions (see also Discussion), we do not distinguish between them in this study.

Appearance of 34-kDa Active cdc2 Kinase in Mature Oocytes

In a previous study using immunoblotting with the anti-PSTAIR monoclonal antibody, we were unable to find any remarkable changes in cdc2 kinase during oocyte maturation (Hirai *et al.*, 1992a). However, it has been revealed that the anti-PSTAIR antibody also reacts to cdc2-related proteins, including cdk2 kinase, which has the same electrophoretic mobility as cdc2 kinase (Hirai *et al.*, 1992b). Thus, it is plausible that changes in cdc2 kinase during oocyte maturation were masked behind the cdk2 kinase also visualized by the PSTAIR antibody. We investigated changes in cdc2 kinase more definitively during oocyte maturation, using the new monoclonal antibody specific to cdc2 kinase (Fig. 1). Immature oocytes contained a 35-kDa cdc2 kinase, whereas mature oocytes contained a 34-kDa cdc2 kinase, in addition to the 35-kDa form (Fig. 2B, lane 2). As described, the purified MPF contained the 34- but not the 35-kDa cdc2 kinase, which was eliminated at the first step of the purification with a Q-Sepharose Fast-Flow anion exchange column (Fig. 1, see also Yamashita *et al.*, 1992a). In addition, anti-cyclin B immunoprecipitates from mature oocyte extracts, which contained only the 34-kDa cdc2 kinase (Fig. 2B, lane 1), had high kinase activity (data not shown), whereas anti-PSTAIR immunoprecipitates from mature oocyte extracts, which contained mainly the 35-kDa cdc2 kinase, had no kinase activity (Yamashita *et al.*, 1991). Therefore, it is concluded that the 35- and 34-kDa cdc2 kinase are the inactive and active forms, respectively.

Association of Cyclin B with cdc2 Kinase

The finding that immature goldfish oocytes contain an inactive cdc2 kinase but no cyclin B (Fig. 2) shows that in goldfish the association of inactive cdc2 kinase and cyclin B might be a step regulating MPF activation, whereas in *Xenopus* and starfish the cdc2 kinase–cyclin B complex is already present in immature oocytes (Gautier and Maller, 1991; Kobayashi *et al.*, 1991; Strausfeld *et al.*, 1991). We examined the complex formation of cdc2 kinase and cyclin B during goldfish oocyte maturation by precipitation with $p13^{suc1}$ and anti-cyclin B antibody. Cyclin B was found in the $p13^{suc1}$ precipitates from mature but not from immature oocytes (Figs. 2A and 2B, lane 2), indicating the formation of a complex between cdc2 kinase and cyclin B in mature oocytes. Anti-cyclin B immunoprecipitates from mature oocyte extracts con-

tained the 34-kDa active but not the 35-kDa inactive form (Fig. 2B, lane 1). This result demonstrates that the cdc2 kinase which binds to cyclin B in mature oocytes is mainly the 34-kDa active form.

Changes in cdc2 Kinase and Cyclin B Protein Levels during Oocyte Maturation

To further investigate cdc2 kinase and cyclin B protein levels during oocyte maturation, oocyte extracts at various times after the addition of 17α,20β-DP were precipitated with either p13^{suc1} or anti-cyclin B antibody and immunoblotted with anti-cdc2 kinase and anti-cyclin B antibody. As described above, immature oocytes contained the 35-kDa inactive cdc2 kinase but no cyclin B, and mature oocytes contained both the 35-kDa inactive and the 34-kDa active cdc2 kinases and cyclin B (Figs. 3A and 3C). The appearance of the 34-kDa active cdc2 kinase coincided with the appearance of cyclin B just before GVBD (Figs. 3A and 3C). Anti-cyclin B immunoblots of the p13^{suc1} precipitates (Fig. 3B) and anti-cdc2 kinase immunoblots of anti-cyclin B immunoprecipites (Fig. 3D) showed that the binding of cdc2 kinase and cyclin B coincided with the appearance of cyclin B and the 34-kDa active cdc2 kinase.

We previously showed that the cyclin B which appeared during oocyte maturation was labeled with [^{35}S]-methionine (Hirai *et al.*, 1992a), demonstrating *de novo* synthesis during oocyte maturation. On the other hand, anti-cyclin B immunoprecipitates from mature oocyte extracts sometimes contained the 35-kDa inactive cdc2 kinase (Hirai *et al.*, 1992a), and the 35-kDa cdc2 kinase, as well as the 34-kDa form, can bind to cyclin B in a cell-free system (Yamashita *et al.*, unpublished). Therefore, it is most likely that the 35-kDa inactive cdc2 kinase binds to *de novo* synthesized cyclin B at first and then is rapidly converted into the 34-kDa active form.

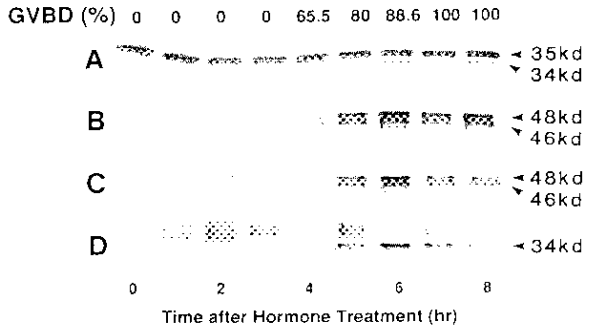

FIG. 3. Changes in cdc2 kinase (A and D) and cyclin B (B and C) protein levels during goldfish oocyte maturation induced by 17α, 20β-DP. Anti-cdc2 (A and D) and anti-cyclin B (B and C) immunoblots of p13^{suc1} (A and B) and anti-cyclin B (C and D) precipitates.

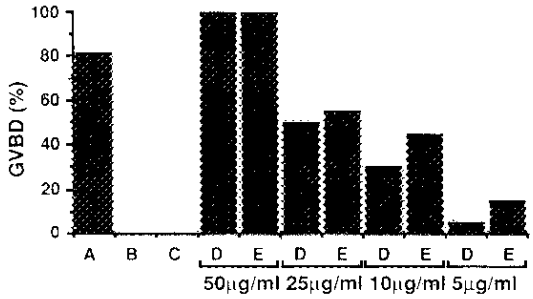

FIG. 4. Induction of oocyte maturation by *E. coli*-produced cyclin B protein in the absence of protein synthesis. Oocytes were treated with 17α,20β-DP or injected with the indicated amount of cyclin B protein and then scored for GVBD 8 hr later. (A) 17α,20β-DP; (B) 17α,20β-DP + cycloheximide; (C) no cyclin B protein (1 m*M* Hepes, pH 7.0); (D) cyclin B protein; (E) cyclin B protein + cycloheximide.

Induction of MPF Activation by Cyclin B Protein

These results indicate that the appearance of cyclin B is required and is sufficient for inducing oocyte maturation in goldfish. To confirm that the appearance of cyclin B is sufficient for inducing oocyte maturation, purified *E. coli*-produced full-length goldfish cyclin B protein was injected into immature oocytes. Even under conditions of inhibited protein synthesis, injected cyclin B induced oocyte maturation within 1 hr after injection, in a dose-dependent manner (Fig. 4). Injection of 1 ng of cyclin B fully induced GVBD in the recipient oocytes. The concentration of cyclin B within the injected oocyte was estimated to be 2 μg/ml. This is about equal to the cyclin B concentration in mature oocytes, as described above.

The introduction of cyclin B protein into immature oocyte extracts also induced MPF activation and the activation occurred in an almost "all or nothing" manner (Fig. 5; see also Hirai *et al.*, 1992a). The threshold concentration of cyclin B for inducing the activation was around 2 μg/ml, equivalent to that for inducing oocyte maturation by injection. These results demonstrated that the presence of 2 μg/ml cyclin B, corresponding to the concentration in mature oocytes, is sufficient for inducing oocyte maturation.

MPF Activity in Cycloheximide-Treated Mature Oocytes

In both *Xenopus* and fish, mature oocytes are arrested at the second meiotic metaphase until fertilized (cf. Fig. 7A). When they are inseminated or artificially activated, the kinase activity maintained at a high level during arrest is precipitated, accompanying cyclin B destruction (Minshull *et al.*, 1989; Murray and Kirschner, 1989; Murray *et al.*, 1989; Hirai *et al.*, 1992a; Yamashita *et al.*, 1992b). Unfertilized mature *Xenopus* oocytes can

maintain a high MPF level, even if protein synthesis is inhibited (Gerhart *et al.*, 1984). We examined whether unfertilized mature goldfish oocytes can resist protein synthesis inhibition. When protein synthesis was inhibited by 20 μg/ml cycloheximide, the kinase activity fell to 50%–70%, according to the batch, of the original activity within 30 min after the exposure (Fig. 6A). The control mature oocytes without cycloheximide maintained high kinase activity level under the same culture conditions (data not shown). In accordance with the drop in kinase activity, the cyclin B protein level also decreased in the cycloheximide-treated oocytes, but did not disappear completely (Fig. 6B). After the sudden drop in the kinase activity and cyclin B protein level within 30 min after the treatment, the remaining activity and cyclin B were maintained even after a prolonged incubation of the oocytes with cycloheximide (Fig. 6B). When mature goldfish oocytes are activated, the kinase activity decreases to 1/10 of the initial value (Yamashita *et al.*, 1992b), and the cyclin B level drops below the detection limit of immunoblotting (Hirai *et al.*, 1992a). Thus, the decreases in the kinase activity and the cyclin B level induced by cycloheximide treatment are partial compared with that occurring upon normal activation. Cytological examination of the cycloheximide-treated mature oocytes showed that the cell cycle was liberated from the arrest at the second meiotic metaphase within 30 min and proceeded to the second meiotic anaphase, where it was arrested again (Figs. 7B and 7C). Therefore, the cell cycle progression is also partial in the cycloheximide-treated oocytes compared with normally activated mature oocytes, in which the cell cycle proceeds to interphase (Yamashita *et al.*, 1990). These results show that cdc2 kinase activity, cyclin B protein level, and cell cycle progression are closely linked. Furthermore, it is suggested that the mechanisms of maintaining MPF activity in unfertilized mature goldfish oocytes differ from those in mature *Xenopus* oocytes, in addition to a difference in the mechanisms of MPF activation.

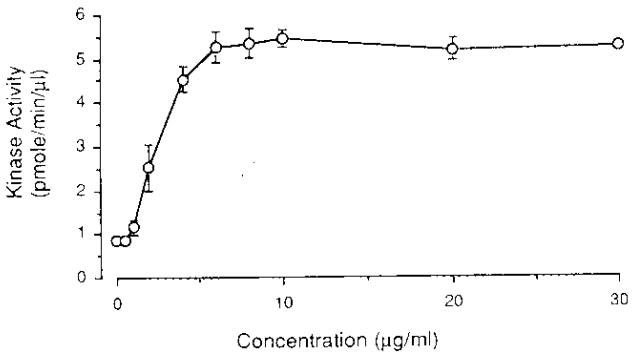

FIG. 5. Activation of cdc2 kinase with *E. coli*-produced cyclin B in a cell-free system. Immature oocyte extracts were treated with varying concentrations of full-length cyclin B for 30 min at 25°C, and then the kinase activity was measured using a synthetic peptide (SP-peptide), which is specifically phosphorylated with cdc2 and its related kinases. Mean ± standard deviation (n = 2).

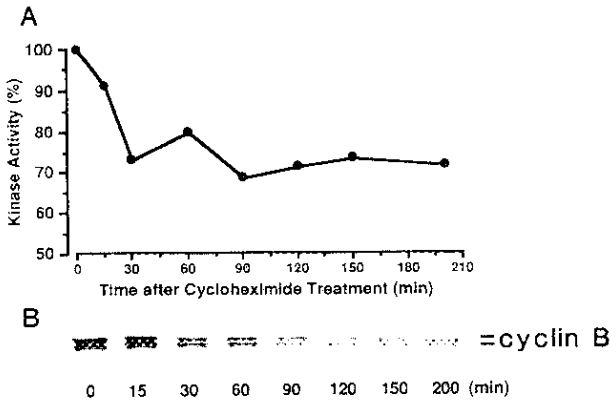

FIG. 6. Decrease in cdc2 kinase activity (A) and cyclin B protein level (B) in mature oocytes treated with protein synthesis inhibitor. Oocytes matured *in vitro* were continuously treated with 20 μg/ml cycloheximide and the kinase activity and cyclin B protein levels were examined at the indicated times.

DISCUSSION

MPF consists of cdc2 kinase and cyclin B (Draetta *et al.*, 1989; Dunphy *et al.*, 1988; Gautier *et al.*, 1988, 1990; Labbé *et al.*, 1989a,b; Yamashita *et al.*, 1992a). To understand how MPF is activated, we examined the time course of cdc2 kinase and cyclin B protein levels during goldfish oocyte maturation, using monoclonal antibodies specific to goldfish cdc2 kinase and cyclin B (Figs. 1–3). We obtained the following results. Cyclin B is absent in immature oocytes and, probably by means of *de novo* protein synthesis, it appears just before GVBD. Immature oocytes contain only the 35-kDa inactive cdc2 kinase, but in accordance with the appearance of cyclin B, the 34-kDa active cdc2 kinase that forms a complex with cyclin B (MPF) appears in maturing oocytes. These results indicate that cyclin B synthesized during oocyte maturation forms a complex with preexisting 35-kDa inactive cdc2 kinase and activates it, which is accompanied by a shift in the electrophoretic mobility of cdc2 kinase from 35- to 34-kDa. In this case, MPF activation should be controlled by the level of cyclin B accumulation and the complex formation of cdc2 kinase and cyclin B. These mechanisms of MPF activation in fish apparently differ from those in *Xenopus* (Gautier and Maller, 1991; Kobayashi *et al.*, 1991) and starfish (Strausfeld *et al.*, 1991), in which cyclin B is present in immature oocytes and forms a complex with cdc2 kinase (pre-MPF).

The cdc2 kinase is activated by post-translational

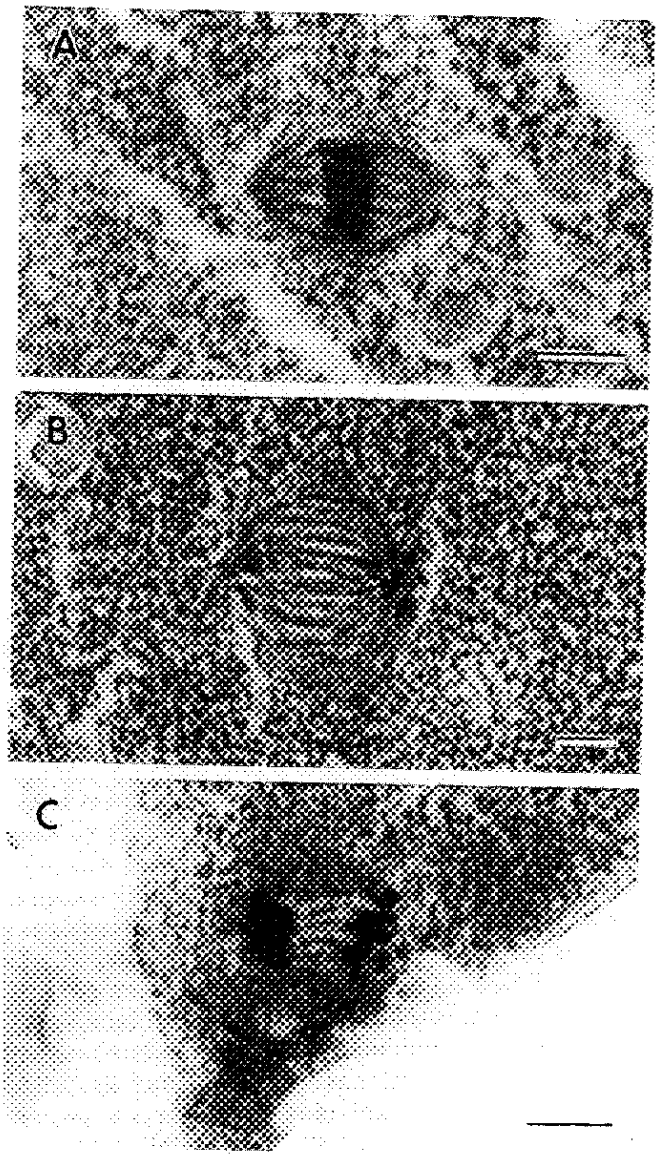

FIG. 7. Cytological examination of mature oocytes treated with protein synthesis inhibitor. The cell cycle arrested at metaphase II (A) was released and proceeded to anaphase (B and C). Bar 5 μm. (A) Untreated, 60 min; (B) cycloheximide, 20 min; (C) cycloheximide, 60 min.

modifications including phosphorylation and dephosphorylation after binding to cyclin (see review by Jacobs, 1992). Therefore, it is plausible that cdc2 kinase undergoes chemical modifications in maturing fish oocytes after binding to cyclin B, although the complex formation of cyclin B and cdc2 kinase during maturation is a critical step for activating cdc2 kinase in these oocytes. Actually, this has shown that the activation of cdc2 kinase is accompanied by a shift in its electrophoretic mobility from 35- to 34-kDa (Figs. 2 and 3). We previously showed that the 34-kDa cdc2 kinase, but not the 35-kDa form, was labeled on threonine residues when oocytes matured in the presence of ^{32}P (Yamashita *et al.*, 1992a). Thus, it is likely that threonine phosphorylation of cdc2 kinase, which may cause the electrophoretic mobility shift, is involved in the activation of cdc2 kinase. Indeed, recent studies using mutant cdc2 kinases have demonstrated that in addition to dephosphorylation of Thr14 and Tyr15, phosphorylation of Thr161 is required for cdc2 activation (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Solomon *et al.*, 1992). We are now investigating whether similar control mechanisms for MPF activation work after cdc2 kinase binds to cyclin B in maturing fish oocytes, using mutant goldfish cdc2 kinases.

In contrast to *Xenopus* (Gautier and Maller, 1991; Kobayashi *et al.*, 1991), goldfish cyclin B is not stockpiled in immature oocytes (this study), and it is abruptly synthesized during oocyte maturation (Hirai *et al.*, 1992a). Thus, cyclin B is still a potential initiator of goldfish oocyte maturation. If so, synthesis of cyclin B is required and is sufficient for inducing oocyte maturation in goldfish. Cyclin B is sufficient for inducing goldfish oocyte maturation, as demonstrated by inducing MPF activation by cyclin B proteins both *in vivo* (Fig. 4) and *in vitro* (Fig. 5). To confirm the requirement of cyclin B during goldfish oocyte maturation, we investigated the effects of antisense oligonucleotides on 17α,20β-DP-induced oocyte maturation. So far, however, we have been unable to obtain an inhibitory effect that is significantly different between sense and antisense oligonucleotide injections. Therefore, it is premature to say that cyclin B is an initiator of goldfish oocyte maturation. Furthermore, the involvement of c-mos, one of the candidates for the initiator of *Xenopus* oocyte maturation, in goldfish oocyte maturation remains unknown.

