BioOne COMPLETE

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

Authors: Nakahata, Shingo, Mita, Koichi, Katsu, Yoshinao, Nagahama, Yoshitaka, and Yamashita, Masakane

Source: Zoological Science, 18(3) : 337-343

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.18.337

BioOne Complete (complete.BioOne.org) is a full-text database of 200 subscribed and open-access titles in the biological, ecological, and environmental sciences published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Complete website, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Complete content is strictly limited to personal, educational, and non - commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

Downloaded From: https://bioone.org/journals/Zoological-Science on 13 Sep 2024
Terms of Use: https://bioone.org/terms-of-use

ZOOLOGICAL SCIENCE **18**: 337–343 (2001) © 2001 Zoological Society of Japan

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

Shingo Nakahata[1], Koichi Mita[1], Yoshinao Katsu[2,3], Yoshitaka Nagahama[2] and Masakane Yamashita[1,*]

[1]*Laboratory of Molecular and Cellular Interactions, Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan*
[2]*Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan*
[3]*Center for Integrative Bioscience, Okazaki National Research Institutes, Okazaki 444-8585, Japan*

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)

* Corresponding author: Tel. +81-11-706-4454;
FAX. +81-11-706-4456.
E-mail: myama@sci.hokudai.ac.jp

Downloaded From: https://bioone.org/journals/Zoological-Science on 13 Sep 2024
Terms of Use: https://bioone.org/terms-of-use

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 GGR-TTNGGCCAYTCCCAYTT (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 ARRCANACR-AANCCRAANCC. 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%.

Downloaded From: https://bioone.org/journals/Zoological-Science on 13 Sep 2024
Terms of Use: https://bioone.org/terms-of-use

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 (CGGGATCCATGTGGTCGCAG-AGAGAT) 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 ATGCTCGAGGCTGGAGT-CACGACTTTTCTG (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-GFP-ABP-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*

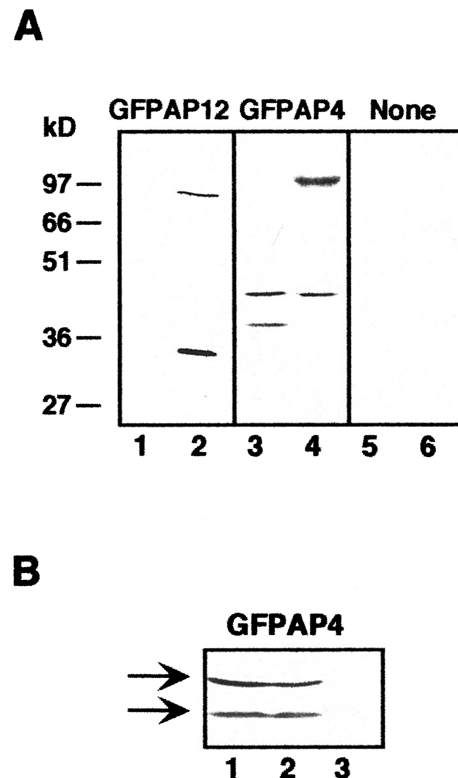

Fig. 1. (A) Immunoblots of immature *Xenopus* (lanes 1, 3 and 5) and goldfish (lanes 2, 4 and 6) oocyte extracts by anti-goldfish PAP monoclonal antibodies (GFPAP12, lanes 1 and 2; GFPAP4, lanes 3 and 4; negative controls without the primary antibodies; lanes 5 and 6). (B) GFPAP4-immunoblots of *Xenopus* 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.

Downloaded From: https://bioone.org/journals/Zoological-Science on 13 Sep 2024
Terms of Use: https://bioone.org/terms-of-use

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

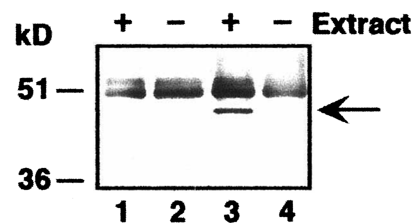

Fig. 2. Anti-goldfish PABP immunoblots of immunoprecipitates from *Xenopus* ovary (50 mg) extracts (+) or extraction buffer alone (-) with preimmune serum (lanes 1 and 2) or anti-goldfish PABP antiserum (lanes 3 and 4). Immunoprecipitates without extracts show the positions of immunoglobulins (lanes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

Downloaded From: https://bioone.org/journals/Zoological-Science on 13 Sep 2024
Terms of Use: https://bioone.org/terms-of-use

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.

