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Immunological Detection and Characterization of Poly(A) Polymerase, Poly(A)-Binding Protein and Cytoplasmic Polyadenylation Element-Binding Protein in Goldfish and *Xenopus* Oocytes

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ABSTRACT—Cytoplasmic polyadenylation regulates translational activation of dormant cyclin B1 mRNA stored in immature oocytes, a process required for the initiation of oocyte maturation in goldfish and *Xenopus*. As a first step towards understanding the molecular mechanisms of translational activation of cyclin B1 during oocyte maturation, we have isolated cDNA clones encoding proteins involved in cytoplasmic polyadenylation and produced specific antibodies against recombinant proteins. These include poly(A) polymerase (PAP), poly(A)-binding protein (PABP) and cytoplasmic polyadenylation element-binding protein (CPEB). Monoclonal antibodies raised against goldfish PAP recognized several forms of PAP in goldfish and *Xenopus* oocytes. Besides ordinary PAPs with high molecular weight (ca. 100 kDa), the antibodies also detected those with low molecular weight (ca. 40 kDa) that are present specifically in the cytoplasm, raising new players that might be responsible for cytoplasmic polyadenylation. An antibody against goldfish PABP showed for the first time in *Xenopus* oocytes the protein expression of PABPII, another PABP distinct from the well-characterized PABPI. Monoclonal antibodies raised against *Xenopus* CPEB recognized both unphosphorylated 62-kDa and phosphorylated 64-kDa forms but did not cross-react with goldfish CPEB, which was specifically detected by anti-goldfish CPEB monoclonal antibodies produced previously. The cDNAs, recombinant proteins and antibodies produced in this study are expected to provide useful tools for investigating the regulatory mechanisms of cyclin B1 translation during oocyte maturation in goldfish and *Xenopus*.

INTRODUCTION

Oocyte maturation is induced by maturation-promoting factor (MPF), which is formed and activated within the oocyte cytoplasm upon stimulation of maturation-inducing hormones (MIH) secreted from follicle cells surrounding the oocytes (for review, see Masui and Clarke, 1979; Nagahama *et al.*, 1995; Yamashita *et al.*, 2000). MPF has been purified from the eggs of several species and shown to consist of Cdc2 and cyclin B (Lohka *et al.*, 1988; Gautier *et al.*, 1988, 1990; Labbé *et al.*, 1988, 1989; Yamashita *et al.*, 1992a, b; Katsu *et al.*, 1993). To date, the molecular mechanisms of MPF formation and activation during oocyte maturation have been investigated in detail for *Xenopus* (Gautier and Maller, 1991; Kobayashi *et al.*, 1991; Minshull *et al.*, 1991) and goldfish (Hirai *et al.*, 1992; Kajiura *et al.*, 1993; Katsu *et al.*, 1993) (see also review by Yamashita, 1998). Cyclin B is absent in immature goldfish oocytes, and its synthesis through translational activation of dormant mRNA in immature oocytes by stimulation of MIH (17α,20β-dihydroxy-4-pregnen-3-one) is prerequisite for initiating oocyte maturation in this species (Yamashita *et al.*, 1995; Katsu *et al.*, 1999). In *Xenopus*, MIH (progesterone) also induces the translational activation-mediated syntheses of cyclin B1 and $p33^{ringo}$, both of which bind to and activate maternally stockpiled Cdc2, and these processes are thought to be necessary to activate MPF during normal (progesterone-induced)

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oocyte maturation (Nebreda *et al.*, 1995; Ferby *et al.*, 1999; Frank-Vaillant *et al.*, 1999). Cyclin B1 synthesis is a common phenomenon during oocyte maturation in all species examined so far and probably a general and essential process for the initiation of oocyte maturation, at least in lower vertebrates (see review by Yamashita, 2000).

During *Xenopus* oocyte maturation, the translation of cyclin B1 mRNA stored (masked) in oocytes is activated by cytoplasmic polyadenylation (Richter, 1996), which requires two *cis* elements in the 3' untranslated region (UTR), the highly conserved hexanucleotide AAUAAA and a nearby U-rich sequence termed the cytoplasmic polyadenylation element (CPE) (Fox *et al.*, 1989; McGrew *et al.*, 1989; McGrew and Richter, 1990; Stebbins-Boaz and Richter, 1994). The importance of cytoplasmic polyadenylation for translational activation of cyclin B1 mRNA has been confirmed by the finding that recruitment of endogenous cyclin B1 mRNA onto polysomes is specifically prevented by inhibition of cytoplasmic polyadenylation or by removal of its 3' UTR (Barkoff *et al.*, 2000).