In unfertilized mature *Xenopus* oocytes, MPF activity is maintained at a high level, even if protein synthesis is inhibited by cycloheximide (Gerhart *et al.*, 1984). The stability of MPF activity depends on a cytostatic factor (CSF; Gerhart *et al.*, 1984; Newport and Kirschner, 1984; Murray *et al.*, 1989). Since c-mos, a probable component of CSF, is not affected by protein synthesis inhibition (Watanabe *et al.*, 1989), this protein seems to be involved in stabilizing MPF in cycloheximide-treated mature oocytes. Unlike *Xenopus*, the cycloheximide-treated unfertilized mature goldfish oocytes caused a drop in MPF activity to 50–70% of its initial level (Fig. 6) and an exit from metaphase followed by arrest at anaphase (Fig. 7). Release from metaphase arrest caused by protein synthesis inhibitors has been reported in the mouse (Clarke and Masui, 1983) and mollusk (*Patella*, Loon *et al.*, 1991). However, in those species, the cell cycle is not arrested at anaphase but reaches interphase. The difference in

the sensitivity of MPF to protein synthesis inhibitors indicates the existence of species-specific mechanisms that maintain MPF activity in unfertilized mature oocytes. The mechanisms functioning in *Xenopus* are independent of protein synthesis, whereas those in the mouse and mollusk are dependent. Mature goldfish oocytes may be equipped with both mechanisms. Since cyclin B continues to be synthesized in unfertilized mature goldfish oocytes (Hirai *et al.*, 1992a), the partial decrease in the cyclin B protein level found in cycloheximide-treated oocytes may be due to an interruption of the continuous supply of cyclin B. The presence of molecular mechanisms of maintaining MPF activity independent of protein synthesis is not certain, but c-mos may contribute to them, as it works in unfertilized mature *Xenopus* oocytes (Watanabe *et al.*, 1989).

It is notable that a 30–50% decrease in the kinase activity and cyclin B protein level induces cell cycle progression to some extent (from metaphase to anaphase). This finding indicates a close correlation between the kinase activity/cyclin B protein level and cell cycle progression. The mechanism that links the kinase activity and the cell cycle progression is not certain, but, as proposed for the cell cycle control in somatic cells (see review by Jacobs, 1992), one possibility is that plural cyclins are involved at each step of the cell cycle. It is possible that goldfish oocytes at the second meiotic metaphase contain two types of cyclin B. One is sensitive to protein synthesis inhibition and its destruction may be responsible for inducing an exit from metaphase, and the other is insensitive to the inhibitors and its destruction may induce further progression of the cell cycle toward interphase. Indeed, we isolated two species of cyclin B, the homology between which is more than 95% (Hirai *et al.*, 1992a). These cyclins may have different functions in initiating oocyte maturation and maintaining MPF activity, irrespective of their high homology. Further studies are required to understand the precise roles of each player involved in oocyte maturation, as well as to identify new players.

MPF, consisting of a cdc2 kinase-cyclin B complex, is a universal factor that promotes oocyte maturation (Kishimoto, 1988). As this study shows, however, the mechanisms of MPF activation and its stabilization vary among species, in spite of the involvement of the same players. Despite the impressive progress in recent years, there is still much to be learned about the control of oocyte maturation. Further studies using various species should provide comprehensive understanding of the control mechanisms of oocyte maturation.

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Isolation and Characterization of Goldfish cdc2, a Catalytic Component of Maturation-Promoting Factor

(oocyte maturation/cell cycle/molecular cloning/monoclonal antibody/gel filtration)

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We have isolated a cdc2 cDNA from a library constructed from immature goldfish oocytes. The isolated clone has a PSTAVR sequence, instead of the PSTAIR sequence common to cdc2 in other species. Its product was characterized by monoclonal antibodies against its C-terminal amino acid sequence. The antibodies recognized an anti-PSTAIR-reactive 35 kDa protein in immature oocyte extracts, which was not recognized by anti-goldfish cdk2 antibody. In addition to the 35 kDa cdc2, mature oocytes contained a 34 kDa cdc2, which was a component of MPF purified from carp eggs. Upon gel filtration column, the 35 kDa cdc2 migrated at monomeric position, while the 34 kDa cdc2 migrated at around 100 kDa, where cyclin B also comigrated. These results strongly suggest that the 35 kDa protein is monomeric inactive cdc2, while the 34 kDa protein is cyclin B-bound active cdc2. The finding that the 35 kDa inactive cdc2 does not form a complex with any other proteins in immature oocytes is in contrast to the situation in *Xenopus* and starfish, in which cdc2-cyclin B complex exists already as pre-MPF in immature oocytes.

Introduction

Oocyte maturation is induced by maturation-promoting factor (MPF) under the influence of maturation-inducing hormone secreted from follicle cells (19, 20, 21). MPF has no species specificity, and it is not only responsible for inducing oocyte maturation but is a universal regulator of the G2/M transition in eukaryotic cell cycle (8). To date, MPF has been purified from eggs of *Xenopus* (13), starfish (10, 11) and carp (31). MPF is composed of two subunits. A catalytic subunit is a serine/threonine protein kinase, which is a homolog of the fission yeast (*Schizosaccharomyces pombe*) $cdc2^+$ gene product, and a regulatory subunit is cyclin B, which was first discovered in the early embryos of marine invertebrates (6, 15, 23).

The behavior of the components of MPF, cdc2 and cyclin B, during oocyte maturation must be known to understand the molecular mechanisms of MPF activation that lead the oocytes to mature, *via* germinal vesicle breakdown, chromosome condensation and spindle formation. To do this, we produced a monoclonal antibody against the PSTAIR sequence of cdc2, which recognized all eukaryotic cdc2 proteins examined so far (30). Using this antibody, we examined changes in anti-PSTAIR-reactive proteins during oocyte maturation of goldfish, but we were unable to find any remarkable changes (4). However, the PSTAIR sequence, which was thought to be a hallmark of cdc2, is also conserved in cdc2-like proteins, such as cdk2 and cdk3 (16). Therefore, it should be necessary to use antibodies specific to cdc2 for the detailed examination of changes in cdc2 proteins during oocyte maturation.

To obtain antibodies specific to goldfish cdc2 protein, we first isolated a partial cdc2 cDNA clone by polymerase chain reaction (PCR), then isolated a full-length cDNA from a library constructed from immature goldfish oocytes. In this report, we describe the isolation and sequence of goldfish cdc2 cDNA clone, and characterize its product with monoclonal antibodies against the C-terminal amino acid sequence.

Materials and Methods

Extraction of oocytes: Goldfish (*Carassius auratus*) was purchased from a dealer, and raised at 15°C until use. Full-grown immature oocytes were isolated from ovaries by pipetting. Mature oocytes (ovulated eggs) were obtained by injecting 200 IU of human chorionic gonadotropin (Puberogen, Sankyo Zoki; 29). Immature and mature oocytes were extracted with an extraction buffer containing 100 mM β-glycerophosphate and 5 mM EGTA by ultracentrifugation, as described previously (30).

PCR: Total RNA was prepared from full-grown immature goldfish oocytes by acid guanidium thiocyanate-phenol-chloroform (AGPC) extraction method (1). Poly(A)$^+$ RNA was isolated by oligo(dT)-Latex beads (Oligotex-dT30, Takara). Single-strand cDNA was synthesized by incubating poly(A)$^+$ RNA with 1 μg of oligo-dT primer (12–18dT), 0.5 mM each of dATP, dCTP, dGTP and dTTP, 10 mM dithiothreitol (DTT), 5 U of reverse transcriptase (RAV-2, Takara), and 10 U of human placental ribonuclease inhibitor.

To isolate cdc2 homolog from goldfish cDNA library, we produced two degenerate PCR primers, which contains *Bam*HI recognition site at the 5′ end, with Applied Biosystems DNA Synthesizer model 391: 5′ primer, 5′-TAAGGATCCGGNACNTAYGGNGTNGTNTAYAA-3′, and 3′ primer, 5′-TAAGGATCCRTTYTGNGGYTTNARRTCNCKRTG-3′ (Y=C+T, R=A+G, N=A+C+G+T, K=G+T, *Bam*HI site is underlined). The 5′ and 3′ primers correspond to the amino acid sequences, GTYGVVYK and HRDLKPQN, respectively (Fig. 1A).

PCR was carried out in 50 μl reaction mixture consisting of 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 2.5 U Taq DNA polymerase (Perkin Elmer), 50 ng single strand cDNA, and 1 μM each primers. The first run of PCR was 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. After the run, 2.5 U Taq DNA polymerase was added to the reaction mixture, and further reacted at the same condition as the first run.

Isolation of goldfish cdc2 cDNA: PCR products were fractionated on 5% polyacrylamide gel, and digested with *Bam*HI. The isolated DNA fragments were subcloned into a plasmid vector pBluescript II KS M13$^-$ (Stratagene) and sequenced using the dideoxy-sequencing methods (BcaBEST sequencing kit, Takara). The PCR product, which had the strongest homology to the conserved domain of cdc2, was used to isolate a full-length cDNA clone.

To isolate a full-length cDNA, a λgt10 cDNA library constructed from full-grown immature goldfish oocytes was screened with a 24-mer oligonucleotide, which was selected from the sequence of the PCR product (Fig. 1A). Approximately 2×10^5 of plaques were transferred onto a nylon membrane (Hybond-N$^+$, Amersham), and hybridized with 2×10^6 cpm ^{32}P end-labeled probe at 60°C overnight in 20 ml of hybridization solution containing 5×SSC (1×SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5×Denhardt's reagent (1 mg/ml each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS), and 200 μg/ml herring sperm DNA. Membrane were washed twice at 55°C for 30 min with 1×SSC containing 0.1% SDS. Isolated clones were subcloned into pBluescript II KS M13$^+$, and serial deletion mutants were produced with exonuclease III. The sequence data obtained with the dideoxy sequencing method were analyzed on GENETYX software (SDC).

Northern blotting: Poly(A)$^+$ RNA was prepared from full-grown immature and mature oocytes, as described above. One microgram of poly(A)$^+$ RNA was electrophoresed in 1.1% agarose gel containing 6% formaldehyde, with a running buffer consisting of 20 mM MOPS (pH 7.0), 5 mM EDTA and 8 mM sodium acetate. RNA was transferred onto Hybond-N$^+$, and hybridized with 1×10^7 cpm of 35-mer oligonucleotide probe (Fig. 1A) at 60°C overnight. Membrane was washed twice at 55°C for 30 min with 1×SSC containing 0.1% SDS, and analyzed by a BAS 2000 Bio-Image Analyzer (Fuji).

Monoclonal antibodies against the C-terminal sequence of cdc2: A peptide, CPYFDDLDKSTLPASNLKI, which corresponds to the C-terminal sequence of goldfish cdc2 with an additional cysteine in the N-terminus (Fig. 1A), was synthesized using the F-moc protocol on an Applied Biosystem model 431A peptide synthesizer. The peptide was purified by reverse-phase HPLC and conjugated to bovine serum albumin and keyhole limpet hemocyanin through its N-terminal cysteine by N-(ε-maleimidocaproyloxy)succinimide (Dojin). The coupled peptides were injected to 6-week-old female BALB/c mice to obtain monoclonal antibodies, according to the procedures described

A

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                                                                                CTCACGCTCTCTCTGTTCT   19

CAAAGACTTTCATAAAGATATTGGAAGGAAAATAGAAGAGTTTGGTTATTCATCCATATTCGTATATAGTTACAGAGAAGTATTGAGAAA  109

ATGGATGACTATCTGAAGATAGAGAAAATTGGTGAAGGTACATATGGTGTGGTATATAAGGGCAGGAATAAAACCACTGGACAAGTGGTT  199
 M  D  D  Y  L  K  I  E  K  I  G  E  G  T  Y  G  V  V  Y  K  G  R  M  K  T  T  G  Q  V  V   30

GCTATGAAGAAGATCCGCCTGGAGAGTGAGGAGGAGGGAGTTCCCAGCACCGCTGTCAGAGAAATCTCCCTCCTCAAGGAGCTCCAGCAT  289
 A  M  K  K  I  R  L  E  S  E  E  E  G  V  P  S  T  A  V  R  E  I  S  L  L  K  E  L  Q  H   60

CCCAACGTTGTACGCCTGCTGGATGTATTGATGCAGGAGTCAAAGTTATACCTGGTGTTTGAATTTCTGTCCATGGATCTGAAGAAGTAC  379
 P  N  V  V  R  L  L  D  V  L  M  Q  E  S  K  L  Y  L  V  F  E  F  L  S  M  D  L  K  K  Y   90

TTGGACTCCATCCCATCAGGCCAGTTCATGGATCCCATGCTTGTCAAGAGTTACCTGTATCAGATCCTTGAGGGGATTCTGTTCTGCCAC  469
 L  D  S  I  P  S  G  Q  F  M  D  P  M  L  V  K  S  Y  L  Y  Q  I  L  E  G  I  L  F  C  H  120

TGTCGCAGGGTTCTGCATCGTGACCTAAAGCCCCAGAATCTGCTGATTGATAATAAAGGTGTGATTAAGCTGGCAGACTTTGGGCTGGCA  559
 C  R  R  V  L  H  R  D  L  K  P  Q  N  L  L  I  D  N  K  G  V  I  K  L  A  D  F  G  L  A  150

CGTGCCTTTGGAGTCCCGGTCAGAGTGTACACACATGAGGTGGTCACTCTGTGGTACAGAGCTCCAGAAGTCTTGCTGGGGGCCTCACGT  649
 R  A  F  G  V  P  V  R  V  Y  T  H  E  V  V  T  L  W  Y  R  A  P  E  V  L  L  G  A  S  R  180

TATTCCACACCAGTAGATGTCTGGAGTATTGGTACCATCTTTGCCGAACTCGCTACAAAGAAACCTCTCTTCCATGGAGACTCAGAAATA  739
 Y  S  T  P  V  D  V  W  S  I  G  T  I  F  A  E  L  A  T  K  K  P  L  F  H  G  D  S  E  I  210

GACCAGCTCTTCAGGATCTTCAGGACTCTTGGAACCCCTAACAATGAGGTCTGGCCAGATGTTGAGTCGCTGCCAGATTATAAGAATACC  829
 D  Q  L  F  R  I  F  R  T  L  G  T  P  N  E  V  W  P  D  V  E  S  L  P  D  Y  K  N  T  240

TTCCCAAAGTGGAAATCTGGGAATCTGGCCAGTACTGTGAAAAACCTGGACAAGAACGGCATTGACCTGCTCACGAAAATGCTGATTTAT  919
 F  P  K  W  K  S  G  N  L  A  S  T  V  K  N  L  D  K  N  G  I  D  L  L  T  K  M  L  I  Y  270

GACCCTCCTAAGCGGATCTCAGCACGGCAAGCAATGACACATCCATATTTTGACGATTTAGACAAGAGCACTCTTCCAGCCAGTAACCTG 1009
 D  P  P  K  R  I  S  A  R  Q  A  M  T  H  P  Y  F  D  D  L  D  K  S  T  L  P  A  S  N  L  300

AAGATATAGACTTCAGTTCTAAAGCAATGGAAGCTTAGTCTTTTGACATGCAATAATTAACCTTTTGCTCATTTGTTTATTGGGTGGCTT 1099
 K  I  *                                                                                      302

TTGTACTTTTCTACTTTTTCTACTTTTGTTTTGTCTTTTTGTATGTCTTTTTCCTATCCTGTTTTATGTTTCATCAAACACCCGTTTGTA 1189

AATAAACATATATATTTTTTTGTGGACCCAAAGTGGCATATTACAAAGGTAAACTGTTCATAATAAAGAACAAAAAAAAAAAAAAAAAAA 1279

AAAAA                                                                                        1284
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B

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Goldfish cdc2       M  D  DYLKIEKIGEGTYGVVYKGRNK  TTGQ VVAMKKIRLESEEEGVPSTAVREISLLKELQHP    NVVRLLDVLMQES KLYLVFEFLS   84
Xenopus cdc2        -  -  E-T-----------------H-  A--- T---------N---------I-----------    -I-C--------D- R---I-----   84
Human cdc2          -  E  --T-----------------H-  ---- -------------------I---------R--    -I-S-Q-----D- R---I-----   84
Goldfish cdk2       -  E  SPQ-V-------------AK--  V--E T--L-----DT-T-------I---------N--    -I-K-H--IHT-N ---------H   84
Xenopus cdk2 (Eg1)  -  E  NPQ-V-------------AR-R  E--E I--L-----DT-T-------I---------N--    -I-K----IHT-N ---------N   84
Human cdk2          -  E  NPQ-V-------------AR--  L--E V--L-----DT-T-------I---------N--    -I-K----IHT-N ---------H   84

Goldfish cdc2       MDLKKYLDSIPSGQF MD PMLVKSYLYQILEGILFCHCRRVLHRDLKPQNLLIDNKGVI KLADFGLARAFGVPVRVYTHEVVTLWYRAPEVLLG  177
Xenopus cdc2        --------------Y I- T--------------V---S----------------S---- ------------I---------------------  177
Human cdc2          ------------P--F -- SS-------------V---S----------------D--T- ------------I-I-------------S------  177
Goldfish cdk2       Q---RFM--STVTGI SL - ------F-L-Q-LA---SH--------------NAQ-E- ----------------T--------------I---  176
Xenopus cdk2 (Eg1)  Q----FM-GSNISGI SL A ------F-L-Q-LA---SH--------------NSD-A- -------------T--TF-------------I---  176
Human cdk2          Q----FM-ASALTGI PL - -I----F-L-Q-LA---SH--------------NTE-A- ----------------T--------------I---  176

Goldfish cdc2       ASRYSTPVDVWSIGTIFAELATKKPLFHGDSEIDQLFRIFRTLGTPNNEVWPDVESLPDYKNTFPKWKSGNLASTVKNLDKNGIDLLTKMLIYDPP  273
Xenopus cdc2        SV----------------I---------------------A----------E----Q----S-----G-S-SAN---I--D-L----------A  273
Human cdc2          SA-------I-------------------------------A----------E----Q----------P-S---H-----E--L---S-------A  273
Goldfish cdk2       CKY---A--I--L-C----MI-R-A--P-----------------DESI--G-T-MP---PS-----ARQD-SKV-PP--ED-R---GQ--I---N  272
Xenopus cdk2 (Eg1)  CKF---A--I--L-C----MI-RRA--P-----------------DEVS--G-TTM----S-----IRQDFSKV-PP--ED-R---AQ--Q--SN  272
Human cdk2          SKY---A--I--L-C----MV-RRA--P-----------------DEV---G-T-M----PS----ARQDFSKV-PP--ED-RS--SQ--H---N  272

Goldfish cdc2       KRISARQAMTHPYFDDLDKSTLPASNLKI              302
Xenopus cdc2        -----RK-LL----------S--DNQIRN              302
Human cdc2          ----GKM-LN----N---NQIKKM                   297
Goldfish cdk2       -----KN-LV-RF-R-VTMPVP-LRL                 298
Xenopus cdk2 (Eg1)  -----KV-L---F-R-VSRPTPHLI                 297
Human cdk2          -----KA-LA--F-Q-VT-PVPHLRL                 298
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Fig. 1 A) Nucleotide and predicted amino acid sequences of goldfish cdc2. Amino acid sequences indicated by underline correspond to PCR primers. Nucleotide sequences remarked by underline are used as probe to isolate full-length cDNA clone. B) Comparison of amino acid sequences of the goldfish cdc2 with cdc2 homologs from *Xenopus* (18) and human (12) and cdk2 homologs from goldfish (5), *Xenopus* (24) and human (2, 22, 26). Identical residues are shown by dashes.

previously (30).

