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ARTICLE *in* MECHANISMS OF DEVELOPMENT · SEPTEMBER 2003

Impact Factor: 2.44 · DOI: 10.1016/S0925-4773(03)00160-6 · Source: PubMed

Mechanisms of Development 120 (2003) 865–880

www.elsevier.com/locate/modo

Involvement of *Xenopus* Pumilio in the translational regulation that is specific to cyclin B1 mRNA during oocyte maturation

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Received 20 January 2003; received in revised form 21 June 2003; accepted 23 June 2003

Abstract

Protein synthesis of cyclin B by translational activation of the dormant mRNA stored in oocytes is required for normal progression of maturation. In this study, we investigated the involvement of *Xenopus* Pumilio (XPum), a cyclin B1 mRNA-binding protein, in the mRNA-specific translational activation. XPum exhibits high homology to mammalian counterparts, with amino acid identity close to 90%, even if the conserved RNA-binding domain is excluded. XPum is bound to cytoplasmic polyadenylation element (CPE)-binding protein (CPEB) through the RNA-binding domain but not to its phosphorylated form in mature oocytes. In addition to the CPE, the XPum-binding sequence of cyclin B1 mRNA acts as a *cis*-element for translational repression. Injection of anti-XPum antibody accelerated oocyte maturation and synthesis of cyclin B1, and, conversely, over-expression of XPum retarded oocyte maturation and translation of cyclin B1 mRNA, which was accompanied by inhibition of poly(A) tail elongation. The injection of antibody and the over-expression of XPum, however, had no effect on translation of Mos mRNA, which also contains the CPE. These findings provide the first evidence that XPum is a translational repressor specific to cyclin B1 in vertebrates. We propose that in cooperation with the CPEB-maskin complex, the master regulator common to the CPE-containing mRNAs, XPum acts as a specific regulator that determines the timing of translational activation of cyclin B1 mRNA by its release from phosphorylated CPEB during oocyte maturation.

Keywords: Cyclin B1; Pumilio; CPEB; Cdc2; Mos; Translational control; 3′ UTR; *Xenopus*; Oocyte maturation; Phosphorylation

1. Introduction

Oocytes of many animal species stop their meiosis at the first prophase, during which they grow by accumulation of proteins and mRNAs necessary for embryonic development. Upon stimulation of the oocyte surface by a hormone, e.g. progesterone in frogs, the resting oocytes resume meiosis and become fertilizable, a process called oocyte maturation (Yamashita et al., 2000). The final inducer of oocyte maturation is the maturation-promoting factor (MPF), which consists of cyclin B-bound Cdc2. MPF is stockpiled in immature oocytes as an inactive form called pre-MPF, although its amount varies from species to species. In some mammals and many lower vertebrates pre-MPF is absent, and new synthesis of cyclin B is therefore necessary for initiating oocyte maturation in these species (Taieb et al., 1997; Yamashita, 2000; Yamashita et al., 2000). Since pre-MPF is stockpiled in immature *Xenopus* oocytes, its activation is required for initiating maturation. Despite intensive investigations, however, the actual biochemical pathways that link the hormonal stimulation to the activation of pre-MPF, including the extent to which neosynthesis of Mos and cyclin B is involved in pre-MPF activation, are still obscure (De Smedt et al., 2002; Dupré et al., 2002; Frank-Vaillant et al., 1999; Hochegger et al., 2001; Peter et al., 2002). In addition to the activation of pre-MPF, normal progression of oocyte maturation is ensured by the continued synthesis of cyclin B protein, which is dependent

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doi:10.1016/S0925-4773(03)00160-6

on translational activation of the dormant mRNA stored in the oocytes in all species examined so far (Hochegger et al., 2001; Iwabuchi et al., 2000; Ledan et al., 2001; Polanski et al., 1998; see also review, Yamashita et al., 2000).

It is well established in *Xenopus* that the cytoplasmic polyadenylation element (CPE) in the 3′-untranslated region (UTR) of cyclin B1 mRNA is responsible for both translational repression (masking) and activation (unmasking) of the mRNA (Mendez and Richter, 2001; Richter, 2000). The CPE is bound by a CPE-binding protein, CPEB (Hake and Richter, 1994), to which an eIF4E-binding protein, maskin, is bound (Stebbins-Boaz et al., 1999). The resulting CPEB-maskin-eIF4E complex on the CPE-containing mRNA precludes the formation of an active initiation complex including eIF4E, eIF4G and 40S ribosomal subunit, thereby repressing translation of the mRNA (Cao and Richter, 2002). Upon progesterone stimulation, CPEB is phosphorylated on Ser174 by Eg2 (also known as Aurora A) (Mendez et al., 2000a). This event allows CPEB to bind to a cleavage and polyadenylation specificity factor (CPSF), which promotes poly(A) elongation by recruiting poly(A) polymerase (Mendez et al., 2000b). Poly(A)-binding proteins (PABP) then bind to the elongating poly(A) tail, and this binding facilitates interaction between PABP and eIF4G, an event necessary for translational activation (Wakiyama et al., 2000, 2001). Finally, the PABP-bound eIF4G induces replacement of eIF4E from the CPEB-maskin complex to the initiation complex, and thereby the mRNA becomes translated (Cao and Richter, 2002). Although these events remain to be elucidated, it is clear that the CPEB-maskin complex plays an essential role in translational repression and activation of the CPE-containing mRNA in *Xenopus* oocytes.

Similar to cyclin B1 mRNA, Mos and Wee1 mRNAs contain the CPE in their 3′ UTR. Nonetheless, translational activations of these mRNAs during *Xenopus* oocyte maturation do not coincide. Mos is translated soon after progesterone stimulation prior to germinal vesicle breakdown (GVBD) (Ballantyne et al., 1997; de Moor and Richter, 1997), whereas cyclin B1 and Wee1 are translated later, around the time of GVBD and thereafter, respectively (Charlesworth et al., 2000; Kobayashi et al., 1991; Murakami and Vande Woude, 1998; Nakajo et al., 2000). Accordingly, oocytes must be equipped with mechanisms that define the precise timing of translational activation of each CPE-containing mRNA during maturation. Two papers published recently have shed light on these mechanisms. Charlesworth et al. (2002) have revealed a new *cis*-element that selectively determines the timing of translational activation of Mos mRNA, irrespective of cyclin B1 and Wee1 mRNAs. *Trans*-acting factors for this *cis*-element, however, have not been identified. Mendez et al. (2002) have shown that later phosphorylation of CPEB on Ser210 by Cdc2 is sufficient to induce CPEB degradation and that this event activates translation of cyclin B1 mRNA but not Mos mRNA. Based on these findings, they have suggested that the CPEB:CPE ratio is responsible for selective activation of CPE-containing mRNAs during *Xenopus* oocyte maturation. Nevertheless, it is also possible that the requirement of CPEB Ser210 phosphorylation for translational activation of cyclin B1 mRNA is not due to degradation of CPEB but due to release of a factor that is required for maintaining translational repression and is tethered to cyclin B1 mRNA via CPEB. Although some clues have been obtained, many things remain to be elucidated for understanding the precise temporal control of translation of the CPE-containing mRNAs encoding cyclin B1, Mos and Wee1, which is necessary to ensure the normal progression of oocyte maturation (Hochegger et al., 2001; Ledan et al., 2001; Nakajo et al., 2000).

Pumilio was first discovered as an essential protein for the formation of abdominal segments during early *Drosophila* embryogenesis. Pumilio homologs have been identified in various organisms, from yeast to human, and they constitute a large and evolutionarily conserved protein family, the Puf family (Wickens et al., 2002). Members of the Puf family regulate translation by binding to the 3′ UTR of their target mRNAs, including cyclin B mRNA, in *Drosophila* (Asaoka-Taguchi et al., 1999; Murata and Wharton, 1995; Sonoda and Wharton, 2001), *C. elegans* (Crittenden et al., 2002; Kraemer et al., 1999; Zhang et al., 1997), *Dictyostelium* (Souza et al., 1999) and *Saccharomyces cerevisiae* (Olivas and Parker, 2000; Tadauchi et al., 2001). In vertebrates, however, the functions of Puf proteins are unknown. The core sequence in the 3′ UTRs of target mRNAs that is required for recognition by the Puf family proteins is UGU triplets (Wang et al., 2002). Since this sequence is conserved in the 3′ UTRs of cyclin B1 mRNAs of many species, we have started to examine whether Pumilio is involved in translational control of cyclin B1 mRNA during oocyte maturation. We have already shown the specific binding of *Xenopus* Pumilio (XPum) to cyclin B1 mRNA via the UGUA(A) sequence in the 3′ UTR and the physical association of XPum with CPEB in oocytes (Nakahata et al., 2001a), but the biological roles of XPum are still unclear.

In the present study, we have investigated the effects of injection of anti-XPum antibody and over-expression of XPum on oocyte maturation and translation of cyclin B1 mRNA in order to gain insights into the function of XPum during oocyte maturation. We found that manipulations of XPum affect oocyte maturation and cyclin B1 translation but have no effect on Mos translation; the enhancement and inhibition of XPum function result in retardation and acceleration of oocyte maturation, respectively, and the over-expression of XPum delays translational activation of cyclin B1 mRNA via inhibition of the poly(A) tail elongation. We also obtained evidence that XPum is bound to unphosphorylated CPEB in immature oocytes but not to phosphorylated CPEB in mature oocytes, implying the liberation of XPum from phosphorylated CPEB during oocyte maturation. On the basis of these findings, we propose that XPum contributes to a selective

control of the timing of translational activation of cyclin B1 mRNA, which might be triggered by release of XPum from the CPEB-maskin complex, the major masking and unmasking element common to the CPE-containing cyclin B1, Mos and Wee1 mRNAs, all of which are translated after progesterone stimulation but at different times.

2. Results

2.1. Xenopus Pumilio is highly homologous to mammalian counterparts

The full-length XPum cDNA, about 5 kb in length, is consistent with the size of the endogenous mRNA in oocytes detected by Northern blotting (data not shown). The cDNA encodes two putative ATG start codons, one at nucleotide 361 and one at nucleotide 430. According to Kozak's rule (Kozak, 1986, 1989) and based on a comparison with mammalian homologs (Fig. 1A), it is likely that the second ATG, at nucleotide 430, is the genuine start codon. In this case, the cDNA encodes 1185 amino acids with a calculated molecular mass of 127 kDa, which is comparable to the native XPum (137 kDa) in oocytes detected by Western blotting (Nakahata et al., 2001a).

Throughout the entire molecule, XPum exhibits high amino acid identity to mammalian counterparts (Fig. 1A). Besides the Puf domain (the hallmark sequence of the Puf family), which shows the highest amino acid identity, the identity is still close to 90% even in the region excluding the Puf domain, despite the fact that this region is hardly conserved between vertebrates and *Drosophila* (Fig. 1B).