Although the molecular basis for translational activation during oocyte maturation has not been fully elucidated, several factors involved in cytoplasmic polyadenylation have been identified in *Xenopus*. One is a poly(A) polymerase (PAP), which catalyzes polymerization of the poly(A) tail (Fox *et al.*, 1992; Martin and Keller, 1996; Martin *et al.*, 2000). Several forms of PAP have been isolated to date (Ballantyne *et al.*, 1995; Gebauer and Richter, 1995; Kashiwabara *et al.*, 2000), but their roles have not yet been determined. Cytoplasmic polyadenylation is also thought to be regulated by a poly(A)-binding protein (PABP) that binds to and stabilizes the elongated poly(A) tails (Stambuk and Moon, 1992; Wormington *et al.*, 1996; Borman *et al.*, 2000), on the basis of the findings in *Xenopus* oocytes that excess PABP inhibits deadenylation and translational inactivation of mRNA (Wormington *et al.*, 1996) and that tethered PABP causes translational stimulation (Gallie *et al.*, 2000; Gray *et al.*, 2000). Another factor responsible for cytoplasmic polyadenylation is a CPE-binding protein (CPEB) (Hake and Richter, 1994). The necessity of CPEB for cytoplasmic polyadenylation has been demonstrated by an experiment in which injection of a neutralizing antibody against CPEB into oocytes not only abrogated cyclin B1 mRNA polyadenylation but also inhibited progesterone-induced oocyte maturation (Stebbins-Boaz *et al.*, 1996).

Elongation in the poly(A) tail of cyclin B mRNA is required for its translational activation during goldfish oocyte maturation (Katsu *et al.*, 1999). Because the 3' UTR of goldfish cyclin B possesses CPE-like sequences and a homolog of CPEB is present in goldfish oocytes (Katsu *et al.*, submitted), it is likely that translation of goldfish cyclin B mRNA is controlled by CPE-mediated cytoplasmic polyadenylation. In both fishes and amphibians, therefore, CPE-mediated cytoplasmic polyadenylation seems to commonly regulate translational activation of cyclin B1 mRNA during oocyte maturation. As a first step toward understanding the mechanisms of translational activation of cyclin B1 mRNA in goldfish and *Xenopus* oocytes, we have isolated cDNA clones encoding goldfish PAP and PABP and *Xenopus* CPEB, produced bacterially expressed proteins, and raised specific antibodies against the recombinant proteins. In this report, immunological detection and characterization of PAP, PABP and CPEB in goldfish and *Xenopus* oocytes are described.

MATERIALS AND METHODS

Oocyte extraction

Goldfish (*Carassius auratus*) and South African clawed frogs (*Xenopus laevis*) were obtained from a local fish farm in Yatomi (Aichi, Japan) and from a dealer in Hamamatsu (Shizuoka, Japan), respectively. The animals were maintained in the laboratory until use. Full-grown immature oocytes of goldfish and *Xenopus* were freed from follicle cells by pipetting and collagenase treatment, respectively (Yoshida *et al.*, 1995). Mature *Xenopus* oocytes were obtained by incubating the oocytes with 10 μg/ml of progesterone.

For preparing small-scale extracts, 50 oocytes were homogenized in 50 μl of extraction buffer (EB: 100 mM β-glycerophosphate, 20 mM HEPES, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 100 μM (*p*-amidinophenyl)methanesulfonyl fluoride, 3 μg/ml leupeptin, pH 7.5) and centrifuged at 15,000 g for 10 min at 4°C. Bulk extracts were prepared by ultracentrifugation of oocytes (100,000 g, 30 min, 4°C) in EB (Yamashita *et al.*, 1992a). Aliquots were frozen in liquid nitrogen and stored at –80°C until use.