We obtained 11 monoclonal antibodies. Although all antibodies recognized 34 and 35 kDa proteins in mature oocyte extracts in common, one antibody (GFC3-9) recognized these proteins with high specificity (cf. Fig. 2). Thus, this clone was used for further analysis of cdc2 proteins by immunoblotting.

Immunoblotting and p13^{suc1} precipitation: Precipitates from immature and mature oocyte extracts with p13^{suc1}-conjugated beads (30) were analyzed with SDS-polyacrylamide gel electrophoresis (PAGE), followed by immunoblotting with anti-PSTAIR (30), anti-cdk2 (5), or anti-cdc2 C-terminal (GFC3-9) antibodies, as described previously (30).

Gel filtration of oocyte extracts: Five hundred microliters of oocyte extracts were loaded onto a Superose 12 gel filtration column (1×30 cm, Pharmacia) equilibrated in the extraction buffer at a flow rate of 0.5 ml/min, and 0.5 ml fractions were collected. Protein cdc2 in each fraction was precipitated with p13^{suc1} beads and immunoblotted with anti-cdc2 C-terminal antibody and anti-goldfish cyclin B antibody (4).

Results

Isolation of goldfish cdc2 cDNA

To amplify cdc2 cDNA by PCR, we used a pair of degenerate oligonucleotide primers corresponding to highly conserved sequences in cdc2 family, GTYGVVYK and HRDLKPQN (Fig. 1B). According to their sequence homology, the obtained PCR products were categorized into 3 species; cdc2, cdk2 (5), and cdk5 (16, 28). Using the partial cdc2 clone obtained by PCR, we isolated full-length cdc2 cDNA from a λgt10 library constructed from full-grown immature goldfish oocytes. The isolated clone had an insert of 1284 bp containing a poly(A)$^+$ tail and an open reading frame encoding 302 amino acids (Fig. 1A). Northern blot analysis showed a hybridization signal at the 1.3 kbp position (data not shown), indicating that the clone is nearly full-length.

The PSTAIR sequence motif (EGVP-STAIREISLLKE) is a hallmark of cdc2, cdk2 and cdk3 (16), but the isolated clone encodes a PSTAVR sequence, in which isoleucine changes to valine (Fig. 1). To examine whether the PSTAVR sequence is a genuine sequence in goldfish cdc2 or merely an artifact obtained during the cloning procedures, we also used another cDNA library in λZAPII vector (Stratagene), which was independently constructed from mRNA different from that use to construct the λgt10 library. Nevertheless, we obtained the same cDNA clone with the PSTAVR sequence (data not shown). Furthermore, a 2.2 kbp cdc2 genomic DNA fragment isolated from goldfish blood cells also had the PSTAVR sequence (data not shown). Therefore, the PSTAVR sequence is not an artifact but an authentic sequence present in goldfish cdc2.

The predicted molecular weight of the protein encoded by this gene is 34,499. As shown in Fig. 1B, this clone had higher homology with cdc2 (85% for *Xenopus*, 85% for human and 84% for mouse) than cdk2 (67% for goldfish, 66% for

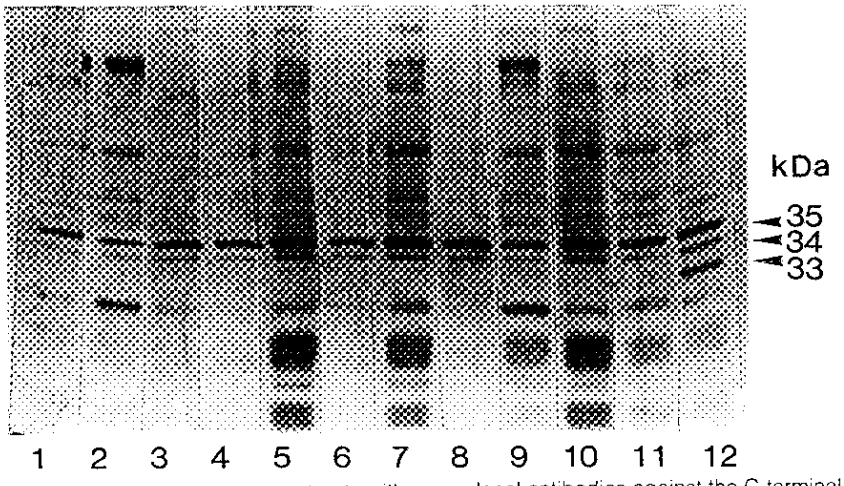

Fig. 2 Immunoblots of crude mature oocyte extracts with monoclonal antibodies against the C-terminal sequence of goldfish cdc2 and the PSTAIR sequence. Lanes 1–11, anti-C-terminal antibodies (GFC3-1, 2, 3, 5, 6, 9, 10, 17, 18, 21, and 22); Lane 12, anti-PSTAIR antibody.

Xenopus and 67% for human) at amino acid sequence level. Therefore, we refer to this clone as goldfish cdc2 cDNA, which was also confirmed by several immunological studies described below.

Characterization of goldfish cdc2

To confirm that the product of cDNA that we identified is cdc2, we produced monoclonal antibodies against the deduced C-terminal amino acid sequence of the cDNA. We obtained 11 monoclonal antibodies, all of which recognized a 35 kDa protein in immature oocyte extracts and 34 and 35 kDa proteins in mature oocyte extracts (Figs. 2 and 3). Both 34 and 35 kDa proteins were recognized by anti-PSTAIR antibody and precipitated with $p13^{suc1}$ (Fig. 3), indicating that they both belong to the cdc2 family. MPF highly purified from carp eggs contained anti-PSTAIR-reactive 33 and 34 kDa proteins (Fig. 4, cf., 31). The 33 kDa protein was cdk2, which did not correspond to MPF activity, whereas the 34 kDa protein was most likely cdc2, since it closely corresponded to the MPF activity throughout the purification steps (32). The anti-cdc2 C-terminal antibodies recognized the 34 kDa protein found in highly-purified MPF (Fig. 4). The 34 kDa protein recognized by the anti-C-terminal antibodies was also found in anti-cyclin B immunoprecipitates from mature oocyte extracts (7), indicating that a complex between the 34 kDa protein and cyclin B can be formed *in vivo*. These immunological studies demonstrated that the product encoded by the cDNA we have isolated has all characteristics belonging to cdc2, and probably encodes goldfish cdc2.

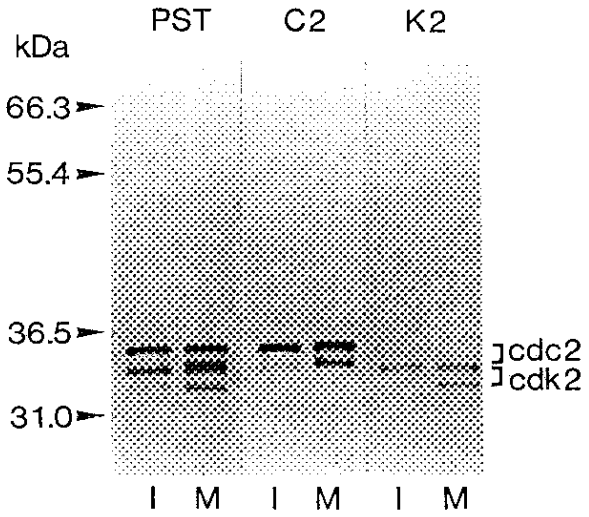

Fig. 3 Immunoblots of $p13^{suc1}$ precipitates from immature (I) and mature (M) goldfish oocyte extracts, probed with anti-PSTAIR antibody (PST), anti-cdc2 C-terminal antibody (C2) and anti-cdk2 C-terminal antibody (K2).

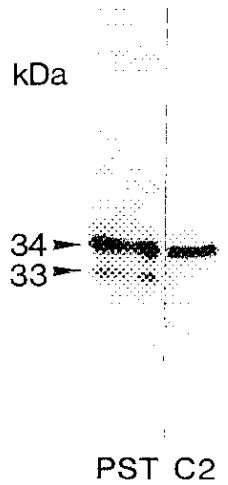

Fig. 4 Immunoblots of MPF purified from carp eggs with anti-PSTAIR antibody (PST) and anti-cdc2 C-terminal antibody (C2).

Anti-PSTAIR reactive 34 and 35 kDa proteins are active and inactive cdc2, respectively

We previously reported that the anti-PSTAIR antibody recognized 33, 34, and 35 kDa bands in crude extracts from immature and mature goldfish oocytes (30, 31). Recently, however, we noticed that the 34 kDa band was sometimes separated into two bands, when SDS-PAGE was carried out at lower current (15 mA instead of 35 mA) (Fig. 3). The anti-PSTAIR-reactive 33 and lower 34 kDa bands were cdk2, as revealed by anti-cdk2 antibody (Fig. 3). The monoclonal antibodies against the C-terminal sequence of goldfish cdc2 recognized the anti-PSTAIR-reactive 35 and upper 34 kDa bands. Since they were also recognized by monoclonal antibodies against mouse cdc2 protein (Yamashita *et al.*, unpublished), it is highly likely that both are cdc2 proteins. Thus, the anti-PSTAIR-reactive 33 and 35 kDa bands are cdk2 and cdc2, respectively, and that the 34 kDa band, which was not well resolved by previous SDS-PAGE, is a mixture of cdk2 and cdc2.

To characterize the two cdc2 proteins found in goldfish oocytes, we examined the changes in these proteins during oocyte maturation. The 35 kDa cdc2 was found in both immature and mature oocyte extracts, but the 34 kDa cdc2 was detectable only in mature oocyte extracts (Fig. 3). Anti-cyclin B immunoprecipitates from mature oocyte extracts, which had high kinase activity, contained mainly the 34 kDa cdc2 (5, 7), while anti-PSTAIR immunoprecipitates, which contained mainly the

35 kDa cdc2, had no kinase activity (30). In addition, the 34 kDa cdc2 was found in highly purified MPF, while the 35 kDa one was not (Fig. 4; cf. 31). Therefore, it is likely that the 34 kDa cdc2 is active and the 35 kDa one is inactive form of cdc2.

Goldfish cdc2 does not form a complex with any proteins in immature oocytes

In immature oocytes of *Xenopus* and starfish, cdc2 protein has already formed a complex with cyclin B as pre-MPF (3, 9, 25). In contrast to these species, we find that there is no detectable cyclin B in immature goldfish oocytes (5, 7). This finding strongly suggests that cdc2 protein in immature goldfish oocytes is monomeric. To confirm this, we examined the consecutive fractions of immature and mature oocyte extracts eluted from gel filtration column, by immunoblotting with anti-cdc2 C-terminal and anti-cyclin B antibodies. When immature oocyte extracts were applied on the gel filtration column, cdc2 protein was eluted as a single peak at the monomeric position around 35 kDa (Fig. 5A). However, cdc2 proteins in mature oocyte extracts were eluted as two peaks at around 100 kDa and 35 kDa. The 34 kDa cdc2 protein was found only in the first peak at 100 kDa, where cyclin B was also detected (Fig. 5B). We have already shown that highly purified MPF and histone H1 kinase consist of a complex of cyclin B and 34 kDa cdc2, which exhibit an apparent molecular weight of 100 kDa on Superose 12 (31, 32). Therefore, the 100 kDa complex of cyclin B and 34 kDa cdc2 found in this study should be active MPF and histone H1 kinase. The vast majority of the inactive 35 kDa cdc2 proteins in mature oocytes migrated at the monomeric position (Fig. 5B). These results demonstrate that most, if not all, cdc2 proteins (inactive 35 kDa form) in immature oocytes are monomeric and that, when oocytes mature, a part of cdc2 proteins form complexes with cyclin B and are activated (active 34 kDa form). It is also notable that a minor fraction of the 35 kDa cdc2 proteins was also detectable at 100 kDa (Fig. 5B). This means that not all cdc2 proteins that bind to cyclin B are activated. Taken together, it is most likely that inactive 35 kDa

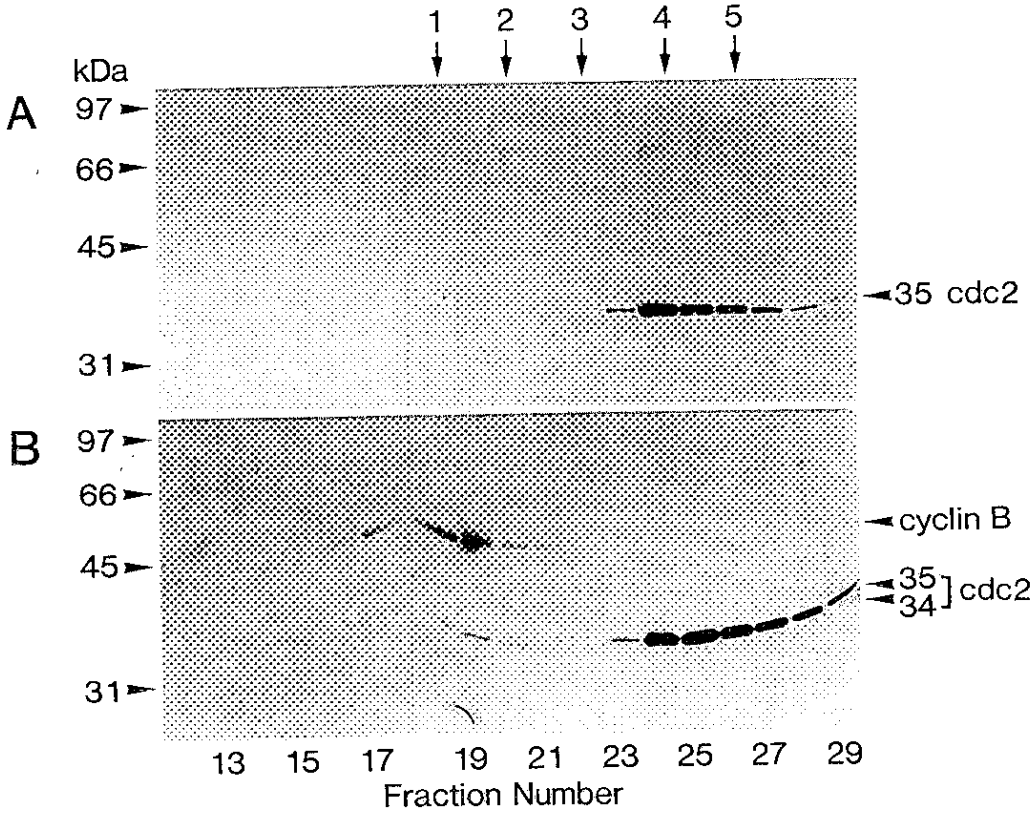

Fig. 5 Immunoblots of consecutive fractions eluted from gel filtration column (Superose 12), as probed with both anti-goldfish cdc2 C-terminal antibody (GFC3-9) and anti-goldfish cyclin B antibody (B63; cf. 4). A) Immature oocyte extracts. B) Mature oocyte extracts. Arrowheads indicate the positions of molecular weight markers: 1, aldorase (158 kDa); 2, bovine serun albumin (67 kDa); 3, ovalbumin (43 kDa); 4, chymotrypsinogen A (25 kDa); 5, ribonuclease A (13.7 kDa).

cdc2 binds to cyclin B at first, then it is activated, which is associated with an electrophoretic mobility shift from 35 to 34 kDa.

Discussion

We isolated goldfish cdc2 cDNA with the aid of PCR. The isolated clone had PSTAVR sequence, instead of PSTAIR sequence that is common to cdc2 in many species. Since the PSTAVR sequence was found in clones isolated from another cDNA library prepared independently and from a genomic DNA, the sequence is a genuine sequence specific to goldfish cdc2. Minor modifications in the PSTAIR sequence have also been reported for a slime mold, *Dictyostelium discoideum* (17).

All monoclonal antibodies raised against the C-terminal sequence of goldfish cdc2 recognized a 35 kDa protein in immature oocyte extracts and 34 and 35 kDa proteins in mature oocyte extracts in common (Figs. 2 and 3). Both the 34 and 35 kDa proteins were also recognized by monoclonal antibodies raised against bacterially expressed mouse cdc2 proteins, which do not react with cdk2 proteins (Yamashita *et al.*, unpublished). In addition, both the 34 and 35 kDa cdc2 bind to cyclin B *in vitro* (Yamashita *et al.*, unpublished; see also Fig. 5B) and the 34 kDa cdc2 was found in MPF highly purified from carp eggs (Fig. 4), although the 35 kDa form was not. These results strongly suggest that both the 34 and 35 kDa proteins are cdc2 gene products with different chemical modifications. What is the difference between the 34 and 35 kDa forms of cdc2? Since the 34 kDa cdc2 is found only in mature oocyte extracts and a component of highly purified MPF, this protein should be active cdc2. In fact, the present fractionation experiment of mature oocyte extracts with gel filtration column has demonstrated that almost all the inactive 35 kDa cdc2 is monomeric and all the active 34 kDa cdc2 forms a complex with other proteins (Fig. 5). Since cyclin B is present in the same fractions as the 34 kDa cdc2 (Fig. 5B), it is highly likely that the active 34 kDa cdc2 binds to cyclin B. The mobility shift from the 35 kDa to 34 kDa upon cdc2 activation seems to be due to changes in phosphorylation state of cdc2. Phosphoamino acid analysis revealed that only the 34 kDa cdc2 was threonine phosphorylated (Yamashita *et al.*, unpublished). Although it is common that the phosphorylation of proteins caused slower electrophoretic mobility, phosphorylation of cdc2 may cause faster mobility on SDS-PAGE (14).