2.2. XPum is bound to unphosphorylated but not phosphorylated CPEB

During oocyte maturation, CPEB undergoes major phosphorylation that is associated with an upward shift (a retardation in electrophoretic mobility) in SDS-PAGE, and this phosphorylation coincides with translational activation of cyclin B1 mRNA (Mendez et al., 2000a, 2002). We examined the binding of XPum to unphosphorylated and phosphorylated CPEB by immunoprecipitation analysis using anti-CPEB antibody and anti-XPum NN antibody (see Section 4). Anti-CPEB and anti-XPum NN immunoprecipitates from immature oocyte extracts contained XPum and CPEB, respectively (Fig. 2A), confirming our previous finding that the two proteins form a complex in the oocytes (Nakahata et al., 2001a). However, similar analysis of mature oocytes revealed that XPum is not bound to phosphorylated CPEB, although it remains bound to unphosphorylated CPEB (Fig. 2A), strongly suggesting that XPum is released from phosphorylated CPEB during oocyte maturation. Since early phosphorylation of CPEB on Ser174 does not induce the electrophoretic mobility shift (Mendez et al., 2000a), it is not clear whether the early phosphorylation affected the interaction between CPEB and XPum in this experiment, but the results of a preliminary experiment using mutant CPEBs indicated that mutations of Ser174 to alanine and aspartic acid have no effect on the interaction (data not shown).

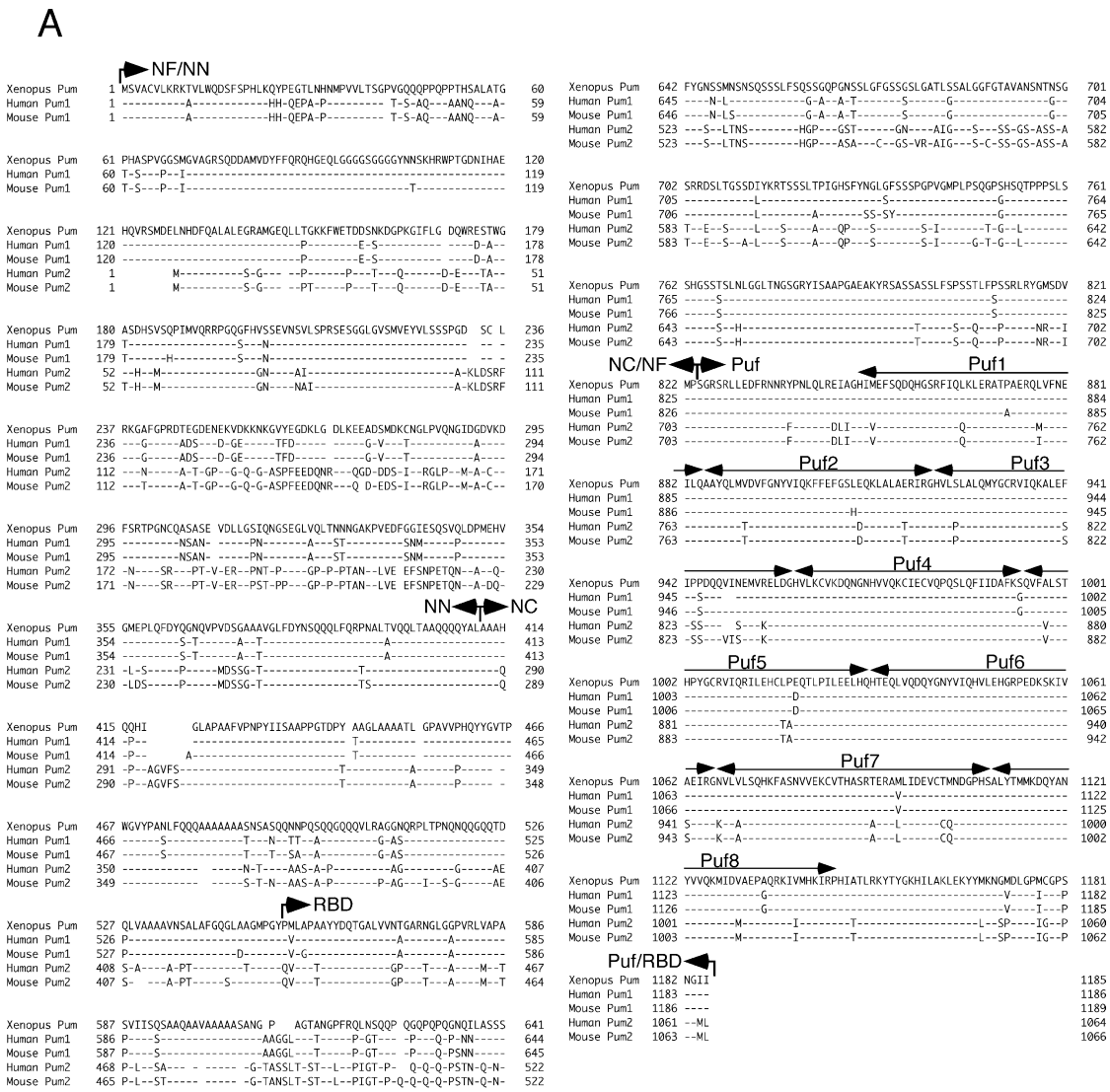
2.3. Puf domain is responsible for binding to CPEB

Using glutathione-S-transferase (GST)-tagged fragments of XPum (GST-XPum NN, NC and Puf, Figs. 1A and 2B), we carried out an experiment to determine which portion of XPum is responsible for the binding to CPEB. mRNA encoding each fragment was injected into oocytes, from which the recombinant proteins were recovered with GSH-Sepharose (Fig. 2C, upper panel). Anti-CPEB immunoblotting of the GSH-Sepharose precipitates showed that CPEB was present in the precipitates from the oocytes injected with Puf but not from those injected with NN and NC or from the uninjected oocytes as a control (Fig. 2C, lower panel). It is therefore apparent that CPEB binds to the Puf domain. Since RNase treatment had no effect, the binding is not due to indirect binding via RNA but due to protein–protein interaction, as demonstrated in the previous paper (Nakahata et al., 2001a). The binding of CPEB to the Puf domain was also confirmed by anti-CPEB immunoprecipitation from the oocytes over-expressing GST-XPum NN, NC and Puf. XPum Puf, as well as endogenous XPum, was detected in the anti-CPEB immunoprecipitate, but XPum NN and NC were not (Fig. 2D). Over-expression of XPum Puf, but not of XPum NN and NC, reduced the binding of CPEB to the endogenous XPum (Fig. 2D), also indicating that the Puf domain is exclusively responsible for the binding to CPEB.

We also examined the binding of XPum Puf to CPEB in mature oocytes. Oocytes were injected with mRNA encoding GST-XPum Puf, and maturation of half of the oocytes was induced with progesterone. After incubation overnight, the progesterone-treated mature oocytes and untreated immature oocytes were extracted and precipitated with GSH-Sepharose. CPEB was detected in the precipitates from immature oocytes but not from mature oocytes, despite the presence of almost equal amounts of GST-XPum Puf in the treated and untreated oocytes (Fig. 2E). These findings are consistent with the above-described results showing that the Puf domain is responsible for the binding to CPEB (Fig. 2C and D) and that XPum does not bind to phosphorylated CPEB (Fig. 2A).

2.4. The XPum-binding sequence acts as a cis-element for translational repression

A tetranucleotide segment, UGUA, in the 3′ UTR of cyclin B1 mRNA is essential for its recognition by XPum (Nakahata et al., 2001a). To determine the involvement of this sequence in translational repression of cyclin B1 mRNA in immature *Xenopus* oocytes, we injected luciferase reporter mRNAs that contain three different sequences (Fig. 3A–C): (1) the wild-type 3′ UTR of cyclin B1 that



B

Amino Acid Identity to *Xenopus* Pumilio (%)

	Whole	Puf	-Puf
Human Pumilio 1	90.3	98.0	87.3
Mouse Pumilio 1	89.3	97.2	85.8
Human Pumilio 2	76.1	90.4	69.7
Mouse Pumilio 2	74.8	89.3	67.4
Drosophila Pumilio	38.9	78.6	18.6

Fig. 1. (A) Amino acid sequence alignment of *Xenopus* Pumilio (AB091091), human Pumilio 1 (AF315592), mouse Pumilio 1 (AF321909), human Pumilio 2 (AF315591), and mouse Pumilio 2 (AF315590). Amino acids identical to *Xenopus* Pumilio are indicated by hyphens. The regions corresponding to the fragments used in this study (XPum NF, NN, NC, RBD, and Puf) are shown. Eight repeats (Puf 1 to Puf 8) that comprise the Puf domain are also indicated (see also Figs. 2B and 6A). (B) Amino acid identity of human, mouse and *Drosophila* Pumilio homologs to *Xenopus* Pumilio at the whole molecule (Whole), the Puf domain (Puf) and the remaining region exclusive of the Puf domain (-Puf).

includes the XPum-binding site and two CPEs (CPE1 and CPE2); (2) its mutated form (the XPum-recognition site UGUA having been mutated to GUGC, which caused disruption of XPum-binding, but not CPEB-binding, as confirmed by UV cross-linking experiments; Fig. 3D, data for CPEB not shown); and (3) the polylinker sequence of pBluescript as a control. The translational activity of the mRNA carrying the wild-type 3′ UTR was less than 1/20 of that of the control mRNA carrying the polylinker sequence (Fig. 3E). This repression is mainly attributable to the CPE (Barkoff et al., 2000; de Moor and Richter, 1999), especially CPE1 rather than CPE2 (Nakahata et al., 2001a). The translational activity of the mRNA that has a mutation at the XPum-binding site but has the two intact CPEs was over two-fold higher than that carrying the wild-type 3′ UTR (Fig. 3E), indicating that the XPum-binding sequence exerts