Isolation of germinal vesicles from full-grown oocytes

Germinal vesicles (GVs) were mechanically isolated from full-grown *Xenopus* oocytes in modified Barth's saline buffered with HEPES (Cyert and Kirschner, 1988), according to the procedure described previously (Dettlaff *et al.*, 1964). The isolated GVs and the enucleated oocytes were separately subjected to immunoblotting.

cDNA cloning

Two oligonucleotides, TGYTTYGAYGGNATHGARAT and GGRTTNGGCCAYTCCCAYTT (M = A + C, R = A + G, Y = C + T, H = A + C + T, N = A + C + G + T), were used to amplify a cDNA fragment of goldfish PAP by reverse transcription-polymerase chain reaction (RT-PCR). The PCR product homologous to PAP in other species was then used as a probe to isolate full-length PAP clones from a mature goldfish oocyte cDNA library. Of approximately 4×10^5 plaques screened, five plaques were isolated. Sequencing of the longest two clones revealed that one (named GFPAP-1) has an insert of 3,299-bp with an open reading frame (ORF) of 747 amino acids (DDBJ/EMBL/GenBank Accession No. AB048540) and that the other (GFPAP-3) has an insert of 3,452-bp with an ORF of 747 amino acids (DDBJ/EMBL/GenBank Accession No. AB048541). The two clones are highly homologous to each other in their ORF (95.9% identity at the amino acid level) but different in their 3' UTR. At the amino acid level, they exhibit about 80% identity to *Xenopus* type 3 PAP (Ballantyne *et al.*, 1995; Gebauer and Richter, 1995) and 60% to type 1 PAP (Ballantyne *et al.*, 1995).

cDNA fragments of goldfish PABP were isolated from poly(A)$^+$ RNA in mature goldfish oocytes by RT-PCR using two degenerate oligonucleotides, ATGTGGTCNCARMGNGAYCC and ARRCANACRAANCCRAANCC. The obtained 462-bp cDNA fragments were inserted into pCRII (Invitrogen, Carlsbad, CA) by TA cloning. DNA sequencing has revealed that the cDNAs can be classified into two clones (named GFPABP-2 and -10, DDBJ/EMBL/GenBank Accession No. AB048538 and AB048539, respectively). Both clones encode RNA-binding domains highly homologous (ca. 90% for GFPABP-2 and ca. 80% for GFPABP-10 at the amino acid level) to those of PABP in humans (Grange *et al.*, 1987), mice (Wang *et al.*, 1992) and *Xenopus* (Zelus *et al.*, 1989). The amino acid homology between GFPABP-2 and -10 is 83.1%.

Production of recombinant proteins

An expression vector for a fusion protein between glutathione S-transferase (GST) and goldfish PAP (GST-GFPAP) was produced as follows: The entire ORFs of goldfish PAPs (GFPAP-1 and -3) in pBluescript SK (Stratagene, La Jolla, CA) in the T3 direction were amplified with GAAGATCTATGAAAGAGATGTCAACG (a 5' primer introducing a *Bgl*II site) and GTAATACGACTCACTATAGGGC (a T7 primer), digested with *Bgl*II, and ligated into *Bam*HI-cut pGEX-KG (Guan and Dixon, 1991). The ORF of GFPAP-3 was successfully ligated into pGEX-KG, whereas that of GFPAP-1 was not. Since the ORFs of GFPAP-1 and -3 are almost identical (Only 5 out of 747 amino acids have biochemical characters completely different from each counterpart.), we used only GST-GFPAP-3 as an antigen to produce antibodies against goldfish PAP. To construct fusion proteins between GST and goldfish PABP (GST-GFPABP-2 and -10), cDNAs in pCRII in the SP6 direction were amplified by PCR with a 5' primer introducing a *Bam*HI site (CGGGATCCATGTGGTCGCAGAGAGAT) and a T7 primer. The resulting cDNA fragments were digested with *Bam*HI and ligated into *Bam*HI-cut pGEX-KG. GST-fusion proteins were expressed in *Escherichia coli* XL1 (Stratagene) and purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroelution in Tris-glycine buffer without SDS, as described previously (Hirai *et al*., 1992).

To construct a histidine-tagged CPEB, the full ORF of *Xenopus* CPEB (Hake and Richter, 1994) was amplified by PCR with two oligonucleotides, GGAATTCCCATGGCCTTCCCACTGAAAGAT (a 5' primer introducing an *Eco*RI site) and ATGCTCGAGGCTGGAGTCACGACTTTTCTG (a 3' primer introducing a *Xho*I site). The PCR product was digested with *Eco*RI and *Xho*I, and ligated into the same cloning site of pET21c (Novagen, Madison, WI). Histidine-tagged *Xenopus* CPEB (T7-XlCPEB-His) was expressed in *E. coli* BL21-(DE3)pLysS and purified by electroelution following SDS-PAGE.