In immature oocytes of *Xenopus* (3, 9) and starfish (25), inactive cdc2 is already in a complex with cyclin B as pre-MPF. We have demonstrated in this study that inactive 35 kDa cdc2 is monomeric in immature goldfish oocytes. This result clearly differs from the situation in *Xenopus* and starfish and seems to resemble the case of clam, in which cdc2 forms a complex with cyclin B that is solubilized in process of oocyte maturation (27). However, we have also found that there is no detectable cyclin B in immature goldfish oocytes and that cyclin B is synhtesized *de novo* during oocyte maturation (5, 7). In goldfish, therefore, the synthesis of cyclin B during oocyte maturation appears to have a crucial role in activating cdc2 and in inducing oocyte maturation. These results have clearly shown that the mechanisms of MPF activation differ from species to species, in spite of the involvement of the same molecules.

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A fish homolog of the cdc2-related protein p40^{MO15}: its cDNA cloning and expression in oocytes

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ABSTRACT

We have previously shown that threonine (probably Thr-161) phosphorylation of cdc2 kinase (p34^{cdc2}), the catalytic subunit of maturation-promoting factor (MPF), is a crucial step for the 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP, a maturation-inducing hormone)-induced activation of MPF in goldfish (*Carassius auratus*) oocytes. In this study, we have cloned a cDNA that encodes a goldfish homolog of p40^{MO15}, the catalytic subunit of a protein kinase which has been shown to activate cdc2 kinase through phosphorylation of Thr-161, from a goldfish oocyte cDNA library. Reverse transcription PCR using oligonucleotide primers corresponding to highly conserved sequences in cdc2-related genes was used to generate a 144 bp cDNA PCR product from goldfish oocyte RNA. This probe was then used to screen the cDNA library for a full-length cDNA encoding a homolog of p40^{MO15} in goldfish. The isolated clone of approximately 1.3 kbp contained an open reading frame encoding a 344 amino acid protein that was 83% and 55% identical to *Xenopus* p40^{MO15} and a homolog of p40^{MO15} in rice, respectively. Using Northern blot techniques, a 1.3 kb *MO15* mRNA was detected in both full-grown immature oocytes and mature oocytes of ginbuna (*Carassius auratus langsdorfii*), a species closely related to goldfish. These data suggest that p40^{MO15} homolog is produced in fish oocytes and plays an important role in the 17α,20β-DP-induced activation of MPF through threonine phosphorylation of cdc2 kinase.

The p34^{cdc2} protein kinase (cdc2 kinase), the catalytic subunit of maturation-promoting factor (MPF), is a key regulator of G_2/M transition in eukaryotes (11). The activity of cdc2 kinase is regulated not only by association with cyclin B, the regulatory subunit of MPF, but also by its phosphorylation and dephosphorylation. For instance, cdc2 kinase can be positively regulated by dephosphorylation on Thr-14 and Tyr-15 and phosphorylation on Thr-161 (14).

Oocyte maturation in goldfish (*Carassius auratus*) is triggered by the release of maturation-inducing hormone (17α,20β-dihydroxy-4-pregnen-3-one, 17α,20β-DP) by the follicle cells surrounding the oocyte under the influence of circulating gonadotropin. 17α,20β-DP acts on the oocyte surface and induces the activation of MPF in the oocyte cytoplasm (10, 17, 18). Our previous findings suggest that in goldfish 17α,20β-DP induces oocytes to synthesize cyclin B, which in turn activates preexisting 35 kD inactive cdc2 kinase through its threonine (probably Thr-161) phosphorylation, producing the 34 kD active cdc2 kinase (9). From the studies in goldfish it is clear that a protein kinase that phosphorylates Thr-161 plays a key role in the activation of cdc2 kinase during the 17α,20β-DP-induced meiotic maturation in fish oocytes. An enzymatic activity that causes the phosphorylation of cdc2 kinase on Thr-161 in the presence of cyclin B has been identified in *Xenopus* egg extracts (16) and mammalian tissue culture cell extracts (2). Most recent biochemical

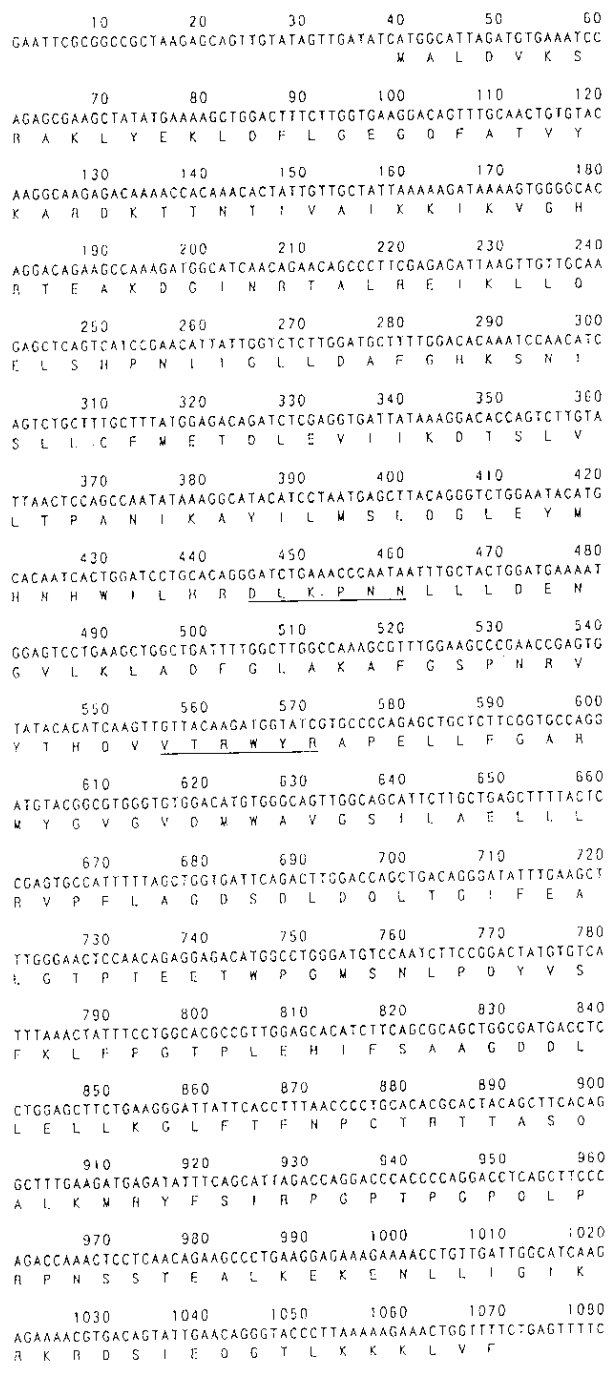

Fig. 1 Nucleotide and deduced amino acid sequences of the cDNA encoding goldfish oocyte p40^{MO15} homolog. Sequences used as PCR primers are underlined.

work has provided evidence to indicate that this kinase corresponds to p40^{MO15} (4, 12, 15), which was originally isolated from *Xenopus* oocytes during a search for homologues of frog cdc2 kinase (13). In this study, a full-length cDNA for p40^{MO15} homolog was isolated from a goldfish oocyte cDNA library, and its nucleotide sequence was determined. In addition, expression of *MO15* mRNA in oocytes of ginbuna (*Carassius auratus langsdorfii*, a species closely related to goldfish) was investigated.

To isolate a p40^{MO15} homolog from a goldfish cDNA library, we produced two degenerate PCR primers corresponding to highly conserved sequences in cdc2-related genes. The 5′ and 3′ primers correspond to the amino acid sequences, DLKPNN and VTRWYR, respectively (3, Fig. 1). Total RNA was prepared from full-grown immature goldfish oocytes by the acid guanidium thiocyanate-phenol-chloroform extraction method (1). Poly(A)$^+$RNA was isolated by oligo(dT)-Latex beads (Oligotex^{TM}-dT30, TaKaRa). cDNA was synthesized by incubating poly(A)$^+$RNA with the 3′ primer with reverse transcriptase (cDNA Synthesis System Plus, Amersham) and amplified by PCR according to the procedure recommended by the manufacturer (AmpliTaq^{TM} DNA polymerase, TaKaRa). Thirty cycles of amplification were performed with 1 min denaturation at 94°C, 1 min annealing at 55°C, and 2 min extension at 72°C. The amplified products of 100–250 bp were subcloned into pBluescript vector (Stratagene). Thirty two clones were obtained and their nucleotide sequences were determined using a DNA sequencing kit (*Bca*BEST^{TM} Dideoxy Sequencing Kit, TaKaRa). Among them, six clones were found to be identical, having 144 bp which are highly homologous at the amino acid level to that of *Xenopus* p40^{MO15} (92% in 48 amino acids).

The PCR-derived probe (the 144 bp fragment) was then used to screen a goldfish oocyte cDNA library (8) for a full-length cDNA clone of goldfish p40^{MO15} homolog. Approximately 3×10^5 plaques were transferred onto a nylon membrane (Hybond-N$^+$, Amersham) in duplicate and hybridized at 65°C overnight with the [^{32}P]labeled probe. One positive clone, containing an insert of approximately 1.3 kbp, was obtained and its cDNA sequence was determined as described above. This clone contained an open reading frame encoding a 344 amino acid protein (Fig. 1). The overall amino acid sequence of goldfish p40^{MO15} homolog exhibits 83% identity with *Xenopus* p40^{MO15} (13) and lower (55%) identity with p40^{MO15} homolog in rice (6,

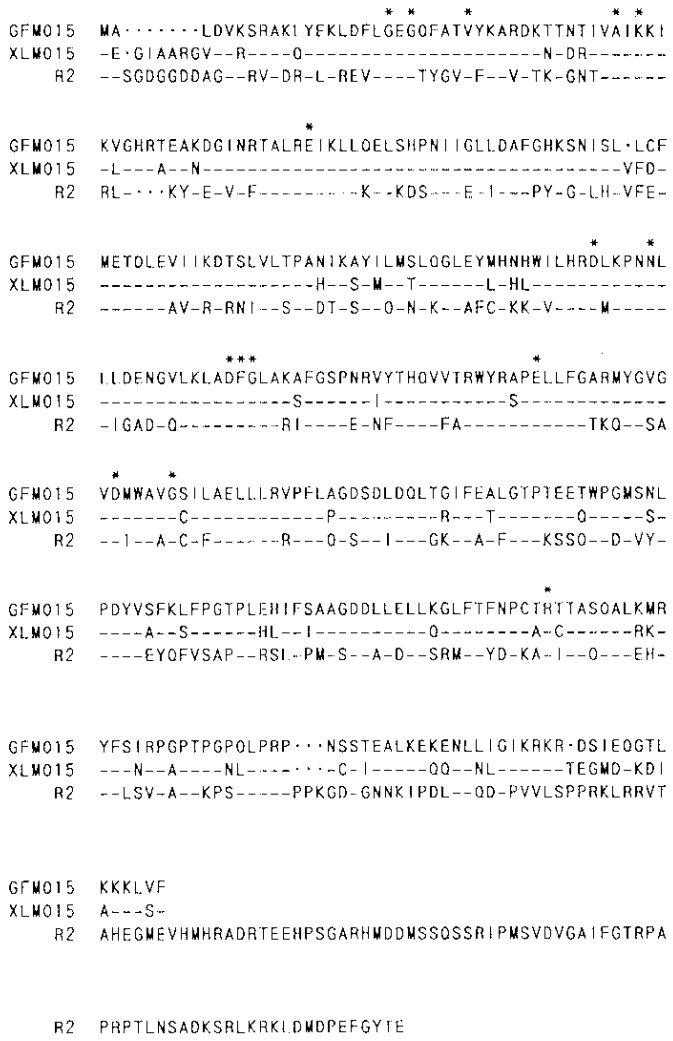

Fig. 2 Comparison of the deduced amino acid sequences for goldfish (GFMO15), *Xenopus* (XLMO15) and rice (R2) p40^{MO15}. Highly conserved amino acids present in all serine/threonine kinases are indicated by asterisks (5).

Fig. 2). Some of this sequence similarity is due to the conserved nucleotide binding and catalytic domains found in all serine/threonine kinases. The sequence of goldfish p40^{MO15} homolog shows 42% identity with goldfish cdc2 kinase (8) and 43% identity with goldfish cdk2 kinase (p33^{cdk2}) (7).

Northern blot analysis was performed using the goldfish cDNA clone as a probe. For this experiment, we used oocytes of ginbuna, since no goldfish oocytes at the appropriate developmental stages, i.e., full-grown immature oocytes and mature oocytes, were available during the time of this experiment. Two μg each of poly(A)$^+$RNA were isolated from full-grown immature oocytes and mature oocytes. As shown in Fig. 3, a single *MO15* mRNA of approximately 1.3 kb was detected in both immature and mature oocytes, indicating that the transcripts are contained in ginbuna oocytes prior to and after meiotic maturation. It has been reported that in *Xenopus*, the *MO15* mRNA is accumulated during oogenesis, and is degraded after the mid-blastula-transition stage of embryogenesis (13). It is noteworthy that in *Xenopus MO15* mRNA is de-adenylated during oocyte maturation. It is of interest to determine if this is the case with goldfish p40^{MO15} homolog in matur-

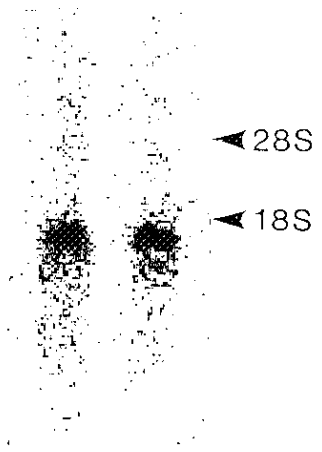

Fig. 3 Northern blot analysis of ginbuna oocyte mRNA. Poly(A)$^+$RNA was isolated from ginbuna full-grown immature oocytes (*left*) and mature oocytes (*right*); 2 μg each were then size fractionated by electrophoresis. The RNA was transferred onto a nylon membrane and probed with [^{32}P]labeled goldfish *MO15* cDNA. The membrane was exposed to an imaging plate for 30 h and analyzed using a BAS 2000 image analyzer (Fuji Photo Film, Tokyo). The mobilities of 28S and 18S RNA are indicated on the right of the figures with arrows.

ing oocytes of fish.

In summary, the data presented in the present study suggest that p40^{MO15} homolog is produced in fish oocytes and plays an important role in the activation of MPF through threonine phosphorylation of cdc2 kinase. The availability of the goldfish cDNA probe should allow questions to be answered regarding the structure, function, and mechanism of action of p40^{MO15} homolog during hormonally induced meiotic maturation of fish oocytes.

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Meiosis-Reinitiation-Inducing Factor of *Tetrahymena* Functions Upstream of M-Phase-Promoting Factor

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ABSTRACT. Reinitiation of meiosis (maturation) of amphibian *Bufo* and *Xenopus* oocytes can be induced if *Tetrahymena* extract is injected into them. The activity differed from M-phase-promoting factor, because action of the former factor on the induction of maturation was inhibited by treatment of the oocytes with cycloheximide. Activity of M-phase-promoting factor was not detected in *Tetrahymena* extract regardless of the presence of cdc2 homologues in the extract. However, cycloheximide-resistant-maturation-inducing activity appeared in the recipients, when the maturation was induced by injection of *Tetrahymena* extract. Immunoblots using antibodies against cdc2 showed that injection of *Tetrahymena* extract induced fast mobility of the recipient cdc2 in the presence of the recipient protein synthesis. The same mobility shift of the cdc2 was also induced when M-phase-promoting factor containing *Xenopus* oocyte extract was injected into immature oocytes or when the immature oocyte extract was treated with alkaline phosphatase. These results indicate that meiosis-reinitiation-inducing factor of *Tetrahymena* functions upstream of M-phase-promoting factor to induce dephosphorylation of the recipient cdc2. *Tetrahymena* cdc2 homologues also showed fast mobility when the *Tetrahymena* extract was treated with alkaline phosphatase. Preliminary experiments showed that the meiosis-reinitiation-inducing factor of *Tetrahymena* was a soluble protein.

Key words. Amphibian oocyte, dephosphorylation of $p34^{cdc2}$, germinal vesicle breakdown, microinjection.

PARAMECIUM caudatum [4] and *Tetrahymena pyriformis* [5] have a germinal vesicle breakdown (GVBD)-inducing factor of amphibian oocytes in their cell extracts. This factor, called meiosis-reinitiation-inducing factor (MRIF), is different from the M-phase-promoting factor (MPF) in three ways: 1) MRIF needs protein synthesis of the recipient oocytes for induction of GVBD [4, 5], whereas MPF does not; 2) MRIF activity appears in the M-phase in the cell cycle as well as in the premeiotic G_1 phase in *P. caudatum* [4], whereas MPF activity appears only in the M-phase; and 3) MRIF of *T. pyriformis* can induce GVBD in amphibian *Bufo bufo japonicum* and *Xenopus laevis* oocytes but not in starfish *Asterina pectinifera* oocytes [5], whereas MPF can induce GVBD in all of them. M-phase-promoting factor activity (cycloheximide-resistant GVBD-inducing activity) has not yet been detected in mitotically growing cells and conjugating cells of *P. caudatum* [4] and even in highly synchronized cells at the cell division stage of *T. pyriformis* [5].

However, it is unknown whether *Tetrahymena* cells have MRIF or MPF activities in the meiotic cell cycle, because *T. pyriformis* used in a previous study [5] cannot conjugate and has no germinal micronucleus. Furthermore, the functions of MRIF in its action for induction of GVBD in the recipient oocytes and functions of MRIF in cell cycle regulation in ciliates themselves are unknown. In the present study, we examined MRIF and MPF activities in various stages of the conjugation and the asexual reproduction in micronucleate strains of *T. thermophila*. Furthermore, since it is known that MPF consists of a cdc2[+] gene product and a B-type cyclin [6, 12] and that dephosphorylation of the cdc2 is needed for active MPF [7, 11], we examined whether MRIF dephosphorylated the recipient oocyte cdc2 to induce active MPF in the oocyte.

MATERIALS AND METHODS

Cells and culture. Cells of *Tetrahymena thermophila* strains B7 and B4051 (mating type II), B112 (mating type III), B19 and B4104 (mating type IV) and 703 (mating type unknown) were grown in a culture medium described previously [5]. In ordinary culture, about 1×10^5 cells were inoculated into 5 ml of fresh culture medium without shaking. For mass culture, about 3×10^5 cells were inoculated into 50 ml of fresh culture medium and grown axenically at 25° C without shaking. Two days after the inoculation, the culture reached a stationary phase of growth at a density of about 1×10^6 cells/ml.