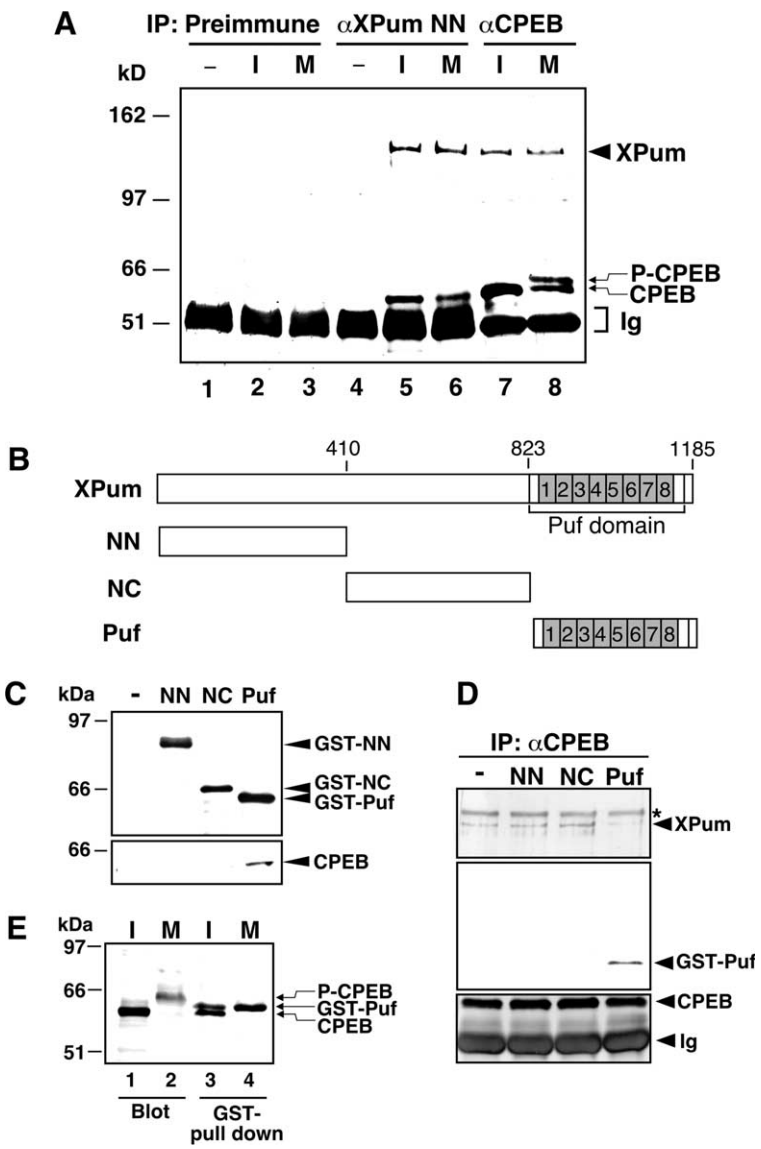


Fig. 2. Binding of XPum to CPEB. (A) Binding of endogenous XPum and CPEB. Buffer (−), extract from 50 progesterone-untreated immature oocytes (I) or extract from 50 progesterone-treated mature oocytes (M) was immunoprecipitated with either preimmune serum (control), anti-XPum NN antibody (αXPum NN) or anti-*Xenopus* CPEB antibody (αCPEB) and immunoblotted with a mixture of anti-XPum NN and anti-CPEB antibodies. XPum and unphosphorylated CPEB (CPEB) were not detected in the control lanes (lanes 1–4) but were detected in the immunoprecipitates from immature and mature oocyte extracts (lanes 5–8), indicating that a complex of XPum and unphosphorylated CPEB is present in both immature and mature oocytes. Phosphorylated CPEB (P-CPEB) was detected in the anti-CPEB immunoprecipitate from mature oocyte extracts (lane 8) but not in the anti-XPum immunoprecipitate (lane 6), demonstrating the absence of a complex of XPum and phosphorylated CPEB in mature oocytes. Ig, immunoglobulins. (B) Fragments used in the XPum-CPEB binding experiments. Amino acid numbers and the Puf domain including eight repeats are indicated. (C) Binding of CPEB to XPum Puf but not to XPum NN and NC. Oocytes were not injected (−) or were injected with mRNA encoding GST-XPum NN (NN), GST-XPum NC (NC) or GST-XPum Puf (Puf) and incubated overnight at 18 °C. All oocytes (10 for each treatment) were extracted, subjected to GSH-Sepharose precipitation, and probed with anti-GST (upper panel) and anti-CPEB (lower panel) antibodies. CPEB was only detected in the precipitates from oocytes injected with XPum Puf. (D) Binding of CPEB to XPum Puf as revealed by anti-CPEB immunoprecipitation. Oocytes were not injected (−) or were injected with mRNA encoding GST-XPum NN (NN), GST-XPum NC (NC) or GST-XPum Puf (Puf) and incubated overnight at 18 °C. Extracts were obtained from 10 oocytes in each treatment, precipitated with anti-CPEB antibody and immunoblotted with anti-XPum 2A5 (top panel), anti-GST (middle panel) and anti-CPEB (bottom panel) antibodies. In spite of similar amount of CPEB in each precipitate, only GST-XPum Puf (GST-Puf) was coprecipitated with CPEB. The amount of CPEB-bound endogenous XPum was reduced when GST-Puf, but not GST-NN and GST-NC, was over-expressed. Asterisk shows a protein non-specifically detected by a secondary antibody. Ig, immunoglobulins. (E) Binding of XPum Puf and CPEB in immature (I) and mature (M) oocytes. The oocytes were not injected (lanes 1 and 2) or were injected with mRNA encoding GST-XPum Puf (lanes 3 and 4) and incubated with (lanes 2 and 4) or without (lanes 1 and 3) progesterone. The uninjected oocytes were extracted and immunoblotted with a mixture of anti-CPEB and anti-GST antibodies (Extract equivalent to one oocyte was loaded on each lane), to show the positions of unphosphorylated CPEB (CPEB, lane 1) and phosphorylated CPEB (P-CPEB, lane 2). Note that all of CPEB is phosphorylated in mature oocytes in this experiment (lane 2), unlike in the experiment for which the results are shown in Fig. 2A, in which a part of CPEB remained unphosphorylated in mature oocytes (Fig. 2A, lane 8). Extracts from the mRNA-injected 10 oocytes were precipitated with GSH-Sepharose before immunoblotting. Unphosphorylated CPEB was detected in the precipitate (lane 3) but phosphorylated CPEB was not (lane 4).

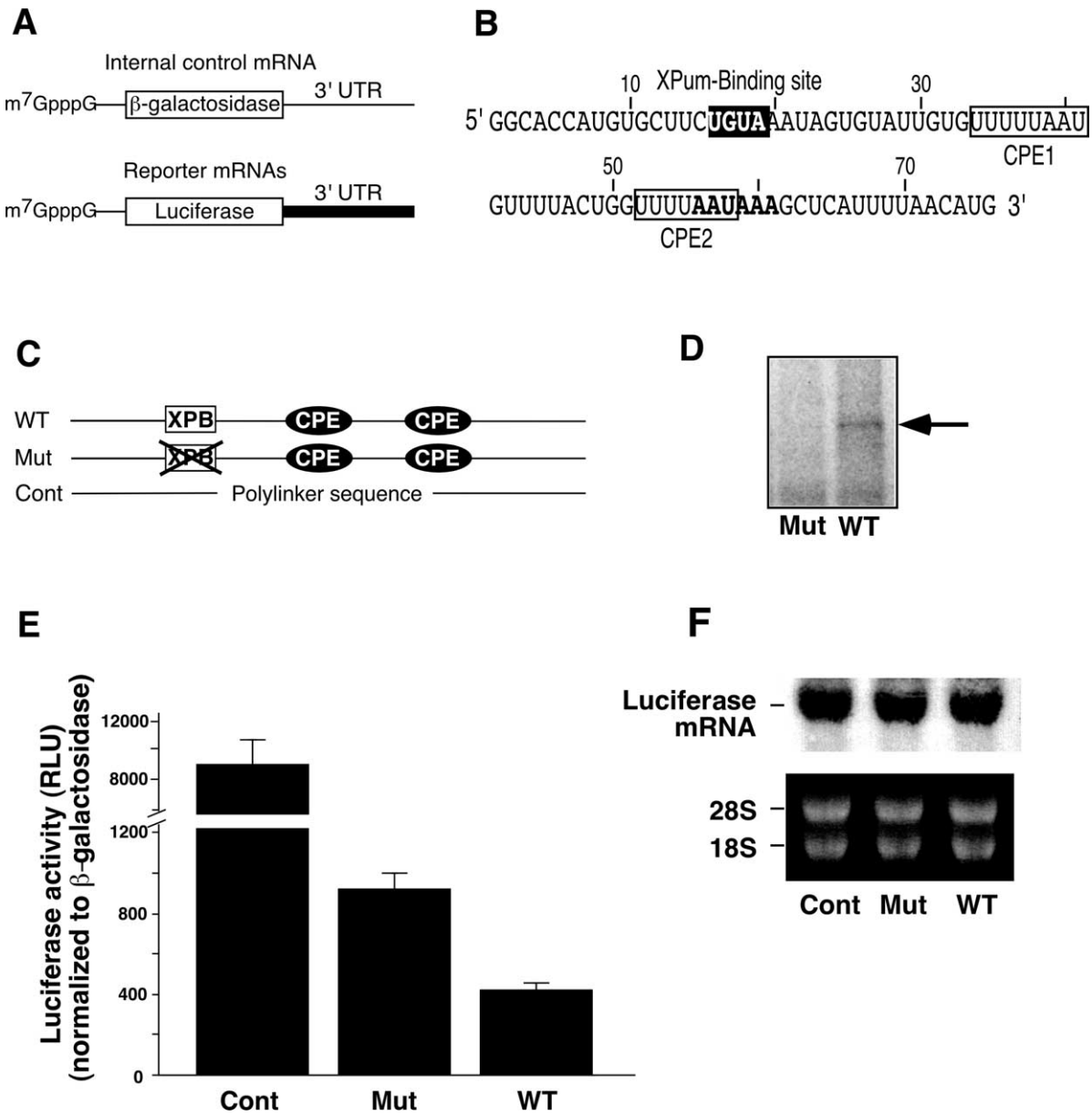


Fig. 3. XPum-recognition sequence as a *cis*-element of translational repression. (A) Structures of two mRNAs used for reporter assay. Reporter mRNAs contain luciferase ORF and various 3′ UTRs (solid bar) as shown in (B) and (C). An internal control mRNA encodes β-galactosidase with an unregulated 3′ UTR. Both control and reporter mRNAs lack a poly(A) tail. (B) Sequence of the cyclin B1 3′ UTR fused to the luciferase coding region. The XPum-binding site is enclosed in a black box, the CPEs are in white boxes, and the polyadenylation signal (AAUAAA) is shown in boldface. (C) Schematic representation of the 3′ UTR of luciferase mRNAs. The XPum-binding site (XPB, open box) and CPEs (shaded oval) are indicated. The mutant XPum-binding site is marked by 'X'. (D) UV cross-linking assay using RNA probes with the wild-type 3′ UTR of cyclin B1 (WT) and its mutated form (Mut), in which the XPum-binding site UGUA has been changed to GUGC. The arrow shows XPum. (E) Luciferase activities of the reporters containing the control polylinker sequence (Cont), the wild-type cyclin B1 3′ UTR (WT) or the 3′ UTR with a mutated XPum-recognition site (Mut). Stage VI oocytes were injected with a mixture of β-galactosidase and either one of the luciferase mRNAs (each 0.01 fmol/oocyte). Following a 3-h incubation, the injected oocytes were pooled into groups of five, and the translational efficiency of each mRNA was measured by luciferase and β-galactosidase assays. Shown is a representative result with relative luciferase activities to the β-galactosidase activities (means ± SEM). The values are significantly different to each other ($P < 0.01$, Student's *t*-test). Similar results were obtained from three different batches of oocytes. (F) Stability of injected reporter mRNAs (1 fmol/oocyte) confirmed by Northern blotting. Ethidium bromide staining of rRNA shows the overall quantity and quality of the extracted RNA and the uniformity of the loading to each lane. Essentially the same result was obtained when 0.01 fmol/oocyte of the reporters were injected.

an additional suppressive effect on the CPE-mediated translational repression. Northern blotting analysis confirmed the stability of the injected reporters (Fig. 3F), indicating that the luciferase activity reflects the translational activity but not the stability of the mRNA. It is therefore concluded that the XPum-binding sequence in the 3′ UTR of cyclin B1 mRNA acts as a *cis*-element responsible for translational repression, in addition to the CPE.