Production of monoclonal antibodies

Purified proteins (GST-GFPAP-3, GST-GFPABP-2, GST-GFPABP-10 and T7-XlCPEB-His) were dialyzed against 1 mM HEPES (pH 7.0), lyophilized, and injected into BALB/c mice to produce monoclonal antibodies, according to the procedures described previously (Yamashita *et al*., 1991). When GST-fusion proteins were used as antigens, monoclonal antibodies were screened with proteins without GST after cleaving the fusion proteins with thrombin (Guan and Dixon, 1991). We obtained hybridomas producing monoclonal antibodies against PAP and CPEB but failed to obtain those against PABP; we thus used polyclonal antibodies (mouse antisera) for detecting PABP. Monoclonal antibodies were prepared as ascites by injecting the hybridomas into the abdominal cavity of BALB/c mice.

Immunoprecipitation and immunoblotting

Extracts (50 μl) were mixed with 20 μl of protein G-Sepharose (Pharmacia, Tokyo, Japan) for 2 hr at 4°C. After centrifugation at 3,000 g for 1 min, the supernatant was mixed with 1 μl of anti-goldfish PAP, anti-goldfish PABP or anti-*Xenopus* CPEB antibodies and 20 μl of protein G-Sepharose, and incubated overnight at 4°C. The beads were washed with EB containing 0.2% Tween 20 and treated with 10 μl of ×2 SDS sample buffer. Proteins associated with the beads were separated by SDS-PAGE using a 12.5% gel, blotted onto Immobilon membranes (Millipore, Tokyo, Japan), and probed with the same antibody used for immunoprecipitation. The antigen-antibody complex was visualized with alkaline phosphatase-conjugated secondary antibodies, as previously described (Yamashita *et al*., 1991).

RESULTS AND DISCUSSION

Immunological detection and characterization of PAP

In this study, we characterized two monoclonal antibodies (GFPAP4 and GFPAP12) that recognize the recombinant full-length goldfish PAP with high specificity (their isotype being IgG1). Immunoblots of goldfish oocyte extracts showed that GFPAP12 detected 94- and 34-kDa bands and GFPAP4 detected 105- and 44-kDa bands (Fig. 1A). The immunoreactive 94- and 105-kDa proteins exhibited relative molecular masses greater than those deduced from goldfish PAP cDNAs isolated in the present study (83.2 kDa for GFPAP-1 and 83.4 kDa for GFPAP-3). Nevertheless, we conclude that these proteins are goldfish PAPs, since anomalous slow electrophoretic mobility due to the carboxy-terminal region of the protein has already been shown in a *Xenopus* PAP (Ballantyne *et al*., 1995). GFPAP4-positive 105-kDa goldfish protein exhibited electrophoretic mobility apparently different from that of GFPAP12-positive 94-kDa goldfish PAP. It is thus likely that goldfish oocytes contain at least two high-molecular-weight forms of PAP. The correspondence between the two forms of PAP protein (94- and 105-kDa) and cDNA (GFPAP-1 and -3) remains to be clarified in further studies. It also remains to be determined whether the immunoreactive 34- and 44-kDa proteins are biochemically modified (truncated) forms of the 94- and 105-kDa PAP or other types of PAP in goldfish, including a cytoplasmic-specific PAP, as discussed in a later paragraph. No positive signals were detected in *Xenopus*