Cell density was counted as follows. Fifty microliters of the culture was mixed with 5 ml of fixative containing 0.01% Alcian Blue, 5% formaldehyde and 10 mM Tris-HCl, pH 7.4. One hundred microliters of the mixture was then observed, using a Nomarski differential interference-contrast microscope (BH2-N, Olympus Co., Tokyo, Japan).

Induction of conjugation. Cells of complementary mating types were grown in 2 days in 50 ml of culture medium, washed 3 times by centrifuging at 170 *g* for 3 min with 10 mM Tris-saline buffer containing 0.2% NaCl, 0.008% KCl, 0.012% $CaCl_2$ and 10 mM Tris-HCl, pH 7.4, and suspended in 200 ml of the buffer in Petri dishes (20 cm in diameter) for 24 h at 25° C (initiation period). Their cell density was then adjusted to 2×10^5 cells/ml by adding the buffer and equal volumes of the cell suspensions were mixed at 25° C. After 30 min of mixing, the first pair appeared and the conjugation ratio increased to about 60% at 1 h and about 90% at 2 h. The first exconjugant cell appeared at 10 h.

Preparation of *Tetrahymena* cell extracts. *Tetrahymena* cells were concentrated by centrifuging at 300 *g* for 3 min and washed 3 times by the same centrifugation with 10 mM Tris-HCl, pH 7.4. The cell pellet was then mixed with an equal or twofold volume of extraction medium I consisting of 0.25 M sucrose, 0.2 M NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, 2 mM EGTA and 10 mM Na, K-phosphate buffer, pH 6.8. In some experiments, the following extraction media were used. Extraction medium IA contained extraction medium I with 30 μM Na-benzoyl-L-arginine methyl ester (BAME, Sigma Chemical Co., St. Louis, MO), 28 μM L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK, Sigma), 300 μM phenylmethylsulfonyl fluoride (PMSF, Sigma), 1 mM dithiothreitol (DTT, Kanto Chemical Co., Tokyo, Japan), 1 mM iodoacetamide (IAA, Sigma), 10 μg/ml leupeptin (Sigma), 80 mM di-sodium β-glycerophosphate (Kanto) and 1 mM ATP-γ-S; extraction medium IB contained extraction medium I with 30 μm BAME, 28 μM TPCK, 1 mM DTT, 1 mM IAA, 10 μg/ml leupeptin, 80 mM di-sodium β-glycerophosphate and 1 mM ATP-γ-S; and extraction medium IC contained extraction medium I with 300 μM PMSF, 1 mM DTT and 10 μg/ml leupeptin.

For alkaline phosphatase treatment experiments, extraction medium II consisting of 0.25 M sucrose, 0.2 M NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, 2 mM EGTA, 30 mM BAME, 28 μM TPCK, 1 mM DTT, 1 mM IAA, 10 μg/ml leupeptin, 300 μM PMSF and

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10 mM Tris-HCl, pH 8.0, was used. Cells were then homogenized by 5 strokes in a Teflon homogenizer at 0° C. The homogenates were centrifuged at either 18,000 *g* for 30 min or 132,000 *g* for 1 h at 2° C, and the supernatants were kept at 0° C until use. Usually, GVBD-inducing activity in the extract was retained for 7 days in the extraction media, if the sample was kept at 0° C.

Preparation of *Bufo* and *Xenopus* oocytes and *Xenopus* oocyte extracts. Oocytes of *Bufo bufo japonicum* and *Xenopus laevis* were prepared as described previously [4, 5]. *Bufo* oocytes were suspended in De Boer's solution (113 mM NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2$, 5.7 mM Tris-HCl, pH 7.4), and *Xenopus* oocytes were suspended in modified Barth's medium [13].

Bufo oocytes containing MPF were prepared by treating immature oocytes with De Boer's solution containing 10 μg/ml progesterone (Tokyo-kasei Chemical Inc., Tokyo, Japan) for 20 h at 18° C. *Xenopus* oocytes containing MPF were prepared by the following two methods. One was the same as the method used for *Bufo* oocytes but substituting modified Barth's medium for De Boer's solution. The other was a modified Lohka and Maller's method [4, 15]. Namely, ovulated unfertilized eggs were obtained by injecting 500 units of human chorionic gonadotropin (HCG, Teikoku-zouki Chemical Co., Tokyo, Japan), and the eggs were dejellied in 2% cysteine and washed three times with 0.1 M NaCl, 50 mM Tris-HCl, pH 7.4.

Xenopus oocyte extracts were obtained as follows. The oocytes were washed once by a hand-operated centrifuge with an extraction medium IA and centrifuged in a twofold volume of the same extraction medium at 109,000 *g* for 15 min at 2° C. The clear supernatants between the lipid cap and the pellet were then harvested and centrifuged at 132,000 *g* for 1 h at 2° C.

Assay for germinal vesicle breakdown-inducing activity. In the case of *Bufo* oocytes, 50 nl of each sample was injected into each oocyte. The injected oocytes were suspended in De Boer's solution and incubated for 20 h at 21–23° C, fixed with 2.5% trichloroacetic acid for 4–6 h at room temperature, and dissected with a razor blade to inspect for germinal vesicle breakdown (GVBD). In the case of *Xenopus* oocytes, 100 nl of each sample was injected into each oocyte, and the injected oocytes were suspended in modified Barth's medium for 5.5 h or 20 h at 21–23° C and fixed for 1–3 h. The *Tetrahymena* extracts used induced 100% GVBD within 5 h after the injection. In each experiment, no GVBD was observed after a control injection of the same volume of the extraction medium. Oocytes which were treated with 10 μg/ml of progesterone showed 100% GVBD, and non-treated oocytes showed 0% GVBD.

Treatment with cycloheximide. Immediately after microinjection, the injected oocytes were suspended in De Boer's solution containing 50 μg/ml cycloheximide (Wako Chemical Co., Tokyo, Japan) for 20 h or modified Barth's medium containing 20 μg/ml cycloheximide for 5.5 h at 21–23° C.

Paraffin section and cytology. Oocytes were fixed in Smith's solution [24] and embedded in paraffin. Twelve-micrometer thick serial sections were stained with Feulgen's reagent and Fast Green and observed at ×400 using a Nomarski differential interference-contrast microscope (Olympus, BH2-N). Nuclear conditions in conjugation of *T. thermophila* were observed in cells stained with a DNA-specific fluorochrome, 4',6-diamidino-2-phenylindole (DAPI) as described previously [5]. About 200 cells were observed at each time of the conjugation process.

Electrophoresis and immunoblotting. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 132,000-*g* supernatants of *T. thermophila* and *X. laevis* oocytes were prepared with extraction medium IA or extraction medium II, in twofold volume relative to the cell pellets. The protein contents of the supernatants were measured spectrophotometrically with a Protein Assay (Bio-Rad, Richmond, CA). The supernatants were boiled for 5 min in Laemmli's lysis buffer [14] and loaded on a slab gel consisting of 3% stacking gel and 12.5% separation gel and electrophoresed at 15 and 30 mA constant current for stacking and separation gels, respectively. After SDS-PAGE, proteins of the gel were transferred to a Immobilon-P membrane (Nihon Millipore, Tokyo, Japan) by a semi-dry Sartoblot (Sartorius GmbH, Goettingen, Germany) at 0.8 mA/cm^2 constant current for 90 min. The membrane was then incubated with a monoclonal antibody against the most conserved amino acid sequence, the PSTAIR sequence (EGVPSTAIREISLLKE) at $p34^{cdc2}$ [30], then biotinylated anti-mouse IgG, and finally with an avidin GH-biotinylated alkaline phosphatase H complex (Vectastain ABC-AP kit; Vector Laboratories, Inc., Burlingame, CA). Molecular weights were measured with a prestain marker kit (Bio-Rad).

Treatment with alkaline phosphatase. *Tetrahymena* cells of 48-h culture were centrifuged at 300 *g* for 3 min, homogenized in a twofold volume of extraction medium II and centrifuged at 123,200 *g* for 1 h at 2° C. *Xenopus* immature oocytes and unfertilized eggs were centrifuged in a twofold volume of the extraction medium at 123,200 *g* for 1 h at 2° C. These supernatants were incubated with an equal volume of 50 units/ml alkaline phosphatase solution (Sigma) containing 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0, for 6 h at 4° C.

RESULTS

Germinal vesicle breakdown-inducing activity in *Tetrahymena* extracts obtained from cells in different phases of culture age. Cells harvested from the log phase of growth 24 h after the inoculation were homogenized in an equal volume of extraction medium I, and their 18,000-*g* supernatant was injected into 11 *Bufo* oocytes. The pellet was mixed with the extraction medium of an equal volume with the supernatants and then injected into 10 oocytes. Germinal vesicle breakdown was induced in 91% (10/11) of the oocytes in the former and 0% (0/10) in the latter. To know whether the supernatant can induce not only GVBD but also the other nuclear events in the oocyte maturation, three oocytes were fixed at 18 h after the injection of the *Tetrahymena* supernatant and serially sectioned (Fig. 1). In each oocyte, a mitotic spindle with condensed chromosomes was observed in the animal pole. However, the first polar body was not observed in them, although progesterone-treated oocytes showed the first polar body 16–17 h after treatment. In the injected oocytes, the first polar body might be mechanically torn off during fixation or during embedding in paraffin, although the possibility that *Tetrahymena* extracts did not induce the first polar body could not be eliminated. However, the result at least shows that *T. thermophila* extracts can induce the first meiotic metaphase of the recipient oocytes.

Germinal vesicle breakdown-inducing activities in different phases of culture age were monitored using strain 703 of *T. thermophila*. About 3×10^5 cells were inoculated into 50 ml of culture medium and cultivated at 25° C. The cells were harvested 24, 48, 72 and 96 h after inoculation and centrifuged at 300 *g* for 3 min. The growth curve of the cells is shown in Fig. 2. The cell pellets were added with an equal volume of extraction medium IC, homogenized and their 132,000-*g* supernatants were prepared. The protein contents at 24, 48, 72 and 96 h were 0.65, 0.675, 0.97 and 0.76 μg/100 nl, respectively. To compare the degrees of GVBD-inducing activity among the cell extracts, each supernatant was diluted to 1:1, 1:2 and 1:3 with the same extraction medium and then injected into 10 *Xenopus* oocytes, 100 nl each, to monitor their %GVBD. The %GVBD of the oocytes in which three levels of concentrations of 24-h culture supernatant were injected were 100%, 60% and 20%, respec-

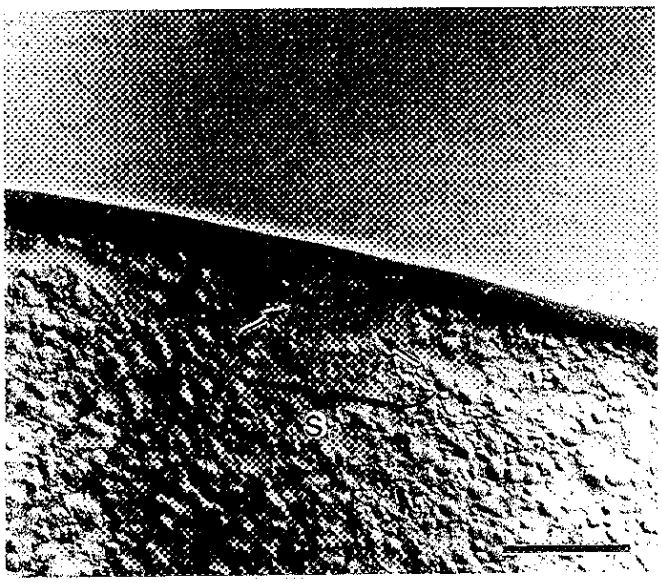

Fig. 1. Chromosome condensation and spindle formation in *Bufo* oocyte induced by microinjection of MRIF-containing *T. thermophila* extract, 18 h after the injection. Paraffin sections of the animal pole half of the recipient oocyte were stained with Feulgen and Fast Green. S, spindle apparatus of the first meiotic metaphase. Arrows indicate condensed chromosomes. Nomarski differential interference-contrast photomicrograph. Bar = 10 μm.

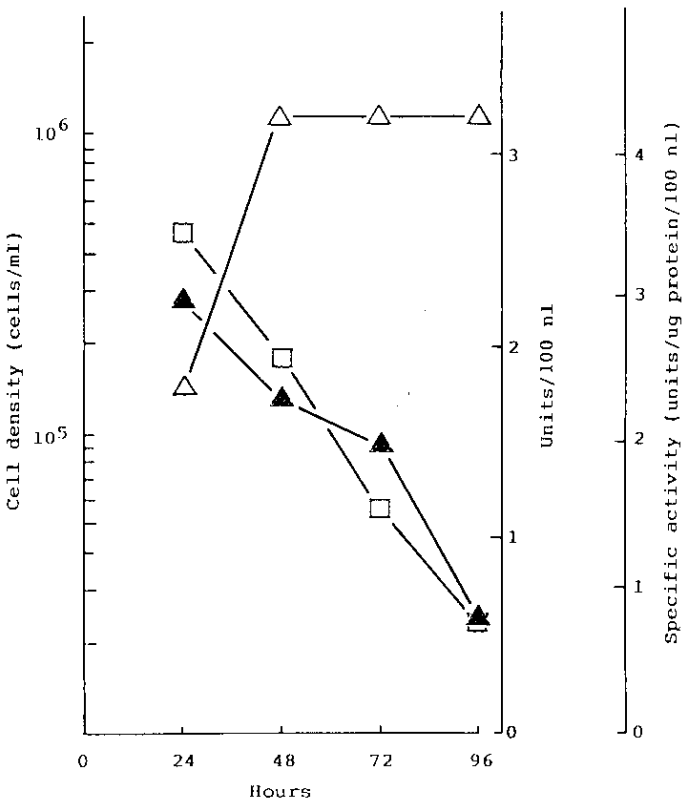

Fig. 2. Germinal vesicle breakdown-inducing activity in *T. thermophila* cells of different phases of culture age. About 3×10^5 cells of strain 703 were inoculated into 50 ml of culture medium and cultivated at 25° C. Their cell densities (△) and GVBD-inducing activity of the cell extracts (units/100 nl, ▲; units/μg protein/100 nl, □) were monitored. The cell extracts were prepared and injected into *Xenopus* oocytes as described in Results. When 50% of the oocytes showed GVBD, the activity of the injected sample was defined as one unit. Note that MRIF-activity decreases in the stationary phase of growth.

tively. Similarly, those of the 48-h culture supernatants were 80%, 40% and 0%, those of the 72-h culture supernatants were 80%, 20% and 0%, and those of the 96-h culture supernatants were 30%, 0% and 0%. When 50% of the recipient oocytes showed GVBD, the activity of the injected sample was defined as one unit. The supernatants of 24-, 48-, 72- and 96-h culture were calculated as 2.25, 1.75, 1.51 and 0.60 units/100 nl, respectively (Fig. 2). When specific activity was defined as units/μg injected protein, the specific activities of the four kinds of samples were 3.46, 2.59, 1.56 and 0.79 units/μg protein/100 nl (Fig. 2). Each experiment was repeated three times, and the results were reproducible. Since the specific activity of MRIF decreased in the stationary phase of growth, MRIF may function for phenomena associated with cell division in *Tetrahymena*.

Germinal vesicle breakdown-inducing activity in cells of initiation, costimulation and various stages of the conjugation process. Sexual cell recognition and conjugation in *T. thermophila* involves two stages, which are termed "initiation" and "costimulation" [2]. Initiation is induced by starvation and occurs in the presence or absence of a complementary mating type [29]. Costimulation follows initiation and requires contact between the mature initiated cells of the complementary mating types. During the costimulation, the cells acquire the ability to unite at their anterior ends to form conjugating pairs. Under our experimental conditions, the costimulation period was about 30 min, and the period was not shortened if the starvation period was prolonged more than 24 h, suggesting that cells which were starved for 24 h were fully initiated. We compared degrees of GVBD-inducing activities in cells of initiation, costimulation, and various stages of the conjugation process.

The initiated cells were prepared by washing and suspending them in Tris-saline buffer for 24 h at 25° C (see Materials and Methods). The cells in the costimulation period were those obtained 0.5 h after mixing the initiated cells of complementary mating types. The conjugating cells used were obtained 2, 3, 4, 6, 7, 8.5 and 10 h after the mixing. *Tetrahymena* cells conjugate with a G_1 macronucleus and a G_2 micronucleus, so that the first morphological change of the micronucleus of conjugating cells shows the beginning of the meiotic prophase. The first pair appears at 0.5 h after mixing; about 90% of the cells formed pairs at 2 h. In about 80% of the conjugating cells the micronucleus began to swell at 2 h. At 3 h, about 80% of the conjugating cells elongated their micronucleus. At 4 h, about 60% of the conjugating cells curved their micronucleus forming a horseshoe shape. At 6 h, about 70% of the conjugating cells entered meiotic metaphases I and II. At 7 h, one of four haploid nuclei divided to form a stationary and a migratory nuclei and exchanged the migratory nuclei in about 50% of the conjugating cells. At 8.5 h, a zygotic nucleus divided once or twice, and two of four post-zygotic nuclei increased their volume and differentiated to macronuclear anlagen in about 60% of the conjugating cells. At 10 h, the macronuclear anlagen increased their volume and an old macronucleus began to contract and degenerate in about 65% of the cells. Exconjugant cells appeared 9.5–10 h after mixing.

The cell pellets from centrifugation at 300 *g* were suspended in a fivefold volume of extraction medium I, homogenized, and their 18,000 *g* supernatants were injected into *Bufo* oocytes

Table 1. Germinal vesicle breakdown-inducing activities of *T. thermophila* extracts obtained from cells in initiation, costimulation and various stages of conjugation.[a]

Source of 18,000-*g* supernatants injected	%GVBD[b]
Cells in initiation period	20%
Cells in conjugation process	
0.5 h (costimulation period)	60%
2 h	90%
3 h	60%
4 h	50%
6 h	45%
7 h	35%
8.5 h	35%
10 h	45%
Control experiments	
Injection of extraction medium I	0%
Oocytes treated with progesterone	100%
Non-treated oocytes	0%

[a] Conditions for initiation and conjugation are given in Materials and Methods section.
[b] In all cases, 20 oocytes were examined.

Table 2. Effects of cycloheximide on GVBD-inducing activity of *Tetrahymena* extracts and MPF-containing cytoplasm of *Bufo* oocytes.