2.5. The anti-XPum monoclonal antibody accelerates progesterone-induced maturation by specific acceleration of cyclin B1 protein synthesis

Since a monoclonal antibody, XPum 2A5, has been raised against the XPum RNA-binding domain (XPum RBD), an essential domain for its function (Nakahata et al., 2001a), it is plausible that injection of this antibody into oocytes will neutralize the activity of endogenous XPum. Generally, pregnant mare serum gonadotropin (PMSG)-primed full-grown *Xenopus* oocytes contain pre-MPF that allows the oocyte to undergo GVBD without new synthesis of cyclin B proteins (Hochegger et al., 2001). It is thus expected that the kinetics of GVBD might be unchanged even if the antibody has effect on cyclin B1 translation. We therefore used PMSG-unprimed *Xenopus* oocytes that contain only a small amount of or no pre-MPF (Yamashita, 2000), to monitor the effect of the antibody on cyclin B1 mRNA translation through the kinetics of GVBD. The unprimed oocytes were able to mature in response to progesterone, but, as expected, the kinetics of maturation were slower than those typically seen in the primed oocytes, and GVBD did not reach 100% sometimes. Furthermore, the kinetics varied from batch to batch. Using these oocytes, however, we obtained clear effect of the antibody on the timing of GVBD. Oocytes were injected with anti-XPum 2A5 antibody, anti-GST antibody or buffer, and the time course of GVBD was observed after progesterone treatment. The oocytes injected with anti-XPum 2A5 antibody underwent progesterone-induced GVBD more rapidly than did the control oocytes injected with GST antibody or buffer (Fig. 4A). In four independent experiments, two-fold acceleration of GVBD by injection of anti-XPum 2A5 antibody was observed (Fig. 4B).

To confirm that the antibody-induced acceleration of GVBD is due to accelerated synthesis of cyclin B1 protein and that this phenomenon is specific to cyclin B1, we examined changes in the protein level of cyclin B1 in the antibody-injected oocytes, together with the protein

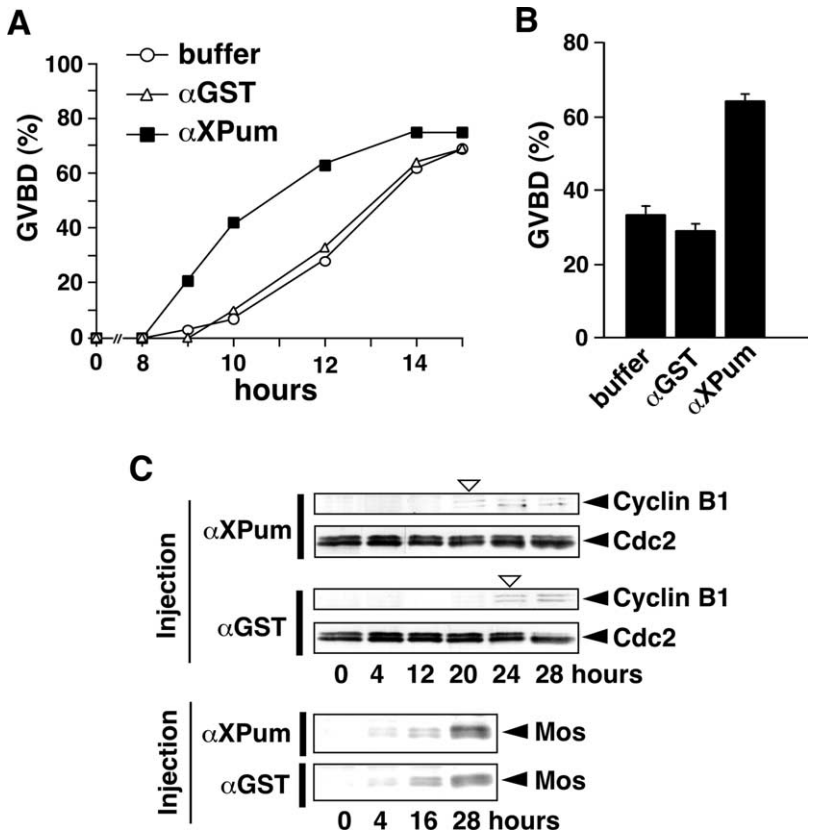


Fig. 4. Acceleration of GVBD and cyclin B1 protein synthesis by injection of anti-XPum 2A5 antibody. Oocytes were injected with buffer, anti-GST antibody (αGST) or anti-XPum 2A5 antibody (αXPum), incubated overnight, and then stimulated with progesterone. (A) Kinetics of maturation of oocytes injected with buffer, anti-GST antibody or anti-XPum 2A5 antibody prior to progesterone stimulation. Shown is a representative of four independent experiments. (B) A quantitative analysis of the acceleration of GVBD by anti-XPum 2A5 antibody. Shown is the percentage of GVBD in the antibody-injected oocytes at the time when the control buffer-injected oocytes showed ~30% GVBD. Data are shown as means ± SEM for four independent experiments using ~100 oocytes in each experiment. The rate of GVBD in the XPum antibody-injected oocytes is significantly higher than that in the buffer- or GST antibody-injected controls ($P < 0.0001$, Student's *t*-test). (C) Immunoblots of oocytes injected with anti-XPum 2A5 antibody or anti-GST antibody as a control, showing changes in the protein contents of cyclin B1, Cdc2 and Mos during progesterone-induced oocyte maturation. Suc1 precipitate from extract equivalent to 9 oocytes was loaded on each lane for detecting cyclin B1 and Cdc2, and crude extract equivalent to one oocyte was loaded for detecting Mos. The constant Cdc2 protein contents confirmed equal sample loading to each lane. White triangles show the initiation of GVBD. Note that the appearance of cyclin B1 in the oocytes injected with anti-XPum 2A5 antibody is faster than that in those injected with anti-GST, whereas the timing of the Mos appearance is almost the same.

level of Mos, which is newly synthesized prior to cyclin B1 and required for Cdc2 activation and cyclin B1 mRNA translation (Ballantyne et al., 1997; de Moor and Richter, 1997; Mendez et al., 2002; Sagata et al., 1988, 1989; Sheets et al., 1995). In immature oocytes, the level of cyclin B1 protein was under the detection limit of the antibody (Fig. 4C; see also Fig. 5D, top panel). The low cyclin B1 protein level may have been due to unpriming of the oocytes used in this study. Compared with the control oocytes injected with anti-GST antibody, the appearance of cyclin B1 protein was fast in the oocytes injected with anti-XPum 2A5 antibody (Fig. 4C). This finding strongly suggests that the antibody-induced acceleration of GVBD is due to accelerated synthesis of cyclin B1 protein, although we cannot exclude the possibility that XPum regulates translation of cyclins other than cyclin B1, such as cyclins B2, B4 and B5 (Hochegger et al., 2001), and that their enhanced synthesis also accelerates GVBD.

Similarly to cyclin B1, the Mos protein level increased as maturation proceeded (Fig. 4C; see also Fig. 5D, bottom panel). In contrast to cyclin B1, however, the timing of the appearance of Mos protein in the oocytes injected with anti-XPum 2A5 antibody was similar to that in the control oocytes (Fig. 4C). These results indicate that anti-XPum 2A5 antibody enhanced cyclin B1 protein synthesis without affecting Mos protein synthesis.

2.6. Over-expression of XPum delays oocyte maturation by specific inhibition of cyclin B1 mRNA translation

The most likely explanation for the acceleration of cyclin B1 protein synthesis by injection of anti-XPum 2A5

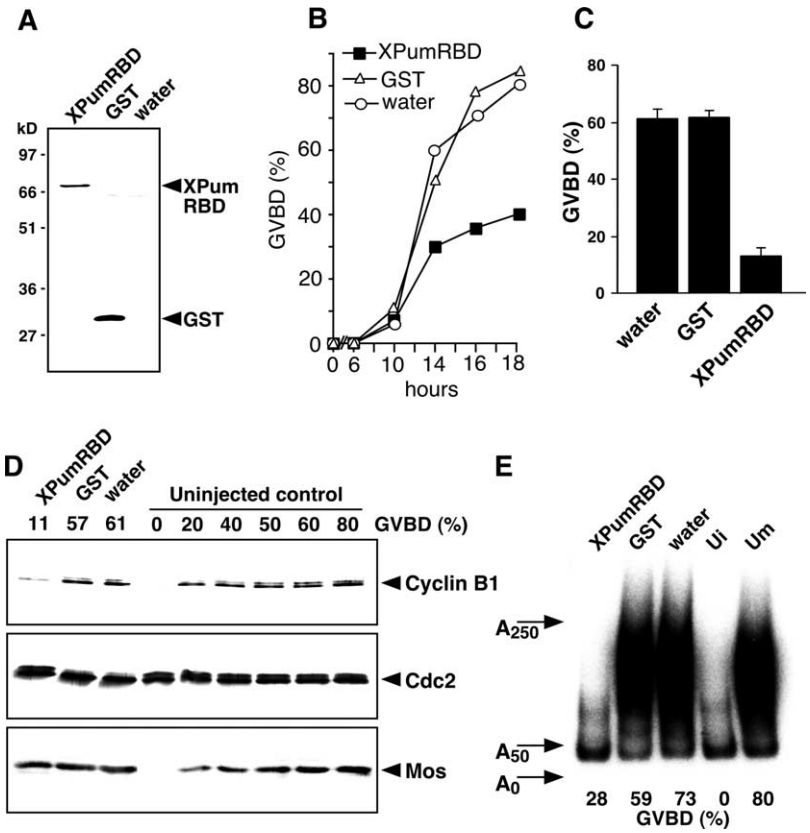


Fig. 5. Retardation of GVBD and cyclin B1 protein synthesis in XPum over-expressing and progesterone-stimulated oocytes. Oocytes were injected with water, GST mRNA or XPum RBD mRNA, incubated overnight, and stimulated with progesterone. (A) Immunoblot with anti-T7 tag antibody that recognizes the N-terminal tag of recombinant proteins, showing protein expression from the injected mRNAs. Extract equivalent to 0.75 oocyte was loaded. (B) Time course of GVBD in the control water- or GST-injected oocytes and in the XPum RBD-injected oocytes. Shown is a representative of six independent experiments. The percentage of GVBD in the XPum RBD-injected oocytes reached to the level similar to that in the control oocytes when incubated for a long time. (C) Percentage of GVBD in the GST- or XPum RBD-injected oocytes at the time when the control water-injected oocytes showed ~50% GVBD. Values are means ± SEM of six independent experiments using ~100 oocytes in each experiment. The XPum RBD injection, but not GST injection, resulted in significant delay in oocyte maturation ($P < 0.0001$, Student's t-test). (D) Anti-cyclin B1 (top panel), anti-Cdc2 (middle panel) and anti-Mos (bottom panel) immunoblots of oocytes injected with XPum RBD, GST or water. Immunoblots of oocytes uninjected but treated with progesterone (Uninjected control) are also shown for changes in protein levels of cyclin B1, Cdc2 and Mos during oocyte maturation. The percentage of GVBD at the time of sampling is shown on the top of each lane. Oocyte extract was subjected to Suc1-precipitation and extract equivalent to 10 oocytes was loaded on each lane for detection of cyclin B1 and Cdc2 (as a loading control). Extract equivalent to one oocyte was loaded for Mos. (E) Poly(A) tail analysis of endogenous cyclin B1 mRNA. Total RNA was extracted from oocytes (15 oocytes/sample) injected with water or mRNAs encoding XPum RBD or GST, as well as from uninjected immature (Ui) and mature (Um) oocytes, and subjected to the PAT assay. The percentage of GVBD at the time of sampling is shown at the bottom of each lane, and the length of poly(A) tail is on the left.

antibody is that the antibody neutralized the repressive activity of XPum against the translation of cyclin B1 mRNA. As an alternative approach to verify this possibility, we over-expressed XPum RBD in PMSG-unprimed oocytes and examined its effects on oocyte maturation and translation of cyclin B1 mRNA. Injection of mRNA encoding XPum RBD or GST (as a control) induced the oocytes to produce respective proteins. The protein level of GST was higher than that of XPum RBD (Fig. 5A), and the protein level of XPum RBD was 10- to 15-fold higher than that of endogenous XPum (data not shown). The over-expression of XPum RBD delayed, but did not prevent, GVBD, whereas the injection of GST or water had no inhibitory effect on GVBD (Fig. 5B and C).