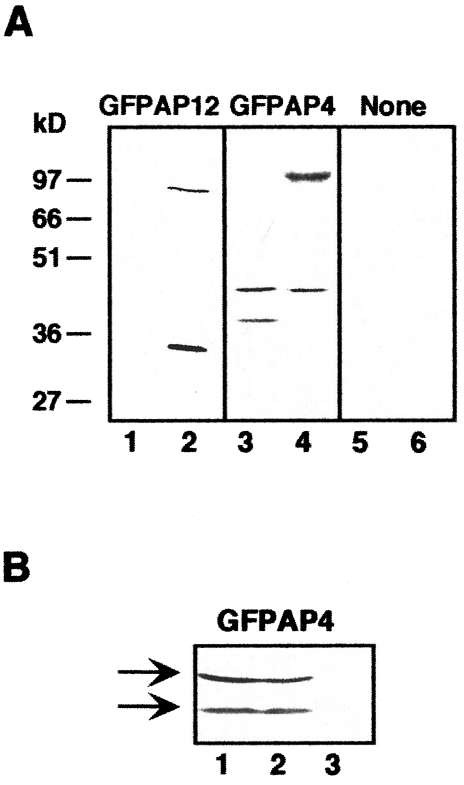

Fig. 1. (A) Immunoblots of immature *Xenopus* (lanes 1, 3 and 5) and goldfish (lanes 2, 4 and 6) oocyte extracts by anti-goldfish PAP monoclonal antibodies (GFPAP12, lanes 1 and 2; GFPAP4, lanes 3 and 4; negative controls without the primary antibodies; lanes 5 and 6). (B) GFPAP4-immunoblots of *Xenopus* extracts from whole oocytes (lane 1), enucleated oocytes (lane 2) and isolated GVs (lane 3). Extracts corresponding to two oocytes were applied to each lane. Arrows indicate the cytoplasmic-specific 39- and 44-kDa PAPs.

oocyte extracts by GFPAP12 (Fig. 1A), indicating that this antibody is specific to goldfish PAPs.

In addition to the 105- and 44-kDa proteins in goldfish oocyte extracts, GFPAP4 recognized 44- and 39-kDa proteins in *Xenopus* oocyte extracts (Fig. 1A). It has been reported that *Xenopus* oocytes contain at least four forms of PAP (106, 103, 96 and 93 kDa); the 106- and 103-kDa forms are present in the nucleus, the 93-kDa form is present in the cytoplasm, and the 96-kDa form is present in both of them (Ballantyne *et al.*, 1995). Besides these PAPs with relatively high molecular weight, a cDNA clone (DDBJ/EMBL/GenBank Accession No. U23456) that encodes *Xenopus* PAP with an ORF of 394 amino acids (calculated molecular weight of 45 kDa) has been isolated from ovary RNA (Ballantyne *et al.*, 1995; Gebauer and Richter, 1995), although its protein expression has not yet been confirmed (The native protein remains unidentified.). GFPAP4-reactive 39- and 44-kDa proteins might correspond to the protein encoded by U23456 cDNA. Since the U23456-encoded protein produced in a rabbit reticulocyte lysate was located in the cytoplasm when injected into oocytes, it has been suggested that this protein is a cytoplasm-specific PAP (Gebauer and Richter, 1995), in contrast to those with high molecular weight present both in the nucleus and cytoplasm.

To determine the subcellular localization of GFPAP4-reactive 39- and 44-kDa proteins in *Xenopus* oocytes, we mechanically isolated GVs from full-grown oocytes and examined the presence of these proteins in the isolated GVs and enucleated oocytes. Both GFPAP4-reactive 39- and 44-kDa proteins were detected in the enucleated oocyte cytoplasm but not in the isolated GVs (Fig. 1B), clearly indicating that they are cytoplasm-specific forms of PAP. Taken together, it is most likely that GFPAP4-reactive 39- and 44-kDa proteins are identical to the U23456-encoded protein or its close relatives, providing immunochemical evidence of the presence of cytoplasm-specific PAPs with low molecular weight for the first time in any species. It remains to be determined whether both GFPAP4-reactive 39- and 44-kDa are encoded by U23456 cDNA (i.e., chemically modified forms of the same protein) or whether only one of them is encoded by U23456 (i.e., different proteins). Although we must await the results of GV-isolation experiments in goldfish (the isolation of GV from goldfish oocytes without destroying the enucleated oocytes being technically impossible at present), it is also likely that GFPAP12-reactive 34-kDa protein, as well as GFPAP4-reactive 44-kDa protein, is a member of cytoplasmic-specific forms of goldfish PAP.

During oocyte maturation, dormant (masked) mRNAs receive poly(A) at characteristic times and to characteristic extents (Sheets *et al.*, 1994; Katsu *et al.*, 1999). Little is known about the mechanism by which coordinated polyadenylation of masked mRNAs is controlled to promote oocyte maturation successfully, but it is possible that different PAPs are involved in polyadenylation of different mRNAs. Consistent with this, our results, together with those of others (Ballantyne *et al.*, 1995; Gebauer and Richter, 1995), indicated that several PAPs are present in both *Xenopus* and goldfish oocytes. Our current aim is to identify PAPs responsible for polyadenylation of cyclin B1 mRNA, using the probes produced in this study.