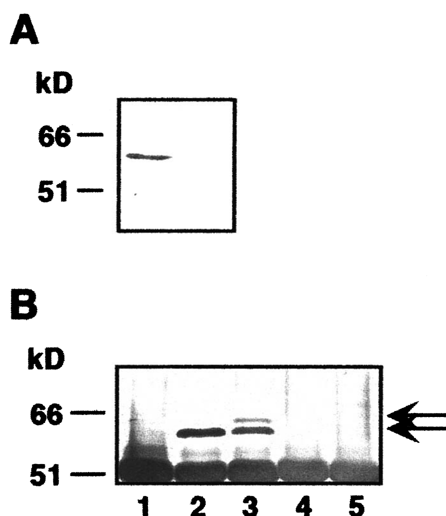

Fig. 3. (A) Immunoblots of immature *Xenopus* oocytes extracts with (lane 1) or without (lane 2) anti-CPEB monoclonal antibody (XlCPEB12). (B) Anti-*Xenopus* CPEB (XlCPEB12) immunoblots of immature (I) and mature (M) *Xenopus* oocyte extracts precipitated with XlCPEB12 (lanes 2 and 3) or anti-goldfish 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.

ACKNOWLEDGMENTS

This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan to M.Y. and JSPS-RFTF 96L00401 and BioDesign Program from the Ministry of Agriculture, Forestry and Fisheries, Japan to Y.N.

REFERENCES

Adam SA, Nakagawa T, Swanson MS, Woodruff TK, Dreyfuss G (1986) mRNA polyadenylate-binding protein: gene isolation and

Downloaded From: https://bioone.org/journals/Zoological-Science on 13 Sep 2024
Terms of Use: https://bioone.org/terms-of-use