Materials injected	%GVBD[a] With 50 μg/ml cyclo-heximide	%GVBD[a] Without 50 μg/ml cyclo-heximide
Tetrahymena extracts[b]		
Log phase of growth (24 h)	0%	100%
Stationary phase of growth (48 h)	0%	100%
Conjugation process (3.5 h)	0%	100%
MPF-containing *Bufo* oocyte cytoplasm	100%	100%
Control experiments		
Injection of extraction medium IA	0%	0%
Oocytes treated with progesterone	0%	100%
Non-treated oocytes	0%	0%

[a] In all cases, 10 oocytes were examined.
[b] Hours in parentheses are culture times after inoculation or hours after mixing of complementary mating type cells.

(Table 1). The supernatant obtained from the initiated cells showed 20% GVBD. This indicates that the GVBD-inducing activity of cells in the 2-day culture (the first day of the stationary phase of growth) decreases when the cells were washed and starved one day for the initiation; whereas, if the cells in the 2-day culture were starved for a further 2 days without washing, they still maintained strong GVBD-inducing activity (Fig. 2). This shows that the activity easily decreases when the cells were compulsorily starved.

The GVBD-inducing activity, however, increased again to 60% GVBD when the initiated cells of complementary mating types were mixed for 0.5 h for costimulation. The activity increased to 90% GVBD at the beginning of the meiotic prophase 2 h after mixing, and then the activity decreased. These results show that the GVBD-inducing activity appears not only in mitotically growing cells or in stationary phase cells but also in cells of initiation, costimulation and the conjugation process. It should be noted that cells at 0.5 h and 2 h after mixing were at the premeiotic G_2 and early meiotic prophase; nevertheless, they showed strong GVBD-inducing activity compared with those in metaphases I and II 6 h after mixing.

Effects of cycloheximide on the action of GVBD-inducing activity of *Tetrahymena* extracts and MPF of maturing *Bufo* oocytes. M-phase-promoting factor does not need protein synthesis of the recipient oocytes to induce GVBD [16, 27]. On the other hand, MRIF of *P. caudatum* and *T. pyriformis* need the recipient protein synthesis for induction of GVBD [4, 5]. To know whether the GVBD-inducing factor of *T. thermophila* requires protein synthesis of the recipient oocytes for induction of GVBD, cells in the log phase (24 h) and early stationary phase (48 h) of growth, and those in the conjugation process 3.5 h after mixing of complementary mating types were washed and their 300 *g* pellets were added with a twofold volume of extraction medium IB and homogenized. Then, 132,000-*g* supernatants of the homogenates were injected into *Bufo* oocytes. In each experiment soon after the injection, 10 *Bufo* oocytes were suspended in De Boer's solution for 20 h at 20° C and 10 other oocytes were suspended in the solution containing 50 μg/ml cycloheximide. *Bufo* oocytes injected with each *Tetrahymena* extract showed 100% GVBD but 0% GVBD if the oocytes were treated with cycloheximide after the injection. On the other hand, if MPF-containing *Bufo* cytoplasm was injected into *Bufo* oocytes, 100% GVBD was induced with or without the presence of 50 μg/ml cycloheximide (Table 2). Thus, we could not detect MPF activity (cycloheximide-resistant-GVBD-inducing activity) in mitotic and meiotic cell cycles of *T. thermophila*. Instead, our results show that, like the MRIF of *P. caudatum* and *T. pyriformis* [4, 5], the GVBD-inducing factor of *T. thermophila* extracts cannot induce GVBD in the absence of protein synthesis of the recipient oocytes. Therefore, hereafter, we call this GVBD-inducing factor the "MRIF" of *T. thermophila*.

Does MRIF function upstream of MPF to induce MPF activity in the recipient oocyte cytoplasm? To know whether the injected MRIF induces MPF activity in the recipient oocytes, we injected MRIF-containing *Tetrahymena* extract (experiment A) and MPF-containing *Bufo* oocyte cytoplasm (experiment B) into the first recipient *Bufo* oocytes and the oocytes were incubated in De Boer's solution for 20 h at 20° C. The *Tetrahymena* extracts were prepared as follows. Cells of the stationary phase of growth (48-h culture) were centrifuged at 300 *g*. The pellet was homogenized in a twofold volume of extraction medium IB and its 132,000-*g* supernatant was obtained. The injected first recipients showed a clear white spot at the animal pole, as in the case of progesterone-treated oocytes. Their cytoplasm was then injected into the second recipient *Bufo* oocytes (50 nl each), the oocytes were suspended in De Boer's solution with or without 50 μg/ml cycloheximide for 20 h at 20° C and their GVBD was examined. It is known that injection of MPF induces MPF activity in the recipient oocyte cytoplasm in the absence of the recipient oocyte protein synthesis [27]. Therefore, if MPF activity was induced in the first recipient by injection of MRIF (experiment A) as well as the first recipients in experiment B, the maturing oocyte cytoplasm in experiment A is expected to induce GVBD in the second recipients in the absence of the oocyte protein synthesis. However, if the injected MRIF did not induce MPF activity in the first recipients, then it is expected that the maturing oocyte cytoplasm is unable to induce GVBD in the second recipients in the absence of the oocyte protein synthesis. As summarized in Table 3, in both experiments A and B, the first recipient cytoplasm could induce GVBD in the second oocytes regardless of the presence or absence of the oocyte protein synthesis. The results strongly suggest that MRIF induced MPF activity in the oocytes.

Table 3. Induction of MPF activity in *Bufo* oocytes by injection of MRIF-containing *Tetrahymena* extracts and MPF-containing oocyte cytoplasm.

Materials injected into 1st recipient	%GVBD of 2nd recipient[a]	
	With 50 μg/ml cyclo-heximide	Without 50 μg/ml cyclo-heximide
Experiment A		
MRIF-containing *Tetrahymena* extract	80%	90%
Experiment B		
Bufo oocyte cytoplasm containing MPF	80%	90%
Non-treated *Bufo* oocyte cytoplasm	ND[b]	0%
Control experiments		
Injection of extraction medium IA	ND	0%
Oocytes treated with progesterone	ND	100%
Non-treated oocytes	ND	0%

[a] In all cases, 10 oocytes were examined.
[b] ND, not done.

To confirm whether the cycloheximide-resistant GVBD-inducing activity induced by injection of MRIF is due to appearance of active MPF, we examined changes in relative mobilities of the oocyte $p34^{cdc2}$ by SDS-PAGE and its immunoblot with a monoclonal anti-cdc2 antibody (PSTAIR antibody) before and after the injection. If active MPF appears in the recipient oocytes, the $p34^{cdc2}$ of the oocytes is expected to be dephosphorylated, and this transition from the phosphorylated form to the dephosphorylated form of $p34^{cdc2}$ is expected to be detected as a different relative mobility of the cdc2. *Tetrahymena* cells containing MRIF were harvested from a 48-h culture and were centrifuged at 300 *g* for 3 min. The cell pellets were added with a twofold volume of extraction medium IA, homogenized and their 132,000-*g* supernatants were prepared. *Xenopus* oocyte extracts containing MPF were prepared from ovulated oocytes (see Materials and Methods). *Tetrahymena* extracts and *Xenopus* oocyte extracts were injected into 220–230 *Xenopus* oocytes, respectively. Soon after the injection, 12 oocytes of the former and 10 oocytes of the latter were suspended in modified Barth's medium containing 20 μg/ml cycloheximide for 5.5 h at 21–23° C, and 12 oocytes of the former and 10 oocytes of the latter were suspended in the cycloheximide-free modified Barth's medium for 5.5 h at 21–23° C to inspect their %GVBD. About 200 remaining oocytes of each group were divided equally and suspended in the modified Barth's media in the presence or absence of 20 μg/ml cycloheximide for 5.5 h at 21–23° C. Their 132,000-*g* supernatants were then prepared using extraction medium II.

Tetrahymena extract induced 0% GVBD (0/12) when the recipient oocytes were treated with cycloheximide but induced 75% GVBD (9/12) when the recipients were not treated with cycloheximide. On the other hand, *Xenopus* extract induced 100% GVBD (10/10) regardless of the presence or absence of cycloheximide. Figure 3 shows an immunoblot with PSTAIR antibody. At least two closely adjacent bands of $p34^{cdc2}$ and another PSTAIR reactive band of molecular weight 32 kDa were detected in both immature oocytes (lane 5) and ovulated oocytes (lane 6). The immature oocyte had no MPF activity but the ovulated oocyte had MPF activity. The p32 is known as a $p34^{cdc2}$ "look-alike" protein encoded by Egl [20, 25]. In lane 6, the upper band of $p34^{cdc2}$ was a trace amount compared with that in lane 5. Instead, the lower band of $p34^{cdc2}$ increased its amount.

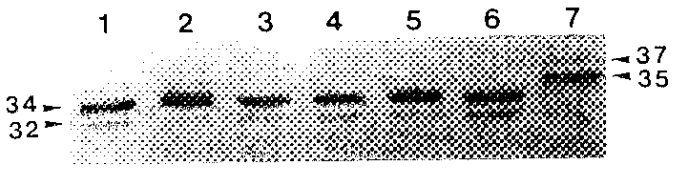

Fig. 3. *Tetrahymena* MRIF induces fast mobility of *Xenopus* oocyte cdc2 in the presence of the oocyte protein synthesis. *Tetrahymena* extracts containing MRIF and *Xenopus* oocyte extracts containing MPF were injected into *Xenopus* oocytes, and the oocytes were suspended in modified Barth's medium for 5.5 h at 21–23° C, with or without 20 μg/ml cycloheximide (CH). The oocyte extracts were then electrophoresed (20.8 μg protein/lane), transferred to an immobilon membrane and immunostained with PSTAIR antibodies. Lane **1**. MRIF-injected oocytes, untreated. Lane **2**. MRIF-injected oocytes, treated with CH. Lane **3**. MPF-injected oocytes, untreated. Lane **4**. MPF-injected oocytes, treated with CH. Lane **5**. Non-injected oocytes, untreated. Lane **6**. Non-injected ovulated oocytes, untreated. Lane **7**. *Tetrahymena*.

It is known that this shift in relative mobility of $p34^{cdc2}$ is caused by dephosphorylation of $p34^{cdc2}$ on tyrosine and threonin residues and that this dephosphorylation is needed for acquisition of MPF activity [3, 6, 19]. Injection of active MPF into immature oocytes induced the mobility shift of the recipient $p34^{cdc2}$ regardless of the presence (lane 3) or the absence (lane 4) of the recipient protein synthesis. However, unlike MPF, MRIF induced the mobility shift of the recipient $p34^{cdc2}$ only in the presence of the recipient protein synthesis (lanes 1 and 2). These results coincide well with the observation that cycloheximide-resistant GVBD-inducing activity had been induced by injection of MRIF in the recipient oocytes. These results strongly suggest that the cycloheximide-resistant GVBD-inducing activity induced by injection of MRIF is MPF activity. The results also suggest that MRIF functions upstream of MPF to induce active MPF in the presence of the recipient protein synthesis.

It should be noted that PSTAIR-reactive polypeptides were also present in *Tetrahymena* extracts (lane 7). Molecular weights of the major cdc2 homologues of *Tetrahymena* were 37 and 35 kDa, and several minor PSTAIR reactive bands which seemed to result from proteolysis of the cdc2 homologues appeared in the low molecular weight region. It seemed that trace amounts of PSTAIR-reactive bands of 37 and 35 kDa in lanes 1 and 2 were *Tetrahymena* cdc2 homologues which had been injected into the oocytes, because these two bands could not be detected in lanes 3–6.

Treatment of *Tetrahymena* and *Xenopus* oocyte extracts with alkaline phosphatase. It is known that alkaline phosphatases selectively dephosphorylate phospho-Tyr-protein [26] and that cdc2 protein kinase must undergo tyrosine dephosphorylation as a prerequisite for the activation of MPF [7, 11]. To confirm that the relative mobility shift of $p34^{cdc2}$ in Fig. 3 is really caused by dephosphorylation of cdc2, *Xenopus* immature oocyte and ovulated oocyte extracts were treated with 50 units/ml alkaline phosphatase for 6 h at 4° C (see Materials and Methods). Immunoblots of SDS-PAGE gels are shown in Fig. 4. When the immature oocyte extract which had no MPF activity was treated with alkaline phosphatase, an upper band of the two cdc2 bands became a trace amount (lane 1) and the lower band increased in amount compared with those of non-treated oocyte extracts (lane 2). On the other hand, when MPF activity bearing ovulated oocyte extract was treated with alkaline phosphatase (lane 3), the upper band of the cdc2 which had been a trace amount in the non-treated extract in lane 4 disappeared. Thus, alkaline phosphatase treatments induced a mobility shift of the cdc2 protein. These results indicate that dephosphorylation of cdc2 is a cause of the mobility shift. It should be noted that the

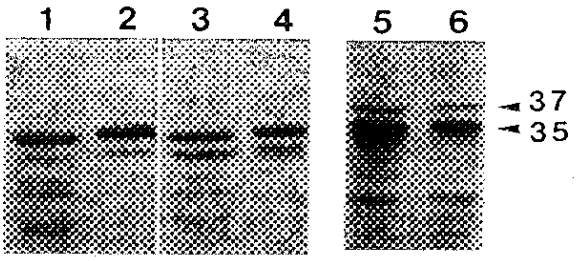

Fig. 4. Alkaline phosphatase induces fast mobility of the *Xenopus* oocyte cdc2 and *Tetrahymena* cdc2 homologues in SDS-PAGE gel. *Xenopus* immature oocyte extract, ovulated oocyte extract and *Tetrahymena* extract were treated or untreated with 50 units/ml alkaline phosphatase (AP) for 6 h at 4° C, and then electrophoresed (14.5 μg protein/lane), transferred to an immobilon membrane and immunostained with PSTAIR antibodies. Lane **1**. Immature oocyte extract, treated with AP. Lane 2. Immature oocyte extract, untreated. Lane 3. Ovulated oocyte extract, treated with AP. Lane **4**. Ovulated oocyte extract, untreated. Lane **5**. *Tetrahymena* extract, treated with AP. Lane **6**. *Tetrahymena* extract, untreated.

mobilities of the lower band of cdc2 in lanes 1–6 were almost the same, but those treated with alkaline phosphatase in lanes 1 and 3 were slightly faster compared with those of the non-treated ones in lanes 2 and 4. The cdc2 of active MPF was also shifted by the treatment (lane 3). Solomon et al. [25] proposed the following model for activation of $p34^{cdc2}$. The cdc2-cyclin complex is inactive in its MPF activity if one tyrosine and on threonin residue of the cdc2 is phosphorylated but is active if one threonin is phosphorylated and one tyrosine is dephosphorylated. Therefore, the enhanced mobility shift of cdc2 by treatment with alkaline phosphatase may be due to dephosphorylation of the phosphorylated threonin residue of the active cdc2. However, another possibility that the enhanced mobility shift of cdc2 may result from partial proteolysis of the cdc2 by contaminated protease in the alkaline phosphatase cannot be eliminated.

Tetrahymena extracts prepared from cells of 48-h culture were also treated with 50 units/ml alkaline phosphatase for 6 h at 4° C. PSTAIR-reactive bands including 37 and 35 kDa increased their mobilities by the treatment (lane 5) compared with the non-treated sample (lane 6), suggesting that phosphorylated and dephosphorylated forms of the *Tetrahymena* cdc2 homologue may have some role in the cell cycle regulation.

Timing of GVBD and mobility shift of cdc2 after injection of *Tetrahymena* MRIF. *Tetrahymena* extract (132,000-*g* supernatant) was prepared with extraction medium II from cells in the 48-h culture as described above. Then, the extract was injected into 312 *Xenopus* immature oocytes and the oocytes were incubated at 22° C. After the injection, each of the 12 oocyte samples was fixed with 2.5% TCA at 15 min and every 30 min up to 6 h, and their %GVBD was observed. At the same time, each of the 12 oocyte samples was washed three times with 50 mM Tris-HCl, pH 7.4, containing 0.1 M NaCl and was quickly frozen at −85° C in 150 μl of extraction medium II to stop the maturation process of the oocytes. The oocytes were then thawed and centrifuged at 132,000 *g* for 1 h at 2° C. The supernatants were then mixed with an equal volume of Laemmli's lysis buffer, boiled for 5 min and electrophoresed. Fifteen micrograms of protein was loaded on each lane. Germinal vesicle breakdown was induced at 2.5 h (16.7%) and thereafter the ratio was increased (Fig. 5). An immunoblot with PSTAIR antibody showed that relative amounts of two closely adjacent bands of the oocyte $p34^{cdc2}$ were almost the same up to 3.5 h after the injection. At 4 h, however, the lower band apparently increased in amount, whereas the upper one decreased. At 5 h, the upper band became a trace amount. These results suggest that the dephosphorylation of the oocyte cdc2 begins at least at 4 h after the injection.

In Coomassie Brilliant Blue-stained gels (Fig. 3–5), bands of oocyte cdc2 were hardly distinguishable (data not shown) indicating that cdc2 proteins were minor components of the cell extracts.

Effects of trypsin and RNase against MRIF activity. Cells in the early stationary phase (48 h) of growth were washed and their 300-*g* pellet was mixed with a twofold volume of extraction medium IB, homogenized and centrifuged at 132,000 *g* for 1 h. Nine volumes of the supernatant were mixed with either one volume of 0.2% (w/v) trypsin (Sigma) dissolved in the extraction medium or one volume of extraction medium and incubated for 30 min at 25° C. Then 0.4% (w/v) trypsin inhibitor (Sigma) dissolved in the extraction medium was added to these mixtures to give a final concentration of the inhibitor of 0.04% (w/v). The mixtures were incubated for 20 min at 25° C and injected into *Bufo* oocytes. Percentages of GVBD induced in the oocytes were 0% (0/10) in the former and 90% (9/10) in the latter. On the other hand, if the cell extract was treated with 1.5 units of insoluble RNase (Sigma) with shaking at 25° C for 1 h and the supernatant was injected into *Bufo* oocytes, 100% (10/10) GVBD was induced. When the extraction medium alone was treated with the RNase and injected into the oocytes, 0% (0/10) GVBD was induced. These results show that the MRIF of *T. thermophila* is a trypsin sensitive, RNase-resistant, soluble protein.