The protein content of cyclin B1 in the oocytes that had been injected with GST or water and induced to mature with progesterone was comparable to that in the uninjected and progesterone-treated oocytes; however, the content was apparently lower in the XPum RBD-injected and progesterone-treated oocytes (Fig. 5D, top panel), in accordance with the lower rate of GVBD in these oocytes. In contrast to cyclin B1, Mos protein level in the oocytes injected with XPum RBD was similar to that in those uninjected or injected with GST or water (Fig. 5D, bottom panel), despite the difference in the rate of GVBD. These results indicate that the over-expression of XPum RBD in PMSG-unprimed *Xenopus* oocytes results in the retardation of GVBD that is accompanied by the impaired cyclin B1 accumulation without affecting Mos accumulation, suggesting the involvement of XPum in translational repression specific to cyclin B1 mRNA.

Translational repression of mRNAs is usually correlated with their short poly(A) tail (Richter, 2000; Wickens et al., 2000). We performed an assay to determine whether the inhibited cyclin B1 synthesis is also accompanied by a short poly(A) tail of the mRNA. The PCR-based poly(A) test (PAT) assay showed that cyclin B1 mRNA was appended with poly(A) tails up to >220 bases during maturation (Compare Ui and Um in Fig. 5E.). In contrast, the poly(A) tail of cyclin B1 mRNA in the XPum-over-expressed oocytes did not extend beyond ~ 30 bases, a situation similar to that in immature oocytes, in spite of the fact that the XPum-over-expressed oocytes underwent $\sim 30\%$ GVBD (Compare Ui and XPum in Fig. 5E.). It is thus likely that the over-expression of XPum delays progesterone-induced GVBD by preventing efficient translation of cyclin B1 mRNA through the maintenance of its short poly(A) tail. These results also suggest that polyadenylation itself is not required for translational activation of cyclin B1 mRNA. This notion has already been represented on the basis of different data (Ballantyne et al., 1997; Frank-Vaillant et al., 1999).

2.7. Regions of XPum that are responsible for translational control of cyclin B1 mRNA

For further analyses to determine the regions responsible for inhibition of cyclin B1 mRNA translation, similar over-expression experiments were performed using fragments other than XPum RBD (Fig. 6A). We examined other two regions, XPum NF and NN. The former contains the N-terminal 2/3 of XPum and overlaps with a part of XPum RBD in the N-terminal region (corresponding to amino acids 552–823) but excludes the Puf domain, while the latter contains the N-terminal 1/3 of XPum without overlapping with XPum RBD (Fig. 6A). Oocytes injected with mRNA encoding XPum NF, NN or RBD produced each recombinant protein. The amount of XPum NF was significantly smaller than that of XPum NN or RBD but greater than that of endogenous XPum (Fig. 6B). XPum NF inhibited progesterone-induced oocyte maturation to an extent similar to the extent of inhibition by XPum RBD (Fig. 6C). In contrast, XPum NN had no inhibitory effect on oocyte maturation, as in the case of oocytes injected with water or GST as controls (Fig. 6C).

To characterize the regions responsible for translational control of cyclin B1 mRNA more precisely, we further carried out another set of experiments, in which XPum NN, NC and Puf were injected to examine the effects of these fragments on GVBD (Fig. 6A). Judging from the result in the above experiments (Fig. 6C), XPum NC is expected to have inhibitory effect on GVBD upon injection. Protein levels of XPum NC and Puf in the mRNA-injected oocytes were similar to each other (Fig. 6D). In contrast to the expectation, however, the XPum NC-injected and progesterone-treated oocytes exhibited no significant delay in GVBD, as in the case of those injected with XPum NN or water as a control, despite the fact that the injection of XPum Puf, like XPum RBD, showed clear inhibitory effect on progesterone-induced GVBD (Fig. 6E).

3. Discussion

3.1. Highly conserved vertebrate Pumilio proteins

Members of the Puf family repress translation through recognition of target mRNAs by the Puf domain that is located at the C-terminus (Wickens et al., 2002). The structure and function of the C-terminal Puf domain have been extensively investigated to date (Edwards et al., 2001; Sonoda and Wharton, 1999; Wang et al., 2001, 2002; Wharton et al., 1998), but those of the N-terminal region, which is about 2 to 3-times longer than the C-terminal Puf domain, remain a mystery. Indeed, introduction of only the Puf domain is sufficient to rescue the abdominal segmentation defects in *pumilio* mutations in *Drosophila*, suggesting that this region is solely responsible for the manifestation of *pumilio* mutant phenotypes. The binding sites for the Nanos and Brain Tumor proteins, the cooperators of *Drosophila* Pumilio, also reside at the C-terminus (Edwards et al., 2001; Sonoda and Wharton, 1999, 2001). Usage of the C-terminal region for binding to both the target mRNAs and the cooperative proteins has also been found in *C. elegans*

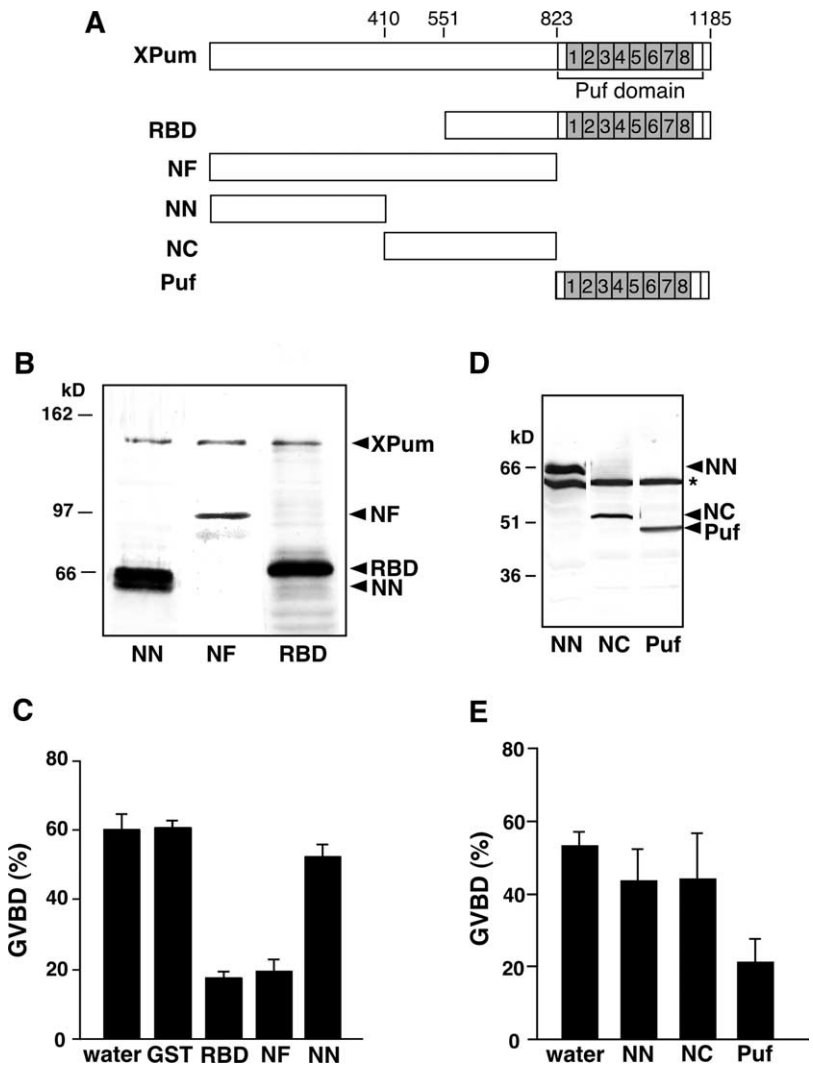


Fig. 6. Effects of over-expression of XPum fragments on progesterone-induced oocyte maturation. (A) A schematic drawing showing the fragments used in the experiments. (B) Immunoblots of extracts from oocytes injected with mRNAs encoding T7-XPum NN, NF or RBD, indicating that the protein level of each fragment is higher than that of endogenous XPum. Extract equivalent to one oocyte was loaded to each lane and immunoblotted with a mixture of anti-XPum NN and anti-XPum 2A5 antibodies. (C) Percentage of GVBD in the control water- or GST-injected oocytes and in the RBD-, NF- and NN-injected oocytes. Values are means ± SEM of four independent experiments using ~200 oocytes in each experiment. To clearly show the difference in the kinetics of GVBD, the experiments were stopped when the control oocytes (water-injected oocytes) had undergone ~50% GVBD. The over-expression of XPum RBD or XPum NF delayed progesterone-induced oocyte maturation significantly ($P < 0.0001$, Student's *t*-test), but the over-expression of XPum NN had no effect, as in the case of the controls (water and GST). (D) Anti-His-tag immunoblots of extracts from oocytes injected with mRNAs encoding T7-XPum NN, NC or Puf. Extract equivalent to one oocyte was loaded to each lane. The protein levels of NC and Puf are similar to each other. An asterisk shows a common band recognized by the anti-His-tag antibody. (E) Percentage of GVBD in the control water-injected oocytes and in the NN-, NC- and Puf-injected oocytes. Values are means ± SEM of seven independent experiments using ~200 oocytes in each experiment. The experiments were stopped when the control oocytes (water-injected oocytes) had undergone ~50% GVBD. The over-expression of XPum Puf delayed oocyte maturation significantly ($P < 0.01$, Student's *t*-test), but the over-expression of XPum NN or NC had no apparent effect.

(Kraemer et al., 1999). Similarly, the Puf domain at the C-terminus of XPum is responsible for binding to both cyclin B1 mRNA (Nakahata et al., 2001a) and CPEB (this study, Fig. 2C and D), and the N-terminal region is dispensable at least for the binding.

We have isolated a full-length cDNA clone for XPum for the first time in any non-mammalian vertebrates. Surprisingly, the cDNA sequence closely resembles the mammalian counterparts, exhibiting around 90% amino acid identity as a whole. Even if the most conserved Puf domain is excluded, the identity is 86–87%. Although little noticed so far, the resemblance between amphibian and mammalian homologs even at the N-terminal region has drawn our attention to this region, in addition to the well-characterized RNA-binding C-terminal region. Actually, we have found in this study that over-expression of XPum NF including the N-terminal region next to the Puf domain (amino acids 552–823) but not the Puf domain itself delays oocyte maturation to an extent similar to that caused by over-expression of the Puf domain (Fig. 6C), implying that

the N-terminal region contributes to translational control of cyclin B1 mRNA, in addition to the contribution by the C-terminal Puf domain, to which the mRNA and CPEB bound. The N-terminal region next to the Puf domain contains two recently identified motifs, the Q/A motif and the S motif, both of which are found in most of the Puf proteins (White et al., 2001). To confirm the importance of this region, we injected mRNA encoding XPum NC that comprises the Q/A and S motifs, but no remarkable inhibitory effect was detectable (Fig. 6E). In this respect, we cannot exclude the possibility that the recombinant XPum NC does not faithfully reproduce the overall structure of the corresponding region of the native XPum, thereby it failed to inhibit GVBD. Further studies are required to clarify the biological role of the N-terminal region of XPum and clarification of the biological role of this region may lead to revelation of new functions of the Puf protein's N-terminal region.