sequencing and identification of a ribonucleoprotein consensus sequence. Mol Cell Biol 6: 2932–2943
Ballantyne S, Bilger A, Astrom J, Virtanen A, Wickens M (1995) Poly(A) polymerases in the nucleus and cytoplasm of frog oocytes: Dynamic changes during oocyte maturation and early development. RNA 1: 64–78
Barkoff AF, Dickson KS, Gray NK, Wickens M (2000) Translational control of cyclin B1 mRNA during meiotic maturation: Coordinated repression and cytoplasmic polyadenylation. Dev Biol 220: 97–109
Borman AM, Michel YM, Kean KM (2000) Biochemical characterisation of cap-poly(A) synergy in rabbit reticulocyte lysates: the eIF4G-PABP interaction increases the functional affinity of eIF4E for the capped mRNA 5’-end. Nucleic Acids Res 28: 4068–4075
Burd CG, Dreyfuss G (1994) Conserved structures and diversity of functions of RNA-binding proteins. Science 265: 615–621
Burd CG, Matunis EL, Dreyfuss G (1991) The multiple RNA-binding domains of the mRNA poly(A)-binding protein have different RNA-binding activities. Mol Cell Biol 9: 3419–3424
Cyert MS, Kirschner MW (1988) Regulation of MPF activity *in vitro*. Cell 53: 185–195
de Moor CH, Richter JD (1997) The Mos pathway regulates cytoplasmic polyadenylation in *Xenopus* oocytes. Mol Cell Biol 17: 6419–6426
de Moor CH, Richter JD (1999) Cytoplasmic polyadenylation elements mediate masking and unmasking of cyclin B1 mRNA. EMBO J 18: 2294–2303
Dettlaff TA, Nikitina LA, Stroeva OG (1964) The role of the germinal vesicle in oocyte maturation in anurans as revealed by the removal and transplantation of nuclei. J Embryol Exp Morph 12: 55–873
Ferby I, Blazquez M, Palmer A, Eritja R, Nebreda AR (1999) A novel $p34^{cdc2}$-binding and activating protein that is necessary and sufficient to trigger G2/M progression in *Xenopus* oocytes. Genes Dev 13: 2177–2189
Frank-Vaillant M, Jessus C, Ozon R, Maller JL, Haccard O (1999) Two distinct mechanisms control the accumulation of cyclin B1 and Mos in *Xenopus* oocytes in response to progesterone. Mol Biol Cell 10: 3279–3288
Fox CA, Sheets MD, Wickens M (1989) Poly(A) addition during maturation of frog oocytes: distinct nuclear and cytoplasmic activities and regulation by the sequence UUUUUAU. Genes Dev 3: 2151–2162
Fox CA, Sheets MD, Wahle E, Wickens M (1992) Polyadenylation of maternal mRNA during oocyte maturation: poly(A) addition *in vitro* requires a regulated RNA binding activity and a poly(A) polymerase. EMBO J 11: 5021–5032
Gallie DR, Ling J, Niepel M, Morley SJ, Pain VM (2000) The role of 5’-leader length, secondary structure and PABP concentration on cap and poly(A) tail function during translation in *Xenopus* oocytes. Nucleic Acids Res 28: 2943–2953
Gautier J, Maller JL (1991) Cyclin B in *Xenopus* oocytes: Implications for the mechanism of pre-MPF activation. EMBO J 10: 177–182
Gautier J, Minshull J, Lohka M, Glotzer M, Hunt T, Maller JL (1990) Cyclin is a component of maturation-promoting factor from *Xenopus*. Cell 60: 487–494
Gautier J, Norbury C, Lohka M, Nurse P, Maller JL (1988) Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene $cdc2^+$. Cell 54: 433–439
Gebauer F, Richter JD (1995) Cloning and characterization of a *Xenopus* poly(A) polymerase. Mol Cell Biol 15: 1422–1430
Grange T, De Sa Martins C, Oddos J, Pictet R (1987) Human mRNA polyadenylate binding protein: evolutionary conservation of a nuclei acid binding motif. Nucleic Acids Res 15: 4771–4787
Gray NK, Coller JM, Dickson KS, Wickens M (2000) Multiple portions of poly(A)-binding protein stimulate translation *in vivo*. EMBO J 19: 4723–4733
Guan K, Dixon JE (1991) Eukaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal Biochem 192: 262–267
Hake LE, Richter JD (1994) CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. Cell 79: 617–627
Hirai T, Yamashita M, Yoshikuni M, Lou YH, Nagahama Y (1992) Cyclin B in fish oocytes: Its cDNA and amino acid sequences, appearance during maturation, and induction of $p34^{cdc2}$ activation. Mol Reprod Dev 33: 131–140
Kajiura H, Yamashita M, Katsu Y, Nagahama Y (1993) Isolation and characterization of goldfish cdc2, a catalytic component of maturation-promoting factor. Dev Growth Differ 35: 647–654
Kashiwabara Si, Zhuang T, Yamagata K, Noguchi J, Fukamizu A, Baba T (2000) Identification of a novel isoform of poly(A) polymerase, TPAP, specifically present in the cytoplasm of spermatogenic cells. Dev Biol 228: 106–115
Katsu Y, Yamashita M, Kajiura H, Nagahama Y (1993) Behavior of the components of maturation-promoting factor, cdc2 kinase and cyclin B, during oocyte maturation of goldfish. Dev Biol 160: 99–107
Katsu Y, Yamashita M, Nagahama Y (1999) Translational regulation of cyclin B mRNA by 17α,20β-dihydroxy-4-pregnen-3-one (maturation-inducing hormone) during oocyte maturation in a teleost fish, the goldfish (*Carassius auratus*). Mol Cell Endocrinol 158, 79–85
Kobayashi H, Minshull J, Ford C, Golsteyn R, Poon R, Hunt T (1991) On the synthesis and destruction of A- and B-type cyclins during oogenesis and meiotic maturation in *Xenopus laevis*. J Cell Biol 114: 755–765
Labbé JC, Capony JP, Caput D, Cavadore JC, Derancourt J, Kaghad M, Lelias JM, Picard A, Dorée M (1989) MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. EMBO J 8: 3053–3058
Labbé JC, Lee MG, Nurse P, Picard A, Dorée M (1988) Activation at M-phase of a protein kinase encoded by a starfish homologue of the cell cycle control gene $cdc2^+$. Nature 335: 251–254
Lohka MJ, Hayes MK, Maller JL (1988) Purification of maturation-promoting factor, an intracellular regulator of early mitotic events. Proc Natl Acad Sci USA 85: 3009–3013
Martin G, Keller W (1996) Mutational analysis of mammalian poly(A) polymerase identifies a region for primer binding and a catalytic domain, homologous to the family X polymerases, and to other nucleotidyltransferases. EMBO J 15: 2593–2603
Martin G, Keller W, Doublie S (2000) Crystal structure of mammalian poly(A) polymerase in complex with an analog of ATP. EMBO J 19: 4193–4203
Masui Y, Clarke HJ (1979) Oocyte maturation. Int Rev Cytol 57: 185–282
McGrew LL, Dworkin-Rastle E, Dworkin MB, Richter JD (1989) Poly(A) elongation during *Xenopus* oocyte maturation is required for translational recruitment and is mediated by a short sequence element. Genes Dev 3: 803–815
McGrew LL, Richter JD (1990) Translational control by cytoplasmic polyadenylation during *Xenopus* oocyte maturation: characterization of *cis* and *trans* elements and regulation by cyclin/MPF. EMBO J 9: 3743–3751
Mendez R, Hake LE, Andresson T, Littlepage LE, Ruderman JV, Richter JD (2000a) Phosphorylation of CPE binding factor by Eg2 regulates translation of c-*mos* mRNA. Nature 404: 302–307
Mendez R, Murthy KGK, Ryan K, Manley JL, Richter JD (2000b) Phosphorylation of CPEB by Eg2 mediates the recruitment of CPSF into an active cytoplasmic polyadenylation complex. Mol Cell 6: 1253–1259