Immunological detection and characterization of PABP

PABP, one of the most conserved ribonucleoproteins, consists of four highly conserved RNA-binding domains (RBDs) and a less-conserved proline-rich carboxy-terminal domain (Adam *et al.*, 1986; Burd *et al.*, 1991; Burd and Dreyfuss, 1994; Okamura *et al.*, 2000). Goldfish cDNA clones obtained by RT-PCR in this study include two of the four RBDs. Using the recombinant proteins produced from these cDNAs as antigens, we raised antibodies against PABP. Because we were unable to isolate monoclonal antibodies, polyclonal antibodies (antisera) were used for detecting PABP in goldfish and *Xenopus* oocytes.

Of four antisera examined, only one serum raised against GST-GFPABP-2 showed a specific reaction. In contrast to the well-characterized 70-kDa PABPI (Zelus *et al.*, 1989; Nietfeld *et al.*, 1990; Wahle *et al.*, 1993), the antibody precipitated a single 49-kDa protein from *Xenopus* ovary extracts (Fig. 2) but not from goldfish ovary extracts (data not shown). The 49-kDa protein was also detected by immunoblotting (data not shown). Judging from its molecular size, which is similar to that of calf PABPII (Wahle *et al.*, 1991; Nemeth *et al.*, 1995), the immunoreactive 49-kDa protein seems to be a *Xenopus* homolog of PABPII. These results provide for the first time evidence, thought not direct, of the existence of PABPII or its cognate in *Xenopus* oocytes, introducing a new player that might be responsible for the regulated polyadenylation specific to oocyte maturation. Further work is required to determine whether the 49-kDa PABPII-like protein has a role in controlling poly(A) tail length, as proposed for PABPI (Sachs and Wahle, 1993). It also remains to be determined whether or not goldfish oocytes contain PABPII, as well as PABPI, like in *Xenopus*.

Although the biological role of PABPI has been analyzed for *Xenopus* over the past decade (Stambuk and Moon, 1992; Wormington *et al.*, 1996; Wakiyama *et al.*, 2000), the involvement of this protein in regulating maturation-specific polyadenylation and translational activation of masked mRNAs is still unclear. Therefore, more extensive analyses on PABPs, especially on PABPII, to which the antibody characterized in

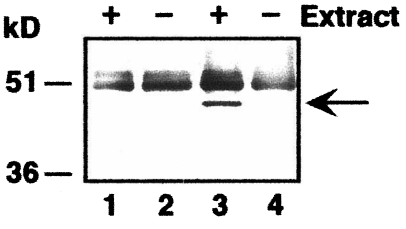

Fig. 2. Anti-goldfish PABP immunoblots of immunoprecipitates from *Xenopus* ovary (50 mg) extracts (+) or extraction buffer alone (-) with preimmune serum (lanes 1 and 2) or anti-goldfish PABP antiserum (lanes 3 and 4). Immunoprecipitates without extracts show the positions of immunoglobulins (lanes 2 and 4). The arrow indicates *Xenopus* PABPII-like protein.

this study would be a great contribution, are needed.

Immunological detection and characterization of CPEB

We have already produced several anti-goldfish CPEB monoclonal antibodies, which recognize the native CPEB in goldfish oocytes with extremely high specificity (Katsu *et al.*, submitted). We examined whether the anti-goldfish CPEB antibodies cross-react with *Xenopus* counterparts, and we found that none of them can work in *Xenopus* (data not shown). Therefore, we raised new antibodies using the recombinant full-length *Xenopus* CPEB as an antigen. Three monoclonal antibodies (XlCPEB5, 10 and 12, their isotype being IgG1) were isolated. None of them cross-reacted with goldfish CPEB (data not shown). The anti-*Xenopus* CPEB monoclonal antibodies recognized a single 62-kDa band in immature *Xenopus* oocytes by immunoblotting and immunoprecipitation (Fig. 3). The immunoreactive 62-kDa protein is comparable with *Xenopus* CPEB characterized previously (Hake and Richter, 1994; de Moor and Richter, 1997). The antibodies also recognized a 64-kDa protein, as well as the 62-kDa form, in mature oocytes by immunoblotting (data not shown) and immunoprecipitation (Fig. 3B), consistent with the previous finding that mature oocytes contain a phosphorylated CPEB that exhibits slower electrophoretic mobility (corresponding to an apparent molecular mass of 64 kDa) in SDS-PAGE (Hake and Richter, 1994; de Moor and Richter, 1997). Thus, the antibodies produced in this study can detect both unphosphorylated 62-kDa and phosphorylated 64-kDa forms of CPEB in *Xenopus* oocytes.