3.2. A new cis-acting element involved in translational repression of cyclin B1 mRNA

The 3′ UTR of cyclin B1 mRNA is responsible for both activation and repression of translation during oocyte maturation (Barkoff et al., 2000; de Moor and Richter, 1999; Tay et al., 2000). A major repressive *cis*-element in the 3′ UTR is the CPE and a *trans*-acting factor is CPEB (Richter and Theurkauf, 2001); however, repression of translation may require other *cis*-elements and/or proteins that act either alone or in combination with CPEB, since the CPE is required but insufficient for repression of cyclin B1 mRNA (Barkoff et al., 2000). In the expectation that XPum is an additional repressor of cyclin B1 mRNA, we performed a reporter assay and found that the reporter mRNA containing a mutation in the XPum-binding site was significantly more active than the reporter mRNA containing the wild-type sequence. However, its extent was only small (about two-fold, Fig. 3E). Both of the reporters harbor the CPE (Fig. 3C), and the CPE plays a major role in translational activation of cyclin B1 mRNA during *Xenopus* oocyte maturation (Ballantyne et al., 1997; Barkoff et al., 1998; de Moor and Richter, 1997). Consequently, the inhibition of XPum might cause only a limited effect on translational activation of cyclin B1 mRNA. On the basis of the result that a mutation in the XPum-recognition sequence causes small but significant restoration of the CPE-mediated translational repression of the reporter mRNA, therefore, we conclude that this sequence is a new *cis*-acting element responsible for translational repression of cyclin B1 mRNA and suggests that XPum and CPEB collaborate to repress the translation of cyclin B1 mRNA and to control the timing of translational activation, as discussed below.

3.3. Translational repression of cyclin B1 mRNA by XPum

An important and central conclusion of this study is that XPum selectively represses translation of cyclin B1 mRNA, although its repressive activity is much weaker than that of CPEB. This conclusion is drawn from the following results: (1) injection of antibody, XPum 2A5, accelerated the timing of GVBD and the appearance of cyclin B1 protein (Fig. 4), and (2) over-expression of the XPum RNA-binding domain delayed the timing of GVBD and the appearance of cyclin B1 protein (Fig. 5). These events are specific to cyclin B1 because the injection of antibody and the over-expression of XPum RBD had no effect on synthesis of Mos that is encoded by the CPE-containing mRNA similar to cyclin B1 (Figs. 4C and 5D).

How can XPum prevent cyclin B1 translation? In the case of *Drosophila* Pumilio, its target appears to be a component of the polyadenylation and/or translation machinery (Wharton and Struhl, 1991; Wreden et al., 1997). A recent study has shown that Pumilio controls the translation of *hunchback* mRNA in *Drosophila* embryos by both poly(A)-independent repression and poly(A) removal (Chagnovich and Lehmann, 2001). In yeast, Puf3p, a Puf protein, promotes deadenylation and degradation of *COX17* mRNA, although Puf3p affects the mRNA decay machinery (Olivas and Parker, 2000). The translational inhibition of cyclin B1 mRNA by over-expression of XPum RBD was associated with the inhibition of poly(A) tail elongation of cyclin B1 mRNA (Fig. 5E), suggesting that XPum represses the translation through a mechanism that maintains a short poly(A) tail of cyclin B1 mRNA, a situation similar to that in *Drosophila* and in yeast.

3.4. Possible involvement of XPum in the timing of translational activation of cyclin B1 mRNA

Recently, Cao and Richter (2002) have proposed a model for polyadenylation-induced translation of CPE-containing mRNAs, including cyclin B1 mRNA, in maturing *Xenopus* oocytes, in which the CPEB-maskin complex is a central component of the translational control. The model might be applicable for not only cyclin B1 mRNA but also other CPE-containing mRNAs such as Mos and Wee1, since these mRNAs are all activated when oocytes are injected with anti-maskin antibody. During *Xenopus* oocyte maturation, however, the timings of translational initiation of these mRNAs apparently differ; Mos is translated first, then cyclin B1 and finally Wee1. Consequently, there must be regulators that specify the different timing of each CPE-containing mRNA. Our findings presented here indicate that XPum is a good candidate for such a regulator specific to cyclin B1 mRNA. In this regard, it is notable that a novel *cis*-element, distinct from the CPE and the XPum-recognition sequence UGUA, exists in the 3′ UTR of Mos mRNA (Charlesworth et al., 2002). A *trans*-acting factor that binds to this sequence may contribute to determination of the timing of translational activation of Mos, like XPum for cyclin B1.

Although we await further analyses, one possible mechanism of translational activation of cyclin B1 mRNA is that a dissociation of XPum from phosphorylated CPEB

during oocyte maturation induces destabilization of the CPEB-maskin-eIF4E complex and provides a cue that leads to unmasking of cyclin B1 mRNA by the mechanism common to CPE-containing mRNAs (Cao and Richter, 2002). In this respect, it is noteworthy that phosphorylation of CPEB on Ser210, which occurs about the time of cyclin B1 translation, is sufficient for selective translational activation of cyclin B1 (Mendez et al., 2002). While this phenomenon has been explained in relation to degradation of CPEB (Mendez et al., 2002), it is also conceivable that the later phosphorylation of CPEB induces release of XPum from the CPEB-maskin-eIF4E complex and that this event triggers translational activation of cyclin B1. Consistent with this possibility, Thom et al. (2003) have recently demonstrated that later phosphorylation of CPEB is required to its dissociation from a large ribonucleoprotein complex upon oocyte maturation, prior to degradation. As a first step to verify this possibility, we are now investigating CPEB mutants, including alanine substitution for Ser210. In addition, our unpublished data indicate phosphorylation of XPum during oocyte maturation, which hints to us that the phosphorylation of XPum is also involved in its release from CPEB, in addition to the CPEB phosphorylation. Further studies that reveal the precise function of XPum should give us an insight into the temporal control of translational activation of the CPE-containing mRNAs, which is essential for ensuring normal progression of oocyte maturation.

4. Experimental procedures

4.1. Animals and oocytes

Xenopus laevis frogs were purchased from a dealer (Hamamatsu Seibutsu Kyozai, Shizuoka, Japan) and maintained in laboratory aquaria at 20 °C. The frogs were not primed by injection of PMSG before any experiments carried out in this study.

Ovaries were surgically removed and rinsed with modified Barth's saline buffered with HEPES (MBS-H) (Cyert and Kirschner, 1988). Full-grown immature oocytes at stage VI (Dumont, 1972) were isolated with 0.2% collagenase (Wako, Osaka, Japan) and treated with 10 μg/ml progesterone (Nacalai, Kyoto, Japan) to induce maturation in vitro. Oocyte maturation was assessed by the appearance of a white spot at the animal pole. In ambiguous cases, oocytes were boiled and cut with a razor blade to examine the occurrence of GVBD.

4.2. Isolation of full-length XPum cDNA

A 800-bp cDNA fragment (named Pum 1-4R) (Nakahata et al., 2001a) that encodes the C-terminal 1/3 of XPum including a part of the Puf domain, the diagnostic hallmark of the Puf family (White et al., 2001), was used as a probe to isolate full-length cDNA clones for XPum. By screening about 2×10^5 plaques, three positive clones were isolated from a λZapII library constructed from *Xenopus* ovary. Sequencing of the longest clone has confirmed that it is full length with stop codons in all three reading frames upstream of the putative ATG start codon (sequence available at DDBJ/EMBL/GenBank with Accession No. AB091091).

4.3. Production of new XPum antibodies

A monoclonal antibody (named XPum 2A5) was raised against a portion of the Puf domain in the C-terminal region of XPum (Nakahata et al., 2001a). This antibody recognizes XPum with high specificity by Western blotting but cannot efficiently precipitate native XPum from crude extracts. Therefore, new antibodies were raised against the N-terminal portion of XPum. Using the full-length XPum cDNA as a template, N-terminal fragments (XPum NN and NC, see Figs. 1 and 2) were amplified with the following primer sets, which introduce sites for *Bam* HI and *Xho* I restriction enzymes: AAGGATCCATGAGCGTTGCATGTGTCTTG and AAACTCGAGTGCATACTGTTGCTGTTGTGC for Pum NN, and AAGGATCCGCCGCGCATCAGCAACACATA and AAACTCGAGAGAGGGCATGACATCTGACAT for XPum NC. The resulting PCR products were digested with *Bam* HI and *Xho* I and ligated into the *Bam* HI/*Xho* I site of pET21a (Novagen, Madison, WI). Recombinant proteins were expressed in *E. coli*, purified by electroelution following SDS-PAGE, and injected into mice to obtain polyclonal antibodies, as described previously (Hirai et al., 1992). Since anti-XPum NN and anti-XPum NC antibodies have similar characteristics, we used anti-XPum NN antibody in this study.

4.4. XPum-CPEB binding assay by immunoprecipitation

Oocytes were homogenized with a pestle (Pellet Pestle, Kontes, Vineland, NJ) in 1 μl/oocyte of ice-cold extraction buffer (EB; 20 mM HEPES, pH 7.5, 100 mM β-glycerophosphate, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 100 μM (*p*-amidinophenyl)methanesulfonyl fluoride, 3 μg/ml leupeptin) and centrifuged at 15,000 g for 10 min at 4 °C. The extracts were mixed with 20 μl of protein G-Sepharose (Pharmacia, Tokyo, Japan) for 2 h at 4 °C for pre-cleaning. After centrifugation at 3000 g for 1 min, the supernatant was mixed with 20 μl of protein G-Sepharose and 1 μl of either anti-XPum NN polyclonal antibody (this study) or anti-*Xenopus* CPEB monoclonal antibody (Nakahata et al., 2001b). Following incubation 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 (100 mM Tris–HCl, pH 6.8, 4% SDS, 12% 2-mercaptoethanol, and 20% glycerol). Proteins associated with the beads were separated by SDS-PAGE, 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).

4.5. cDNA constructs

A construct for luciferase reporter (pRc/CMV-luc) was generated by subcloning the *Hind* III/*Xba* I fragment of pSP-luc + vector (Promega, Madison, WI) into the same cloning site of pRc/CMV (Invitrogen, San Diego, CA) (Maegawa et al., 2002). To produce pRc/CMV-luc/cyclin B1 3′ UTR and its mutant version, the 3′ UTR was amplified from the full-length cyclin B1 cDNA (Minshull et al., 1989) (a gift from Dr Hideki Kobayashi, Kyushu University) by PCR with the following primers: primer 6-x (GCTCTAGAGGCACCATGTGCTTCTGTAA) and primer 2-a (CCGGGCCCCATGTTAAAATGAGCTTTAT) for pRc/CMV-luc/cyclin B1, and primer sub91-94-x (GCTCTAGAGGCACCATGTGCTTCGTGCAATAGTGT) and primer 2-a for pRc/CMV-luc/mutant cyclin B1 (mutated bases shown by underlining). The products were then cloned into *Xba* I/*Apa* I-digested pRc/CMV-luc. A control plasmid for the luciferase reporter assay was created by inserting the polylinker sequence (nucleotides 663–731) of pBluescript II SK(+) (Stratagene, La Jolla, CA) into the *Xba* I/*Apa* I site of pRc/CMV-luc.