DISCUSSION

M-phase-promoting factor is believed to be an ubiquitous cytoplasmic factor in controlling the induction of nuclear membrane breakdown and chromosome condensation in both meiosis and mitosis of various eukaryotic cells, and it can induce

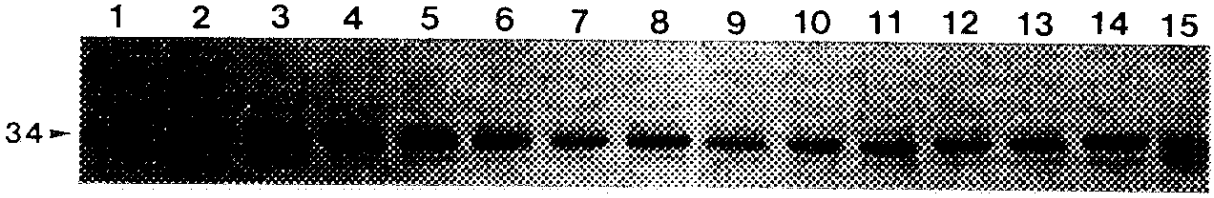

Fig. 5. Timing of GVBD of *Xenopus* oocyte and mobility shift of the oocyte $p34^{cdc2}$ after injection of *Tetrahymena* extracts containing MRIF. At various times after the injection, 12 oocytes were fixed for observation of %GVBD, and 12 oocytes were used for preparation of the oocyte extracts in each time. The extracts were electrophoresed (about 15 μg protein/lane), transferred to an immobilon membrane and immunostained with PSTAIR antibodies. Lane **1**. 15 min, 0% GVBD. Lane **2**. 30 min, 0% GVBD. Lane **3**. 1 h, 0% GVBD. Lane **4**. 1.5 h, 0% GVBD. Lane **5**. 2 h, 0% GVBD. Lane **6**. 2.5 h, 16.7% GVBD. Lane **7**. 3 h, 16.7% GVBD. Lane **8**. 3.5 h, 16.7% GVBD. Lane **9**. 4 h, 50% GVBD. Lane **10**. 4.5 h, 41.7% GVBD. Lane **11**. 5 h, 83.3% GVBD. Lane **12**. 5.5 h, 91.7% GVBD. Lane **13**. 6 h, 100% GVBD. Lane **14**. Non-injected oocytes. Lane **15**. Ovulated oocytes.

GVBD in the absence of oocyte protein synthesis. However, MPF activity has not yet been detected in ciliate *P. caudatum* [4] and *T. pyriformis* [5]. In the present study, we also could not detect the MPF activity in *T. thermophila*. Instead, as in previous studies [4, 5], *T. thermophila* showed MRIF activity in all stages of the mitotic and meiotic stages examined. The activity was detected even if the cells were not in the M-phase in the cell cycle, such as the cells in the stationary phase of growth and in the initiation and costimulation periods. The degrees of the activity changed in culture age and in conjugation process. In the present study, however, we did not compare the activities in units/μg of protein between the cell extracts of log phase of growth and of conjugation process.

Except for MPF, so far, several proteins are known to have activity for inducing reinitiation of meiosis of oocytes; the regulatory subunit of cAMP dependent protein kinase [17], the subunit of G_o protein [9], the $p21^{ras}$ oncogene product (Ras) [1], the $p39^{mos}$ oncogene product (Mos) [22], and the cycloheximide-sensitive meiotic maturation inducing factor (CMF) of *Xenopus* [8, 10]. Among these proteins, the regulatory subunit of cAMP dependent protein kinase [17], the subunit of G_o protein [9], and CMF [8, 10] need protein synthesis to induce GVBD and function upstream of MPF to activate pre-MPF. Therefore, MRIF may be closely related to either of them. Our recent study shows that the molecular weight of MRIF is 100–200 kDa by gel filtration (Katsu, Y. & Fujishima, M., unpubl. data). On the other hand, CMF is 190 kDa by gel filtration and 74 kDa by SDS-PAGE [10]. This suggests that MRIF differs from MPF and may be a molecule similar to CMF.

Our results show that MRIF eventually induces dephosphorylation of $p34^{cdc2}$ of the recipient *Xenopus* oocytes, as did MPF, though the MRIF needs the recipient protein synthesis for this dephosphorylation. Namely, the protein which is newly synthesized by injection of MRIF seems to be responsible for the induction of dephosphorylation of $p34^{cdc2}$. Our recent study shows that a protein recognized by immunoblotting with polyclonal anti-$p39^{mos}$ antibodies appears in the *Xenopus* oocytes when MRIF was injected into the oocytes and fast mobility of the oocyte cdc2 and GVBD were induced in the oocytes (Fujishima, M. & Ogawa, E., unpubl. data). This strongly suggests that the primary function of the injected MRIF in the oocyte may be induction of Mos protein, because Mos is not synthesized in the oocytes in the presence of cycloheximide [23, 28] and Mos phosphorylates cyclin to form active MPF [22] and Mos induces active MPF [23]. We are now examining whether the protein detected with anti-Mos antibodies is really Mos or not. We cannot eliminate the other possibility that MRIF may induce synthesis of cdc2 phosphatase $p80^{cdc25}$ which dephosphorylates cdc2 to activate its protein kinase activity [18].

Recently, Roth et al. [21] showed the presence of a cdc2-like kinase of 36 kDa in *T. thermophila*. They showed that *Tetrahymena* cdc2 homologues are present in isolated macronuclei and that the cdc2 homologue possessed H1 histone kinase activity like that of other cdc2 proteins. In the present study, we also showed that *T. thermophila* had PSTAIR-reactive polypeptides, though the molecular weights of the PSTAIR-reactive major bands were 35 and 37 kDa. The reason why MPF activity could not be detected in *Tetrahymena* extract, besides the presence of cdc2 homologues in the extract, cannot be explained. Recently, we found that relative amounts of phosphorylated and dephosphorylated forms of *T. pyriformis* cdc2 homologue do not change during the cell cycle even if the cell division is highly synchronized (Fujishima, M., Imai, R., Yamashita, M. & Nagahama, K., unpubl. data). This may suggest that the *Tetrahymena* cdc2 homologue does not play an important role in regulation of the cell division. Furthermore, our recent study showed that MRIF-activity was not lost if the *T. thermophila* extract was incubated with $p13^{suc1}$-conjugated sepharose beads, though the majority of the cdc2 homologues was absorbed by the beads (Katsu, Y. & Fujishima, M., unpubl. data). This indicates that MRIF at least differs from MPF.

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Induction of Reinitiation of Meiosis in Amphibian *Bufo* and *Xenopus* Oocytes by Injection of M-Phase Extracts of Ciliate *Tetrahymena* Needs the Recipient Protein Synthesis

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We show here that germinal vesicle breakdown of amphibian *Bufo* and *Xenopus* oocytes can be induced if ciliate *Tetrahymena* extracts are injected into them. The activity of meiosis-reinitiation-inducing factor (MRIF) appeared only at M-phase of a synchronously dividing culture, indicating that this MRIF has an important function for induction of M-phase in the mitotic cell cycle. MRIF of *Tetrahymena* differed from MPF (M-phase-promoting factor), because its action on the induction of GVBD was inhibited by cycloheximide and it could not induce GVBD in starfish oocytes by microinjection. MPF activity was not detected in extracts of vegetatively growing *Tetrahymena*. Preliminary experiments showed that MRIF was a heat-labile, Ca^{2+}-sensitive, and trypsin-sensitive soluble protein. © 1991 Academic Press, Inc.

INTRODUCTION

Previously, Fujishima and Hori [1] reported that ciliate *Paramecium caudatum* had a protein which could induce reinitiation of meiosis in amphibian *Bufo* oocytes if *Paramecium* extracts obtained from cells in conjugation process and cells in log phase of growth were injected into them, and that this meiosis-reinitiation-inducing factor (MRIF) was different from MPF (M-phase-promoting factor) on its action of induction of germinal vesicle breakdown (GVBD): MRIF needed protein synthesis of recipient oocytes for induction of GVBD, whereas MPF did not. MRIF activity fluctuated according to the degrees of micronuclear chromatin condensation in meiotic cell cycle, and cells in the stationary phase of growth did not show MRIF activity, but detailed changes of the MRIF activity in each stage of the mitotic cell cycle could not be examined because of low synchrony of the mitotic cell cycle in *Paramecium*. Therefore, in the present study, we used *Tetrahymena pyriformis* strain W as the source of the GVBD-inducing factor, because highly synchronized mitotic cells can be obtained by a periodic heat-shock treatment in this species [2, 3]. Furthermore, to know differences on the action for induction of GVBD between MPF and GVBD-inducing factor of *Tetrahymena*, we examined whether the factor of *Tetrahymena* needs protein synthesis of the recipient amphibian oocytes for induction of GVBD, and whether it can induce GVBD in starfish oocytes.

MATERIALS AND METHODS

Cells and cell synchrony. Cells of *T. pyriformis* strain W (amicronucleate strain) were grown in a culture medium containing 2% (w/v) proteose peptone (Difco, No. 3), 1% (w/v) yeast extract (Difco), and 0.6% (w/v) glucose (Wako). About 4×10^5 cells were inoculated into 5 ml of fresh culture medium and grown axenically at 25°C without shaking. Two days after the inoculation, the culture reached stationary phase of growth at a density of about 2×10^6 cells/ml. Synchronous cell division was induced according to Scherbaum and Zeuthen [2] with a slight modification. About 300 μl of the 2-day culture was then inoculated to 200-ml flasks containing 50 ml of culture medium and was cultured for 18–20 h with shaking at 26°C, and the cells were subjected to a controlled temperature cycle: eight alternate 30-min exposures to temperatures of 33.8°C (instead of 34°C of original method) and 26°C. Under this condition, dividing cells appeared at 68 min and reached the first peak at 75 min and the second peak at 180–250 min. When cells were treated at 34°C occasional dead cells appeared during the heat treatment; consequently we used 33.8°C in this study.

The percentage of dividing cells was calculated as {[2(number of dividing cells)]/[number of nondividing cells + 2(number of dividing cells)]} × 100.

Observation of synchronous cell division was performed as follows. Fifty microliters of the culture was mixed with 5 ml of fixative containing 0.01% (w/v) Alcian blue, 5% (v/v) formaldehyde, and 10 m*M* Tris–HCl, pH 7.4. One hundred microliters of the mixture was then examined for cell density and percentage of dividing cells, using a differential interference-contrast microscope (Olympus, BH2-N). An aliquot of the fixed cells was stained with 1 μg/ml of a DNA specific fluorochrome, 4′,6-diamidino-2-phenylindole (DAPI), and nuclear conditions of the cells were examined using a fluorescence differential interference-contrast microscope (Olympus, BH2-RFC).

Preparation of Tetrahymena cell extracts. *Tetrahymena* cells were concentrated by centrifugation at 300*g* for 3 min and washed three times by the same centrifugation with 10 m*M* Tris–HCl, pH 7.4, and then the cell pellet was added with an equal volume of the extraction medium containing 0.2 *M* NaCl, 0.25 *M* sucrose, 10 m*M* $MgSO_4$, 2

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mM EGTA, 30 μM Na-benzoyl-L-arginine methyl ester (BAME, Sigma), 28 μM L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK, Sigma), 1 mM dithiothreitol (DTT, Kantokagaku), 1 mM iodoacetamide (IAA, Sigma), 10 μg/ml leupeptin (Sigma), 80 mM disodium β-glycerophosphate (Kantokagaku), 1 mM ATP-γ-S, and 10 mM Na-phosphate buffer, pH 6.8. Cells were then homogenized by five strokes in a Teflon homogenizer at 0°C. The homogenates were centrifuged at 110,000*g*, for 1 h at 2°C, and the supernatants were kept at 0°C until use. Usually, MRIF activity in the extracts was retained for 1 week at 0°C.

Preparation of Bufo and Xenopus oocytes. Oocytes of *Bufo bufo japonicum* were obtained by surgically removing a portion of an ovary and then removing the follicular layers of full-grown oocytes with forceps. All operations on oocytes were conducted using De Boer's solution (113 mM NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2$, 5.7 mM Tris HCl, pH 7.4). Oocytes of *Xenopus laevis* were prepared in the same way, but modified Barth's medium [4] was used instead of De Boer's solution.

Preparation of MPF-containing Xenopus egg extracts. MPF containing *Xenopus* egg extracts were obtained according to Lohka and Maller [5] with slight modifications. Unfertilized eggs were obtained by injecting 500 units human chorionic gonadotropin (HCG, Teikoku-zouki) instead of 750 units HCG, and the eggs were dejellied in 2% (w/v) cystein (pH 7.8) and washed three times with 0.1 M NaCl, 50 mM Tris-HCl, pH 7.4 (instead of pH 7.0). Then, the eggs were washed in an extraction medium used for *Tetrahymena* extracts and transferred to a 1.5-ml centrifuge tube, containing the same medium. After the excess medium was withdrawn, the eggs were crushed by centrifugation at 18,000*g* for 30 min (instead of 10,000*g* for 10 min) at 0°C. The material between the lipid cap and the yolk in the pellet was transferred to a 1-ml centrifuge tube and centrifuged again at 110,000*g* for 1 h (instead of 100,000*g* for 10 min) at 2°C, and the supernatant was kept at 0°C until use. For injection, the supernatant was diluted to $\frac{1}{5}$ by adding extraction medium and used as MPF-containing injection material.

Preparation of starfish oocytes. Starfish, *Asterina pectinifera*, were collected during breeding season and kept at 15–18°C in laboratory aquariums. Ovaries were isolated and cut into small fragments to release immature oocytes and the oocytes were washed with several changes of modified van't Hoff's artificial seawater (ASW) [6] and then suspended in calcium-free seawater (CaFSW) to remove follicles. Calcium-free seawater was prepared by replacing $CaCl_2$ in ASW with NaCl. When follicles were removed, the oocytes were washed with several changes of ASW and suspended for at least 30 min before use.

Assay for GVBD-inducing activity. In the case of *Bufo* oocytes, 100 nl of each sample was injected into each oocyte. The injected oocytes were suspended in De Boer's solution, incubated for 20 h at 20°C, fixed with 2.5% (v/v) trichloroacetic acid for 4–6 h at room temperature, and dissected by a razor blade to inspect for germinal vesicle breakdown. In the case of *Xenopus* oocytes, 50 nl of each sample was injected into each oocyte, and the injected oocytes were suspended in modified Barth's medium for 20 h at 20°C and fixed for 1–3 h. In each experiment, at least three control experiments were performed. The first was an injection of 100 or 50 nl of extraction medium, the second a treatment of oocytes with 10 μg/ml of progesterone (Tokyo-kasei), and the third a nontreatment of the oocytes.

In the case of *Asterina* oocytes, 150 pl of each sample was injected into each oocyte. The injected oocytes were suspended in 80% (v/v) ASW for 2 h at 24–25°C, and then GVBD was observed under a microscope without fixation. In each experiment, at least three control experiments were performed. The first was an injection of 150 pl of extraction medium, the second a treatment of oocytes with 2 μM 1-methyladenine (1-MA, Sigma), and the third a nontreatment of the oocytes. A maturing oocyte containing MPF of *Asterina* was prepared by treating immature oocytes with ASW containing 2 μM 1-MA for 20–40 min at 20–23°C, because during this period strong MPF activity appears [7].

Treatment with cycloheximide. Immediately after microinjection, the injected oocytes were suspended to De Boer's solution or modified Barth's medium containing 50 μg/ml cycloheximide for 20 h at 20°C and GVBD was examined.

Treatment with trypsin. One hundred microliters of *Tetrahymena* extract was incubated with 10 μl of 0.2% (w/v) trypsin (Sigma, type III) and dissolved in extraction medium or 10 μl of extraction medium alone for 30 min at 25°C, and then the mixture was incubated with 11 μl of 0.44% (w/v) trypsin inhibitor (Sigma, type I-S) for 10 min at 25°C and injected into *Bufo* oocytes. To avoid the possibility that trypsin or trypsin inhibitor may affect the induction of GVBD of the oocytes, extraction medium, instead of *Tetrahymena* extracts, was treated with trypsin and trypsin inhibitor in the same way and injected into the oocytes. Furthermore, *Tetrahymena* extracts were treated with extraction medium, instead of trypsin and trypsin inhibitor in the same way, and injected into the oocytes.

RESULTS

Germinal Vesicle Breakdown-Inducing Activity in Mitotic Cell Cycle of Tetrahymena Extracts

To monitor GVBD-inducing activity in mitotic cell cycle of *Tetrahymena*, synchronous cell division was induced by a periodic heat treatment, and changes in the cell density and division index were examined after the heat treatment. Cell division usually began at 68 min after the heat treatment. The first and the second peaks of the synchronous cell divisions appeared at 75 and at 180–250 min, with the maximum division indices being nearly 75 and 15–30%, respectively (Figs. 1 and 2). The timing and the maximum division index of the first peak were almost constant in experiments but those of the second peak were variable in experiments. Therefore, we monitored changes of GVBD-inducing activity from the end of heat treatment (0 min) to just before the second cell division (150 min). Results of the two experiments are summarized in Fig. 2. In Experiment A, cells were harvested at 0, 60, 75, and 150 min after the heat treatment, and their 110,000*g* supernatants were injected into 10 *Bufo* oocytes to compare their GVBD-inducing activities. In Experiment B, the supernatants were obtained from cells of 60, 70, 75, and 85 min and injected into 10 *Bufo* oocytes. Figure 1 and the graphs of division index and cell density in Fig. 2 represent Experiment A. Nuclear conditions of cells from which extracts were prepared showed that a majority of the cells at 75 min were at M-phase in the mitotic cell cycle, because most cells showed an elongated or constricted nucleus as well as a furrow for cell division, while cells at 0, 60, and 150 min did not (Fig. 1). Figure 2 shows that GVBD-inducing activity coincided with the first peak of the synchronous cell divisions. In the two experiments, 10 oocytes in which extraction medium alone was injected showed 0% GVBD, 10 oocytes which were treated with progesterone showed 100% GVBD, and 10 nontreated oocytes showed 0% GVBD, respectively. Since

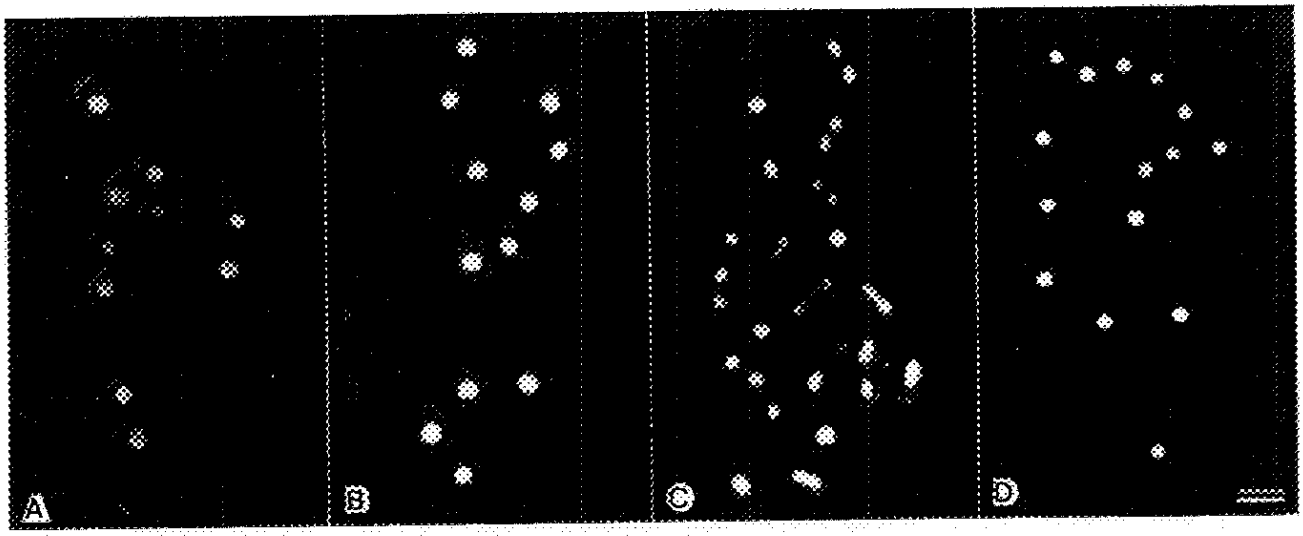

FIG. 1. Fluorescence differential interference-contrast photomicrographs of synchronous cell division of *Tetrahymena pyriformis*. 0 min (A), 60 min (B), 75 min (C), and 150 min (D) after a periodic heat treatment. Cells were fixed in a fixative containing 0.01% (w/v) Alcian blue, 5% (v/v) formaldehyde, and 10 m*M* Tris–HCl, pH 7.4, stained with 1 μg/ml DAPI. Note that cells form a furrow and their nucleus elongates and constricts for cell division and nuclear division in (C), but not in other preparations. Bar, 50 μm.

the GVBD-inducing activity is detectable only at M-phase in the mitotic cell cycle of *Tetrahymena*, it is strongly suggested that the factor has an important role for induction of M-phase in the cell cycle.