Downloaded From: https://bioone.org/journals/Zoological-Science on 13 Sep 2024
Terms of Use: https://bioone.org/terms-of-use

Minshull J, Murray A, Colman A, Hunt T (1991) *Xenopus* oocyte maturation does not require new cyclin synthesis. J. Cell Biol 114: 767–772

Nagahama Y, Yoshikuni M, Yamashita M, Tokumoto T, Katsu Y (1995) Regulation of oocyte growth and maturation in fish. Curr Topics Dev Biol 30: 103–145

Nebreda AR, Gannon JV, Hunt T (1995) Newly synthesized protein(s) must associate with $p34^{cdc2}$ to activate MAP kinase and MPF during progesterone-induced maturation of *Xenopus* oocytes. EMBO J 14: 5597–5607

Nemeth A, Krause S, Blank D, Jenny A, Jeno P, Lustig A, Wahle E (1995) Isolation of genomic and cDNA clones encoding bovine poly(A) binding protein II. Nucleic Acids Res 23: 4034–4041

Nietfeld W, Mentzel H, Pieler T (1990) The *Xenopus laevis* poly(A) binding protein is composed of multiple functionally independent RNA binding domains. EMBO J 9: 3699–3705

Okamura T, Imai Y, Kon Y, Goto M, Yamamoto M, Watanabe T (2000) Molecular cloning and characterization of mouse testis poly(A) binding protein II encoded by the Pabp3 gene, which transcomplements meiotic mutant *sme2* of *S. pombe*. Biochem Genet 38: 1–11

Richter JD (1996) Dynamics of poly(A) addition and removal during development. In Translational Control, J. Hershey, M. Mathews, and N. Sonenberg, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press)

Sachs A, Wahle E (1993) Poly(A) tail metabolism and function in eucaryotes. J Biol Chem 268: 22955–22958

Sheets MD, Fox CA, Hunt T, Vande Woude G, Wickens M (1994) The 3'-untranslated regions of *c-mos* and cyclin mRNAs stimulate translation by regulating cytoplasmic polyadenylation. Genes Dev 8: 926–938

Stambuk RA, Moon RT (1992) Purification and characterization of recombinant *Xenopus* $poly(A)^{+}$-binding protein expressed in a baculovirus system. Biochem J 287: 761–766