GST-fused versions of XPum were produced as follows: Using pGEX-KG (Guan and Dixon, 1991) as a template, the ORF of GST was PCR-amplified with a primer set, which introduces *Bgl* II and *Bam* HI sites (GAAGATCTACCATGTCCCCTATACTAGGT and AAACGCGCGAGGCAGATCGTCAGTCAG). After digestion with *Bgl* II and *Bam* HI, the PCR product was ligated into the *Bam* HI site of pCS2$^+$ (a gift from Drs Kunio Inoue and Shingo Maegawa, Nara Institute of Science and Technology), and the correctly oriented clone was selected (named GST/pCS2$^+$). cDNA fragments of XPum (XPum NN, NC and Puf; see Figs. 1 and 2) were PCR-amplified from the full-length XPum cDNA using the same primer sets as those descried above for XPum NN and XPum NC and the following set for XPum Puf: AAGGATCCGGTAGAAGCAGACTACTTGAA and AAACTCGAGAATGATTCCATTTGAGGGTCC. The resulting PCR products were digested with *Bam* HI and *Xho* I and ligated into the *Bam* HI/*Xho* I site of GST/pCS2$^+$ to produce GST-XPum NN, GST-XPum NC and GST-XPum Puf.

To construct T7-tagged versions of XPum (T7-XPum NF, NN, NC, RBD and Puf), cDNA fragments were PCR-amplified from the full-length cDNA using the following primer sets (The primer sets for XPum NN, NC and Puf were the same as those described above.): AAGGATCCATGAGCGTTGCATGTGTCTTG and AAACTCGAGAGAGGGCATGACATCTGACAT for XPum NF, and CGCGGATCCGATGCTTGCACCTGCTGCT and CCGCTCGAGAATGATTCCATTTGAGGG for XPum RBD. The PCR products were digested with *Bam* HI and *Xho* I and ligated into the *Bam* HI/*Xho* I site of pET21a (for T7-XPum NF, NN, NC and Puf) or pET21b (for T7-XPum RBD). A plasmid encoding T7-tagged GST (T7-GST) was similarly produced by inserting the ORF of GST into pET21b. All of the T7-tagged proteins have a His-tag at the C-terminus.

4.6. Oocyte microinjection

Oocytes used for microinjection experiments were manually isolated from the surrounding follicles with forceps. Collagenase was not used for microinjection experiments, since the treatment has dramatic adverse effects on protein synthesis and progesterone sensitivity of oocytes (Smith et al., 1991). Oocytes were injected with 3 ng of anti-XPum 2A5 monoclonal antibody (Nakahata et al., 2001a), anti-GST monoclonal antibody (Yamashita, unpublished), or buffer (20 mM sodium phosphate, pH 7.0). The antibodies for injection were purified from the ascites by using protein G-Sepharose (Amersham Pharmacia), desalted with Sephadex G-25 and equilibrated to 20 mM sodium phosphate (pH 7.0). The injected oocytes were incubated overnight at 18 °C. After the induction of maturation with progesterone, the oocytes were collected at appropriate intervals and scored for GVBD to examine the effects of antibodies on oocyte maturation. In some experiments, the oocytes were extracted and subjected to immunoblotting analyses, as described in the last paragraph of this section.

GST-XPum NN, NC and Puf cDNAs were linearized with *Not* I and transcribed using an mMESSAGE mMACHINE SP6 kit (Ambion, Austin, TX). T7-XPum RBD, NN, NF, NC, Puf and GST cDNAs were linearized with *Dra* III and transcribed using an mMESSAGE mMACHINE T7 kit. All in vitro synthesized RNAs were phenol/chloroform-extracted, ethanol-precipitated, and dissolved in distilled water. Oocytes were injected with 25 nl of 2 ng/nl mRNA encoding GST-XPum NN, NC or Puf. Half of the oocytes were treated with progesterone and half were not treated, and the treated and untreated oocytes were incubated overnight at 18 °C and subjected to immunoblotting with anti-GST and anti-CPEB antibodies after GSH-Sepharose precipitation as described previously (Nakahata et al., 2001a). For over-expression experiments, oocytes were injected with 25–42.5 ng of mRNA encoding T7-XPum RBD, NN, NF, NC and Puf. After incubation overnight at 18 °C, the oocytes were treated with progesterone. When incubated for a long time after progesterone treatment, the oocytes underwent GVBD irrespective of the proteins injected. To demonstrate the difference in the kinetics of GVBD between the experiment and the control, therefore, GVBD was scored when the control water-injected oocytes reached ~50% GVBD.

At appropriate times after the treatment with progesterone, the oocytes injected with T7-XPum RBD or T7-GST were scored for GVBD, and 15 oocytes were homogenized

in 45 μl of EB followed by centrifugation for 10 min at 4 °C. Thirty microliters of the supernatant was subjected to Suc1-precipitation to detect cyclin B1 in complex with Cdc2, and the remaining supernatant was mixed with 15 μl of × 2 SDS sample buffer to detect Mos and recombinant proteins. The samples were analyzed by Western blotting as described previously (Yamashita et al., 1991) using the following antibodies: monoclonal anti-Cdc2 (MC2-21) (Tanaka and Yamashita, 1995), monoclonal anti-cyclin B1 (Bufo 2F5) (Sakamoto et al., 1998), polyclonal anti-Mos (a gift from Dr Noriyuki Sagata, Kyushu University), and monoclonal anti-T7-tag (purchased from Novagen), and polyclonal anti-His-tag (purchased from Santa Cruz Biotechnology, Santa Cruz, CA).

4.7. Luciferase assay

The luciferase reporters (pRc/CMV-luc/cyclin B1 3′ UTR and pRc/CMV-luc/mutant cyclin B1) were linearized with *Apa* I and transcribed with T7 RNA polymerase using an mCAP RNA capping kit (Stratagene). For normalization of the luciferase assay, β-galactosidase mRNA transcribed from *Not* I-cut pCS2-cβgal (a gift from Drs Kunio Inoue and Shingo Maegawa) was simultaneously injected with luciferase mRNA (each 0.01 fmol/oocyte) and then incubated at 20 °C for 3 h.

The luciferase assay was carried out according to the method of Barkoff et al. (2000). Briefly, oocytes injected with each reporter mRNA were pooled into groups of five and homogenized in 600 μl of 1 × cell lysis buffer (Promega). Fifty microliters of the homogenate was assayed for luciferase activity using a luciferase assay reagent (Promega), and 20 μl of the homogenate was assayed for β-galactosidase activity using Galacto-light Plus (Tropix, Bedford, MA). Luciferase activities were normalized by β-galactosidase activities. Average values of luciferase activity were calculated for each type of sample, from at least three pools of five oocytes.

To confirm stability of the reporter mRNAs, oocytes were injected with either 0.01 or 1 fmol of mRNA. Total RNA was extracted from oocytes using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Total RNA (10 μg) was separated on a 1% agarose gel containing 6% formaldehyde and blotted onto a nylon membrane (Hybond N^+, Pharmacia). The blots were hybridized with the DIG-labeled RNA complementary to the entire ORF of luciferase, and the signal was visualized with alkaline phosphatase-conjugated anti-DIG antibody (Roche, Tokyo, Japan).

4.8. UV cross-linking assay and PAT assay

UV cross-linking assay was carried out as described previously (Nakahata et al., 2001a). Poly(A) tail length of endogenous cyclin B1 mRNA was measured by the PCR-based poly(A) test (PAT) (Salles and Strickland, 1995, 1999) using a cyclin B1-specific oligonucleotide (CATGTGAAGGACTACGTGGCATTCC, corresponding to nucleotides 1281–1305) (Minshull et al., 1989). The PCR conditions were 1 cycle of 5 min at 94 °C and 30 cycles of 45 s at 94 °C, 45 s at 66 °C and 1 min at 72 °C. After the PCR, samples were incubated for 7 min at 72 °C. The products were separated on a 6% non-denaturing polyacrylamide gel.

Acknowledgements

We thank Drs Noriyuki Sagata and Hideki Kobayashi (Kyushu University) for providing the anti-Mos antibody and the *Xenopus* cyclin B1 cDNA, respectively, and Drs Kunio Inoue and Shingo Maegawa (Nara Institute of Science and Technology) for providing pCS2$^+$, pCS2-cβgal, and pRc/CMV-luc and for their helpful discussions. This work was supported in part by a grant-in-aid for scientific research (14340258) and 21st Century COE Program 'Center of Excellence for Advanced Life Science on the Base of Bioscience and Nanotechnology' from the Ministry of Education, Science, Sports and Culture of Japan to M.Y. and for research from CREST, JST (Japan Science and Technology Corporation) to Y.N.

References

Asaoka-Taguchi, M., Yamada, M., Nakamura, A., Hanyu, K., Kobayashi, S., 1999. Maternal Pumilio acts together with Nanos in germline development in *Drosophila* embryos. Nat. Cell Biol. 1, 431–437.

Ballantyne, S., Daniel, D.L.J., Wickens, M., 1997. A dependent pathway of cytoplasmic polyadenylation reactions linked to cell cycle control by c-*mos* and CDK1 activation. Mol. Biol. Cell 8, 1633–1648.

Barkoff, A., Ballantyne, S., Wickens, M., 1998. Meiotic maturation in *Xenopus* requires polyadenylation of multiple mRNA. EMBO J. 17, 3168–3175.

Barkoff, A.F., Dickson, K.S., Gray, N.K., Wickens, M., 2000. Translational control of cyclin B1 mRNA during meiotic maturation: coordinated repression and cytoplasmic polyadenylation. Dev. Biol. 220, 97–109.

Cao, Q., Richter, J.D., 2002. Dissolution of the maskin-eIF4E complex by cytoplasmic polyadenylation and poly(A)-binding protein controls cyclin B1 mRNA translation and oocyte maturation. EMBO J. 21, 3852–3862.

Chagnovich, D., Lehmann, R., 2001. Poly(A)-independent regulation of maternal *hunchback* translation in the *Drosophila* embryo. Proc. Natl. Acad. Sci. USA 98, 11359–11364.

Charlesworth, A., Welk, J., MacNicol, A.M., 2000. The temporal control of *wee*1 mRNA translation during *Xenopus* oocyte maturation is regulated by cytoplasmic polyadenylation elements within the 3′-untranslated region. Dev. Biol. 227, 706–719.

Charlesworth, A., Ridge, J.A., King, L.A., MacNicol, M.C., MacNicol, A.M., 2002. A novel regulatory element determines the timing of Mos mRNA translation during *Xenopus* oocyte maturation. EMBO J. 21, 2798–2806.

Crittenden, S.L., Bernstein, D.S., Bachorik, J.L., Thompson, B.E., Gallegos, M., Petcherski, A.G., Moulder, G., Barstead, R., Wickens, M., Kimble, J., 2002. A conserved RNA-binding protein controls germline stem cells in *Caenorhabditis elegans*. Nature 417, 660–663.

Cyert, M.S., Kirschner, M.W., 1988. Regulation of MPF activity in vitro. Cell 53, 185–195.

de Moor, C.H., Richter, J.D., 1997. The Mos pathway regulates cytoplasmic polyadenylation in *Xenopus* oocytes. Mol. Cell. Biol. 17, 6419–6426.

de Moor, C.H., Richter, J.D., 1999. Cytoplasmic polyadenylation elements mediate masking and unmasking of cyclin B1 mRNA. EMBO J. 18, 2294–2303.