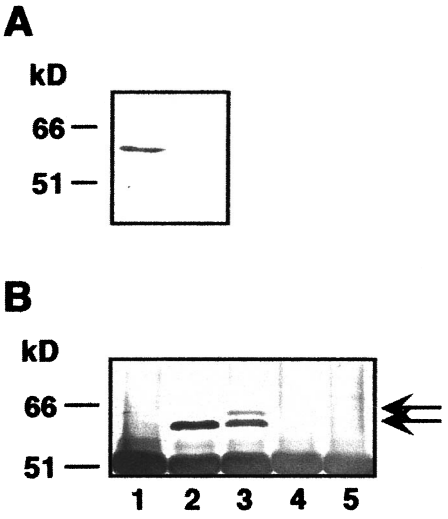

Fig. 3. (A) Immunoblots of immature *Xenopus* oocytes extracts with (lane 1) or without (lane 2) anti-CPEB monoclonal antibody (XlCPEB12). (B) Anti-*Xenopus* CPEB (XlCPEB12) immunoblots of immature (I) and mature (M) *Xenopus* oocyte extracts precipitated with XlCPEB12 (lanes 2 and 3) or anti-goldfish cyclin B1 antibody (B63, Hirai *et al.*, 1992) as a control (lanes 4 and 5). The positions of immunoglobulins are shown by immunoprecipitation without extracts (–) (lane 1). Essentially the same results were obtained when the other antibodies (XlCPEB5 and 10) were used. Arrows indicate two forms (unphosphorylated 62-kDa and phosphorylated 64-kDa forms) of CPEB.

Recent studies have revealed that CPEB is an essential regulator of translational activation of cyclin B1 during oocyte maturation (de Moor and Richter, 1999; Stebbins-Boaz *et al.*, 1999; Barkoff *et al.*, 2000; Tay *et al.*, 2000). CPEB is responsible for both translational repression of cyclin B1 mRNA in immature *Xenopus* oocytes and translational activation through cytoplasmic polyadenylation in maturing oocytes (de Moor and Richter, 1999; Stebbins-Boaz *et al.*, 1999; Barkoff *et al.*, 2000). CPEB is phosphorylated upon progesterone stimulation, which is accompanied by a retardation in electrophoretic mobility (Fig. 3B). The timing of CPEB phosphorylation correlates with the translational activation of cyclin B1 mRNA (Hake and Richter, 1994; de Moor and Richter, 1997, 1999), implying the involvement of this modification in a switch in CPEB activity from a repressor to an activator. However, the biological significance of CPEB phosphorylation in activation of polyadenylation and translation is still a matter of controversy (Stebbins-Boaz *et al.*, 1999; Barkoff *et al.*, 2000; Mendez *et al.*, 2000a, b), and we are still far from understanding the entire mechanism of CPEB-mediated translational control of cyclin B1.

Recently, a protein called maskin has been identified as a CPEB/eIF-4E-associated protein that plays an essential role in regulating translation of CPE-containing mRNAs (Stebbins-Boaz *et al.*, 1999). Although this protein could be involved in the translational control of cyclin B1 mRNA, repression of the translation may require other proteins that act either alone or in combination with CPEB, because CPEs are required but insufficient for repression of cyclin B1 mRNA (Barkoff *et al.*, 2000). Therefore, it is of great importance to identify CPEB-interacting proteins that also bind to the 3' UTR of cyclin B1 mRNA. The antibodies and recombinant proteins produced in this study provide the ultimate biochemical tools to investigate this issue.

Cyclin B synthesis by translational activation of masked mRNA after MIH stimulation is a key event in initiating oocyte maturation in goldfish and *Xenopus* (as well as other fishes and amphibians) (Yamashita, 2000). The cDNAs, recombinant proteins, and antibodies prepared in this study will provide useful experimental tools for further investigation of the molecular mechanisms of translational regulation of cyclin B1 mRNA during oocyte maturation in goldfish and *Xenopus*, the experimental systems that have been used most frequently to date.

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