Stebbins-Boaz B, Cao Q, de Moor CH, Mendez R, Richter JD (1999) Maskin is a CPEB-associated factor that transiently interacts with eIF-4E. Mol Cell 4: 1017–1027

Stebbins-Boaz B, Hake LE, Richter JD (1996) CPEB controls the cytoplasmic polyadenylation of cyclin, Cdk2 and *c-mos* mRNAs and is necessary for oocyte maturation in *Xenopus*. EMBO J 15: 2582–2592

Stebbins-Boaz B, Richter JD (1994) Multiple sequence elements and a maternal mRNA product control cdk2 RNA polyadenylation and translation during early *Xenopus* development. Mol Cell Biol 14: 5870–5880

Tay J, Hodgman R, Richter JD (2000) The control of cyclin B1 mRNA translation during mouse oocyte maturation. Dev Biol 221: 1–9

Wahle E (1991) A novel poly(A)-binding protein acts as a specificity factor in the second phase of messenger RNA polyadenylation. Cell 66: 759–768

Wahle E, Lustig A, Jeno P, Maurer P (1993) Mammalian poly(A)-binding protein II. Physical properties and binding to polynucleotides. J Biol Chem 268: 2937–2945

Wakiyama M, Imataka H, Sonenberg N (2000) Interaction of eIF4G with poly(A)-binding protein stimulates translation and is critical for *Xenopus* oocyte maturation. Curr Biol 10, 1147–1150

Wang MY, Cutler M, Karimpour I, Kleene KC (1992) Nucleotide sequence of a mouse testis poly(A) binding protein cDNA. Nucleic Acids Res 20: 3519

Wormington M, Searfoss AM, Hurney CA (1996) Overexpression of poly(A) binding protein prevents maturation-specific deadenylation and translational inactivation in *Xenopus* oocytes. EMBO J 15: 900–909

Yamashita M (1998) Molecular mechanisms of meiotic maturation and arrest in fish and amphibian oocytes. Semin Cell Dev Biol 9: 569–579

Yamashita M (2000) Toward modeling of a general mechanism of MPF formation during oocyte maturation in vertebrates. Zool Sci 17: 841–851

Yamashita M, Fukada S, Bulet P, Hirai T, Yamaguchi A, Lou YH, Zhao Z, Nagahama, Y (1992a) Purification and characterization of maturation-promoting factor in fish. Dev Biol 149: 8–15

Yamashita M, Fukada S, Yoshikuni M, Bulet P, Hirai T, Yamaguchi A, Yasuda H, Ohba Y, Nagahama Y (1992b) M-phase-specific histone H1 kinase in fish oocytes: Its purification, components and biochemical properties. Eur J Biochem 205: 537–543

Yamashita M, Kajiura H, Tanaka T, Onoe S, Nagahama Y (1995) Molecular mechanisms of the activation of maturation-promoting factor during goldfish oocyte maturation. Dev Biol 168: 62–75

Yamashita M, Mita K, Yoshida, N, Kondo T (2000) Molecular mechanisms of the initiation of oocyte maturation: general and species-specific aspects. Semin Cell Cycle Res 4: 115–129

Yamashita M, Yoshikuni M, Hirai T, Fukada S, Nagahama Y (1991) A monoclonal antibody against the PSTAIR sequence of $p34^{cdc2}$, catalytic subunit of maturation-promoting factor and key regulator of the cell cycle. Dev Growth Differ 33: 617–624

Yoshida N, Tanaka T, Yamashita, M (1995) Changes in phosphorylation activities during goldfish and *Xenopus* oocyte maturation. Zool Sci 12: 599–606

Zelus BD, Giebelhaus DH, Eib DW, Kenner KA, Moon RT (1989) Expression of the poly(A) binding protein during development of *Xenopus laevis*. Mol Cell Biol 9: 2756–2760

(Received October 31, 2000 / Accepted December 1, 2000)

Downloaded From: https://bioone.org/journals/Zoological-Science on 13 Sep 2024
Terms of Use: https://bioone.org/terms-of-use