Hereafter, *Tetrahymena* extracts were obtained from cells at 75 min after the heat treatment.

Effect of Trypsin on GVBD-Inducing Activity of Tetrahymena Cell Extracts

Tetrahymena extracts were treated with either 0.02% (w/v) trypsin or 0.02% (v/v) extraction medium for 30 min at 25°C and then with 0.04% (w/v) trypsin inhibitor for 10 min at 25°C to inactivate the trypsin and were injected into *Bufo* oocytes (Table 1, Experiment A). Percentages of GVBD induced in the oocytes were 0 and 40%, respectively. When extraction medium was treated and injected into oocytes in the same way, however, the oocytes showed 0% GVBD (Table 1, Experiment B). On the other hand, if the extracts which had been kept at 0°C were heated at 25°C for 40 min as well as the time in Experiments A and B and then were injected into the oocytes, 100% GVBD was observed (Table 1, Experiment C). This indicates that MRIF activity does not decrease during the 40-min incubation at 25°C. Injection of extraction medium alone did not induce GVBD in the oocytes (Table 1, Experiment D). These results suggest that MRIF of *Tetrahymena* is a trypsin-sensitive protein. However, the trypsin inhibitor might prevent the action of MRIF on induction of GVBD, as 40% GVBD in the second experiment in (A) of Table 1 was lower than that in (C).

Effect of Ca^{2+} and EDTA on GVBD-Inducing Activity of Tetrahymena Extracts

It is well known that MPF is Ca^{2+}-sensitive and Mg^{2+}-dependent in its activity. To determine the effect of Ca^{2+} and ethylenediaminetetraacetic acid (EDTA) on GVBD-inducing activity of *Tetrahymena*, $CaCl_2$ or EDTA was added to cell extracts to give the final concentrations of 100 and 10 m*M*, respectively, and then were injected into *Bufo* oocytes (Table 2). Addition of $CaCl_2$ to the cell extracts completely inhibited the GVBD-inducing activity, indicating that the activity of *Tetrahymena* is Ca^{2+}-sensitive. Furthermore, the fact that addition of EDTA also induced complete loss of the GVBD-inducing activity of the cell extracts suggests that the activity is Mg^{2+}-dependent. In these points, therefore, GVBD-inducing activity of *Tetrahymena* resembles that of MPF.

Effect of Cycloheximide on the Action of GVBD-Inducing Activity of Tetrahymena Cell Extracts

MPF does not need protein synthesis of the recipient oocytes to induce GVBD [6]. To know whether GVBD-inducing factor of *Tetrahymena* requires protein synthesis of the recipient oocytes for induction of GVBD, *Tetrahymena* extracts were injected into *Bufo* oocytes. After the injection, 10 *Bufo* oocytes were suspended in De Boer's solution for 20 h at 20°C, and another 10 oocytes were suspended in the solution containing 50 μg/ml cycloheximide. *Bufo* oocytes injected with *Tetrahymena* extracts showed 70%, but 0% GVBD if the oocytes were treated with cycloheximide after the injec-

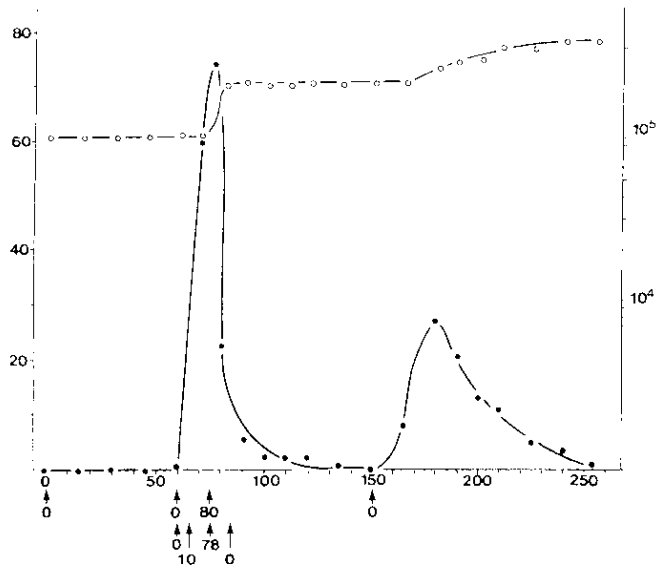

FIG. 2. MRIF activity in mitotic cell cycle of *Tetrahymena pyriformis*. Synchronous cell divisions were induced by a periodic heat treatment. Changes in GVBD-inducing activity from the end of heat treatment (0 min) to just before the second cell division (150 min) were monitored, because timing and the maximum division index of the first peak were almost constant in experiments, but those of the second peak were variable in experiments. In Experiment A, cells were harvested at 0, 60, 75, and 150 min after the heat treatment and homogenized, and then 110,000*g* supernatants of the homogenates were injected into 10 *Bufo* oocytes. In Experiment B, cells were harvested at 60, 70, 75, and 85 min after the heat treatment. Graphs of division index and cell density are those of Experiment A. Numbers under each arrow indicate percentage of oocytes in which GVBD was induced. Note that GVBD-inducing activity appears only at 75 min of a peak of the cell divisions. Abscissa, time after the heat treatment (min). Ordinate (left), percentage of dividing cells (%) (closed circle). Ordinate (right), cell density (cells/ml) (open circle).

tion. On the other hand, if MPF-containing *Xenopus* egg extracts were prepared using the same extraction medium used for *Tetrahymena* extracts (see Materials and Methods), diluted to $\frac{1}{5}$ with the extraction medium and then injected into *Xenopus* oocytes, 90 and 100% GVBD were induced with or without the presence of 50 μg/ml cycloheximide, respectively (Table 3). Therefore, if *Tetrahymena* extracts contained MPF activity, the recipient oocytes were expected to show GVBD in the presence of cycloheximide, because our results showed that the extraction medium and 50 μg/ml cycloheximide did not inactivate MPF activity nor prevent GVBD of the recipient oocytes. Our results suggest that, unlike other eukaryotes, ciliate *Tetrahymena* has no MPF activity. Instead, the results show that, like the meiosis-reinitiation-inducing factor of *Paramecium* [1], the GVBD-inducing factor of *Tetrahymena* extracts cannot induce GVBD in the absence of protein synthesis of the recipient oocytes. Therefore, hereafter, we call this GVBD-inducing factor the MRIF of *Tetrahymena*.

TABLE 1

Effect of Trypsin on GVBD-Inducing Activity of *Tetrahymena* Extracts

Materials injected	% GVBD
(A) Tetrahymena extracts treated with 0.02% (w/v) trypsin, 30 min, 25°C, and 0.04% (w/v) trypsin inhibitor, 10 min, 25°C 0.02% (v/v) extraction medium, 30 min, 25°C, and 0.04% (w/v) trypsin inhibitor, 10 min, 25°C	0 (10) 40 (10)
(B) Extraction medium treated with 0.02% (w/v) trypsin, 30 min, 25°C, and 0.04% (w/v) trypsin inhibitor, 10 min, 25°C	0 (10)
(C) *Tetrahymena* extracts treated with 25°C, 40 min	100 (10)
(D) Extraction medium	0 (10)
Control experiments	
Oocytes treated with progesterone	100 (10)
Nontreated oocytes	0 (10)

Note. Numbers in parentheses are number of oocytes observed. *Tetrahymena* extracts were 110,000*g* supernatants obtained from cells at the first peak of synchronous cell division (75 min). Each *Bufo* oocyte was injected with 100 μl sample, incubated in De Boer's solution for 20 h at 20°C, and fixed with 2.5% (v/v) trichloroacetic acid 20 h for inspecting GVBD. In (A), the cell extracts (100 μl) were treated with 0.2% (w/v) trypsin (10 μl) for 30 min at 25°C and with 0.44% (w/v) trypsin inhibitor (11 μl) for 10 min at 25°C, or treated with 10 μl of extraction medium and trypsin inhibitor as described above. In (B), extraction medium was treated with trypsin and trypsin inhibitor as in (A). In (C), the cell extracts which had been kept at 0°C were heated at 25°C for 40 min before the injection. In (D), extraction medium alone was injected. For control experiments, oocytes were treated with or without 10 μg/ml progesterone.

Effect of Microinjection of Tetrahymena Extracts into Starfish Asterina Oocytes

It is known that MPF acts in a non-species-specific manner across different phyla playing a key role in pro-

TABLE 2

Effects of Ca^{2+} and EDTA on GVBD-Inducing Activity of *Tetrahymena*

Materials injected	% GVBD
Tetrahymena extracts	
Treated with 100 m*M* $CaCl_2$	0 (10)
Treated with 10 m*M* EDTA	0 (10)
Nontreated	100 (10)
Extraction medium	0 (10)
Control experiments	
Oocytes treated with progesterone	100 (10)
Nontreated oocytes	0 (10)

Note. Numbers in parentheses are number of *Bufo* oocytes observed. *Tetrahymena* extracts were 110,000*g* supernatants obtained from cells at the first peak of synchronous cell division (75 min). Each oocyte was injected with 100 μl sample, incubated in De Boer's solution for 20 h at 20°C, and fixed with 2.5% (v/v) trichloroacetic acid for 20 h for inspecting GVBD.

TABLE 3

Effect of Cycloheximide on GVBD-Inducing Activity of *Tetrahymena* Extracts

Materials injected	% GVBD 50 μg/ml cycloheximide	
	Presence	Absence
Tetrahymena extracts[a]	0 (10)	70 (10)
MPF-containing *Xenopus* egg extracts[b]	100 (10)	90 (10)
Extraction medium[a]	0 (10)	0 (10)
Extraction medium[b]	0 (10)	0 (10)
Control experiments		
Bufo oocytes treated with progesterone	0 (10)	100 (10)
Xenopus oocytes treated with progesterone	0 (10)	100 (10)
Nontreated *Bufo* oocytes	0 (10)	0 (10)
Nontreated *Xenopus* oocytes	0 (10)	0 (10)

Note. Numbers in parentheses are number of oocytes observed. *Tetrahymena* extracts were 110,000*g* supernatants obtained from cells at the first peak of synchronous cell division (75 min). MPF-containing oocyte extracts were 110,000*g* supernatants obtained from unfertilized eggs using the same extraction medium used for *Tetrahymena* extracts. Injection volumes for *Bufo* and *Xenopus* oocytes were 100 and 50 μl, respectively. Injected *Bufo* oocytes were incubated in De Boer's solution with or without the presence of 50 μg/ml cycloheximide for 20 h at 20°C and fixed with 2.5% (v/v) trichloroacetic acid for inspecting GVBD. For injected *Xenopus* oocytes, modified Barth's medium was used instead of De Boer's solution.

[a] *Bufo* oocytes were used as recipients.

[b] *Xenopus* oocytes were used as recipients.

moting M-phase within the cytoplasm during both meiosis and mitosis [9]. To examine whether MRIF of *Tetrahymena* can induce GVBD in starfish oocytes, the cell extracts were injected into the starfish *A. pectinifera* oocytes (Table 4). It was found that MRIF of *Tetrahymena* could not induce GVBD in *Asterina* oocytes although it could induce GVBD in *Bufo* oocytes and *Asterina* cytoplasm containing MPF could induce GVBD in *Asterina* oocytes. This and the results so far obtained indicate that MRIF apparently differs from MPF in its action on induction of GVBD.

DISCUSSION

MPF is believed to be a ubiquitous cytoplasmic factor in controlling the induction of nuclear membrane cell extracts. The same had been shown in *Paramecium* cells of conjugation process in our previous work [1].

What is the function of MRIF? The fact that the MRIF activity appeared and disappeared in good accordance with that of dividing cells indicates that MRIF has an important role for induction of M-phase in ciliate cell cycle. Except MPF [7, 10–12], so far, several proteins are known to have an inducing activity of oocyte maturation: cyclins [13], $p34^{cdc2}$ M-phase-specific H1 histone kinase [14, 15], regulatory subunit of cAMP-dependent protein kinase [16], $p21^{ras}$ oncogene product [17], and $pp39^{mos}$ c-*mos* protooncogene product [18]. In these proteins, it is known that the regulatory subunit of cAMP-dependent protein kinase and $p21^{ras}$ need protein synthesis to induce GVBD, and function upstream of MPF to activate pre-MPF [17]. Furthermore, recently, Kuge and Inoue [19] and Inoue and Kuge [20] purified a cycloheximide-sensitive meiotic maturation-inducing factor (CMF) from *Xenopus* oocytes and considered that CMF probably induced synthesis of activator of MPF. Therefore, MRIF may be closely related to the regulatory subunit of cAMP-dependent protein kinase, $p21^{ras}$, or CMF. Recently, we found that *Tetrahymena thermophila* had MRIF, too, and that cycloheximide-resistant GVBD-inducing activity (MPF activity) appeared in the recipient *Bufo* oocyte cytoplasm when GVBD was induced by injection of the MRIF (Fujishima and Sakimura, in preparation). This shows that MRIF functions upstream of MPF to induce MPF activity in *Bufo* oocytes. On the other hand, in this study, we showed that MRIF could not act on induction of GVBD in starfish oocytes. It is known that protein synthesis is required for induction of GVBD with progesterone in amphibian oocytes, but such protein synthesis is not required for induction of GVBD with 1-MA in starfish oocytes [21]. These results suggest that MRIF may induce protein synthesis in recipient oocytes and the protein synthesized may be the same as that synthesized by

TABLE 4

Effect of Microinjection of *Tetrahymena* Extracts into Starfish *Asterina* Oocytes

Materials injected	% GVBD in	
	Asterina oocytes	*Bufo* oocytes
Tetrahymena extracts	0 (10)	80 (10)
Asterina oocyte cytoplasms		
Maturing cytoplasm	100 (20)	—
Immature cytoplasm	0 (20)	—
Extraction medium	0 (10)	0 (10)
Control experiments		
Oocytes treated with progesterone	—	100 (10)
Oocytes treated with 1-MA	100 (25)	—
Nontreated oocytes	0 (22)	0 (10)

Note. Numbers in parentheses are number of oocytes observed. *Tetrahymena* extracts were 110,000*g* supernatants obtained from cells at the first peak of synchronous cell division (75 min). Injection volumes were 150 pl for *Asterina* oocytes and 100 nl for *Bufo* oocytes, respectively. GVBD was observed at 2 h after the injection in *Asterina* oocytes and at 20 hr in *Bufo* oocytes. Maturing oocyte containing MPF of *Asterina* was prepared by treating immature oocytes with ASW containing 2 μ*M* 1-MA for 20–40 min at 20–23°C.

treatment with progesterone and required for induction of GVBD.

However, the question why MPF activity cannot be detected in ciliate cell extracts remains. The following possibilities can be considered. First, MPF is present and functions for induction of M-phase in these ciliates, but the activity is unstable and easily disappears in the cell extracts. It is known that MPF is a Ca^{2+}-sensitive, Mg^{2+}-dependent soluble protein, and that the activity can be stabilized in the presence of ATP, β-glycerophosphate, IAA, and DTT. Therefore, to eliminate the above possibility we used an extraction medium containing 2 m*M* EGTA, 10 m*M* $MgSO_4$, protease inhibitors (30 μM BAME, 28 μM TPCK, 10 μg/ml leupeptin), phosphatase inhibitors (80 m*M* β-glycerophosphate), 1 m*M* ATP-γ-S, and 1 m*M* IAA in this study. The result obtained suggests the second possibility; that is, MPF was not present in the extracts. This possibility was strongly supported by the present experiments. Namely, MPF-containing *Xenopus* egg extracts which were prepared using the extraction medium used for *Tetrahymena* extracts could induce GVBD of the cycloheximide-treated oocytes.

Then, does MRIF induce M-phase in the presence of protein synthesis, or in the absence of it in the ciliate cell? This problem will be clarified if the MRIF is purified using induction of GVBD of amphibian oocytes as a bioassay system and if the purified MRIF is injected into ciliate cells in the presence or absence of cycloheximide. If MRIF needs protein synthesis for induction of M-phase in ciliates, we have to postulate another factor in downstream of MRIF, that appears as a result of protein synthesis after injection of MRIF and functions for induction of M-phase in ciliates but cannot act for induction of GVBD in amphibian and starfish oocytes.

In ciliates, nuclear membrane breakdown never occurs in the whole cell cycle. This may come from differences between the M-phase-inducing factor in ciliates and the MPF, although another possibility that ciliate nuclear membranes may structurally differ from metazoan nuclear membranes cannot be eliminated. Therefore, MRIF or its derivative may affect induction of chromatin condensation of micronucleus or formation of spindle apparatus of the micronucleus in the ciliate cell.

If MRIF or another factor induced by MRIF appears or increases its amounts at or near M-phase in the cell cycle, we expect that the polypeptides can be detected on two-dimensional SDS–polyacrylamide gels. We compared protein compositions of the cell extracts obtained from cells at 0, 45, 60, 75, and 150 min after heat treatment, but such polypeptides have not yet been found on the gels (data are not shown). This suggests that these proteins are probably minor polypeptides on the gels.

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