De Smedt, V., Poulhe, R., Cayla, X., Dessauge, F., Karaiskou, A., Jessus, C., Ozon, R., 2002. Thr-161 phosphorylation of monomeric Cdc2: regulation by protein phosphatase 2C in *Xenopus* oocytes. J. Biol. Chem. 277, 28592–28600.

Dumont, L.W., 1972. Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. J. Morphol. 136, 153–180.

Dupré, A., Jessus, C., Ozon, R., Haccard, O., 2002. Mos is not required for the initiation of meiotic maturation in *Xenopus* oocytes. EMBO J. 21, 4026–4036.

Edwards, T.A., Pyle, S.E., Wharton, R.P., Aggarwal, A.K., 2001. Structure of Pumilio reveals similarity between RNA and peptide binding motifs. Cell 105, 281–289.

Frank-Vaillant, M., Jessus, C., Ozon, R., Maller, J.L., 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.

Guan, K.-L., Dixon, J.E., 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, L.E., Richter, J.D., 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, Y.-H., 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.

Hochegger, H., Klotzbucher, A., Kirk, J., Howell, M., Guellec, K.I., Fletcher, K., Duncan, T., Sohail, M., Hunt, T., 2001. New B-type cyclin synthesis is required between meiosis I and II during *Xenopus* oocyte maturation. Development 128, 3795–3807.

Iwabuchi, M., Ohsumi, K., Yamamoto, T.M., Sawada, W., Kishimoto, T., 2000. Residual cdc2 activity remaining at meiosis I exit is essential for meiotic M–M transition in *Xenopus* oocyte extracts. EMBO J. 19, 4513–4523.

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.

Kozak, M., 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44, 283–292.

Kozak, M., 1989. The scanning model for translation: an update. J. Cell Biol. 108, 229–241.

Kraemer, B., Crittenden, S., Gallegos, M., Moulder, G., Barstead, R., Kimble, J., Wickens, M., 1999. NANOS-3 and FBF proteins physically interacts to control the sperm-oocyte switch in *Caenorhabditis elegans*. Curr. Biol. 9, 1009–1018.

Ledan, E., Polanski, Z., Terret, M., Maro, B., 2001. Meiotic maturation of the mouse oocyte requires an equilibrium between cyclin B synthesis and degradation. Dev. Biol. 232, 400–413.

Maegawa, S., Yamashita, M., Yasuda, K., Inoue, K., 2002. Zebrafish DAZ-like protein controls translation via the sequence 'GUUC'. Genes Cells 7, 971–984.

Mendez, R., Richter, J.D., 2001. Translational control by CPEB: a means to the end. Nat. Rev. Mol. Cell Biol. 2, 521–529.

Mendez, R., Hake, L.E., Andresson, T., Littlepage, L.E., Ruderman, J.V., Richter, J.D., 2000a. Phosphorylation of CPE binding factor by Eg2 regulates translation of c-*mos* mRNA. Nature 404, 302–307.

Mendez, R., Murthy, K.G., Ryan, K., Manley, J.L., Richter, J.D., 2000b. Phosphorylation of CPEB by Eg2 mediates the recruitment of CPSF into an active cytoplasmic polyadenylation complex. Mol. Cell 6, 1253–1259.

Mendez, R., Barnard, D., Richter, J.D., 2002. Differential mRNA translation and meiotic progression require Cdc2-mediated CPEB destruction. EMBO J. 21, 1833–1844.

Minshull, J., Blow, J.J., Hunt, T., 1989. Translation of cyclin mRNA is necessary for extracts of activated *Xenopus* eggs to enter mitosis. Cell 56, 947–956.

Murakami, M.S., Vande Woude, G.F., 1998. Analysis of the early embryonic cell cycles of *Xenopus*; regulation of cell cycle length by Xe-weel and Mos. Development 125, 237–248.

Murata, Y., Wharton, R.P., 1995. Binding of pumilio to maternal *hunchback* mRNA is required for posterior patterning in *Drosophila* embryos. Cell 80, 747–756.

Nakahata, S., Katsu, Y., Mita, K., Inoue, K., Nagahama, Y., Yamashita, M., 2001a. Biochemical identification of *Xenopus* pumilio as a sequence-specific cyclin B1 mRNA-binding protein that physically interacts with a nanos homolog, Xcat-2, and a cytoplasmic polyadenylation element-binding protein. J. Biol. Chem. 276, 20945–20953.

Nakahata, S., Mita, K., Katsu, Y., Nagahama, Y., Yamashita, M., 2001b. Immunological detection and characterization of poly(A) polymerase, poly(A)-binding protein and cytoplasmic polyadenylation element-binding protein in goldfish and *Xenopus* oocytes. Zool. Sci. 18, 337–343.

Nakajo, N., Yoshitome, S., Iwashita, J., Iida, M., Uto, K., Ueno, S., Okamoto, K., Sagata, N., 2000. Absence of Wee1 ensures the meiotic cell cycle in *Xenopus* oocytes. Genes Dev. 14, 328–338.

Olivas, W., Parker, R., 2000. The Puf3 protein is a transcript-specific regulator of mRNA degradation in yeast. EMBO J. 19, 6602–6611.

Peter, M., Labbé, J.-C., Dorée, M., Mandart, E., 2002. A new role for Mos in *Xenopus* oocyte maturation: targeting Myt1 independently of MAPK. Development 129, 2129–2139.

Polanski, Z., Ledan, E., Brunet, S., Louvet, S., Verlhac, M.-H., Kubiak, J.Z., Maro, B., 1998. Cyclin synthesis controls the progression of meiotic maturation in mouse oocytes. Development 125, 4989–4997.

Richter, J.D., 2000. Influence of polyadenylation-induced translation on metazoan development and neuronal synaptic function. In: Sonenberg, N., Hershey, J.W.B., Mathews, M.B. (Eds.), Translational Control of Gene Expression, Cold Spring Harbor Laboratory Press, New York, pp. 785–805.

Richter, J.D., Theurkauf, W.E., 2001. The message is in the translation. Science 293, 60–62.

Sagata, N., Oskarsson, M., Copeland, T., Brumbaugh, J., Vande Woude, G.F., 1988. Function of c-*mos* proto-oncogene product in meiotic maturation in *Xenopus* oocytes. Nature 335, 519–525.

Sagata, N., Daar, I., Oskarsson, M., Showalter, S.D., Vande Woude, G.F., 1989. The product of the *mos* proto-oncogene as a candidate 'Initiator' for oocyte maturation. Science 245, 643–646.

Sakamoto, I., Takahara, K., Yamashita, M., Iwao, Y., 1998. Changes in cyclin B during oocyte maturation and early embryonic cell cycle in the newt, *Cynops pyrrhogaster*: requirement of germinal vesicle for MPF activation. Dev. Biol. 195, 60–69.

Salles, F.J., Strickland, S., 1995. Rapid and sensitive analysis of mRNA polyadenylation states by PCR. PCR Meth. Appl. 4, 317–321.

Salles, F.J., Strickland, S., 1999. Assessment of poly(A) tail lengths on specific mRNAs during development. In: Richter, J.D., (Ed.), A Comparative Methods Approach to the Study of Oocytes and Embryos, Oxford University Press, New York, pp. 457–470.

Sheets, M.D., Wu, M., Wickens, M., 1995. Polyadenylation of c-*mos* mRNA as a control point in *Xenopus* meiotic maturation. Nature 374, 511–516.

Smith, L.D., Xu, W., Varnold, R.L., 1991. Oogenesis and oocyte isolation. Meth. Cell Biol. 36, 45–60.

Sonoda, J., Wharton, R.P., 1999. Recruitment of Nanos to *hunchback* mRNA by Pumilio. Genes Dev. 13, 2704–2712.

Sonoda, J., Wharton, R.P., 2001. *Drosophila* brain tumor is a translational repressor. Genes Dev. 15, 762–773.

Souza, G.M., da Silva, A.M., Kuspa, A., 1999. Starvation promotes *Dictyostelium* development by relieving PufA inhibition of PKA translation through the YakA kinase pathway. Development 126, 3263–3274.

Stebbins-Boaz, B., Cao, Q., de Moor, C.H., Mendez, R., Richter, J.D., 1999. Maskin is a CPEB-associated factor that transiently interacts with eIF-4E. Mol. Cell 4, 1017–1027.

Tadauchi, T., Matsumoto, K., Herskowitz, I., Irie, K., 2001. Post-transcriptional regulation through the HO 3′-UTR by Mpt5, a yeast homolog of Pumilio and FBF. EMBO J. 20, 552–561.

Taieb, F., Thibier, C., Jessus, C., 1997. On cyclins, oocytes, and eggs. Mol. Reprod. Dev. 48, 397–411.

Tanaka, T., Yamashita, M., 1995. Pre-MPF is absent in immature oocytes of fishes and amphibians except *Xenopus*. Dev. Growth Differ. 37, 387–393.

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

Thom, G., Minshall, N., Git, A., Argasinska, J., Standart, N., 2003. Role of cdc2 kinase phosphorylation and conserved N-terminal proteolysis motifs in CPEB complex dissociation and degradation. Biochem. J. 370, 91–100.

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.

Wakiyama, M., Honkura, N., Miura, K., 2001. Interference with interaction between eukaryotic translation initiation factor 4G and poly(A)-binding protein in *Xenopus* oocytes leads to inhibition of polyadenylated mRNA translation and oocyte maturation. J. Biochem. 130, 737–740.

Wang, X., Zamore, P.D., Hall, T.M., 2001. Crystal structure of a Pumilio homology domain. Mol. Cell 7, 855–865.

Wang, X., McLachlan, J., Zamore, P.D., Tanaka Hall, T.M., 2002. Modular recognition of RNA by a human Pumilio-homology domain. Cell 110, 501–512.

Wharton, R.P., Struhl, G., 1991. RNA regulatory elements mediate control of *Drosophila* body pattern by the posterior morphogen nanos. Cell 67, 955–967.

Wharton, R.P., Sonoda, J., Lee, T., Patterson, M., Murata, Y., 1998. The Pumilio RNA-binding domain is also a translational regulator. Mol. Cell 1, 863–872.

White, E.K., Moore-Jarrett, T., Ruley, H.E., 2001. PUM2, a novel murine Puf protein, and its consensus RNA-binding site. RNA 7, 1855–1866.

Wickens, M., Goodwin, E.B., Kimble, J., Strickland, S., Hentze, M., 2000. Translational control of developmental decisions. In: Sonenberg, N., Hershey, J.W.B., Mathews, M.B. (Eds.), Translational Control of Gene Expression, Cold Spring Harbor Laboratory Press, New York, pp. 295–370.

Wickens, M., Bemstein, D.S., Kimble, J., Parker, R., 2002. A PUF family portrait: 3′ UTR regulation as a way of life. Trends Genet. 18, 150–157.

Wreden, C., Verrotti, A.C., Schisa, J.A., Lieberfarb, M.E., Strickland, S., 1997. Nanos and pumilio establish embryonic polarity in *Drosophila* by promoting posterior deadenylation of *hunchback* mRNA. Development 124, 3015–3023.

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

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.

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

Zhang, B., Gallegos, M., Puoti, A., Durkin, E., Fields, S., Kimble, J., Wickens, M.P., 1997. A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. Nature 390, 477–484.