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The clam 3′ UTR masking element-binding protein p82 is a member of the CPEB family

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ABSTRACT

During early development gene expression is controlled principally at the translational level. Oocytes of the surf clam *Spisula solidissima* contain large stockpiles of maternal mRNAs that are translationally dormant or masked until meiotic maturation. Activation of the oocyte by fertilization leads to translational activation of the abundant cyclin and ribonucleotide reductase mRNAs at a time when they undergo cytoplasmic polyadenylation. In vitro unmasking assays have defined U-rich regions located approximately centrally in the 3′ UTRs of these mRNAs as translational masking elements. A clam oocyte protein of 82 kDa, p82, which selectively binds the masking elements, has been proposed to act as a translational repressor. Importantly, mRNA-specific unmasking in vitro occurs in the absence of poly(A) extension. Here we show that clam p82 is related to *Xenopus* CPEB, an RNA-binding protein that interacts with the U-rich cytoplasmic polyadenylation elements (CPEs) of maternal mRNAs and promotes their polyadenylation. Cloned clam p82/CPEB shows extensive homology to *Xenopus* CPEB and related polypeptides from mouse, goldfish, *Drosophila* and *Caenorhabditis elegans*, particularly in their RNA-binding C-terminal halves. Two short N-terminal islands of sequence, of unknown function, are common to vertebrate CPEBs and clam p82. p82 undergoes rapid phosphorylation either directly or indirectly by cdc2 kinase after fertilization in meiotically maturing clam oocytes, prior to its degradation during the first cell cleavage. Phosphorylation precedes and, according to inhibitor studies, may be required for translational activation of maternal mRNA. These data suggest that clam p82 may be a functional homolog of *Xenopus* CPEB.

Keywords: masked mRNA; polyadenylation; RNA-binding proteins; translational control

INTRODUCTION

Gene expression during meiotic maturation and early embryogenesis, at a time when transcription is silent, is regulated predominantly at the level of translation. The spatial and temporal regulation of maternal mRNAs governs resumption of the cell cycle, establishes embryonic axes and determines cell fate (Curtis et al., 1995; Wickens et al., 1996, 1997). A striking example is provided by c-mos mRNA whose polyadenylation and consequent translation is a pivotal regulatory step in meiotic maturation of *Xenopus* and mouse oocytes (Gebauer et al., 1994; Sheets et al., 1995).

We study the regulatory mechanisms responsible for translational activation of surf clam maternal mRNAs at fertilization. Upon fertilization of clam oocytes, arrested in prophase I, the cells rapidly complete meiosis and proceed directly into the mitotic cell division cycles (Hunt et al., 1992, and references therein). During meiotic maturation, the rate of protein synthesis increases two- to fourfold and this is largely due to de novo translation of three abundant maternal mRNAs, encoding cyclins A and B and the small subunit of ribonucleotide reductase (RR), whose products enable cell cycle progression and DNA synthesis (Standart, 1992). Clam maternal mRNAs, in common with mRNAs from other organisms, undergo changes in poly(A) tail length at fertilization, and these changes essentially correlate with translational activity (Rosenthal & Ruderman, 1987).

Cyclin A and RR mRNAs, masked in oocyte lysates, can be specifically translationally activated in vitro using antisense RNAs directed to the "masking elements" located approximately in the center of their 3′ UTRs.

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The antisense RNAs, by forming double-stranded structures, are postulated to prevent binding of masking or repressor proteins thus leading to translational activation. Importantly, in the in vitro unmasking assay, activation of translation occurred in the absence of polyadenylation of these messages, leading us to propose that the two events are not obligatorily coupled (Standart et al., 1990).

Using UV-crosslinking and gel-retardation assays, we recently identified an 82 kDa oocyte protein, denoted p82, that selectively binds the U-rich masking elements in the 3′ UTRs of RR and cyclin A mRNAs. This protein is phosphorylated to a 92 kDa species in activated egg extracts (p92); this modification alters its complex formation with RNA as seen in gel retardation assays and may be an important step in the regulation of translational unmasking (Walker et al., 1996). p82 is associated with masked mRNAs in low-salt gel filtration columns, but is removed from the RNP peak by 0.5 M KCl, conditions known to activate these mRNAs (Standart et al., 1990; Walker et al., 1996). Thus p82 has several of the characteristics expected of a translational masking/repressor protein.

Other examples of regulatory RNA elements residing within 3′ noncoding regions that are proposed to bind translational repressors and act independently of poly(A) tail changes include human β-interferon mRNA (Kruys et al., 1989), *Drosophila* nanos (Gavis & Lehmann, 1994), and *Xenopus* FGF receptor 1-maternal mRNA (Culp & Musci, 1998). In the case of rabbit erythroid 15-lipoxygenase mRNA, direct evidence was obtained both in vitro and in vivo that 3′ UTR binding proteins, namely hnRNP K and E1, result in the specific down-regulation of any mRNA containing their binding sites, in the absence of discernible poly(A) tail changes (Ostareck-Lederer et al., 1994; Ostareck et al., 1997).

On the other hand, some maternal mRNAs are translationally regulated solely or primarily by the evolutionarily conserved mechanism of cytoplasmic polyadenylation. Regulatory sequences that control cytoplasmic poly(A) addition during meiotic maturation comprise a 3′ UTR U-rich consensus motif, $U_{4-6}A_{1-2}U$, designated the cytoplasmic polyadenylation element (CPE), near the ubiquitous nuclear polyadenylation signal, AAUAAA. Both elements are required to support cytoplasmic poly(A) extension and to stimulate translation during oocyte maturation and early development in *Xenopus* and mouse. mRNAs lacking a CPE-motif in their 3′ UTR are deadenylated at maturation by default, and are concomitantly released from polysomes, providing further evidence of the tight connection between poly(A) tail length and translation (Varnum & Wormington, 1990; Richter, 1996; Verrotti et al., 1996; Wickens et al., 1996). Quite how poly(A) tail length affects ribosome binding at the 5′ cap for these mRNAs is not yet understood; ribose methylation of certain mRNAs requires ongoing polyadenylation, implying a functional role for such cap modification (Kuge & Richter, 1995).

CPEB (cytoplasmic polyadenylation element-binding protein), a *Xenopus* oocyte protein of 62 kDa, was originally defined as the protein that specifically bound the CPE motif of B4 mRNA. CPEB becomes phosphorylated during meiotic maturation, either directly or indirectly by cdc2 kinase (Paris et al., 1991). Use of antibodies to the cloned protein demonstrated the positive role of CPEB in cytoplasmic polyadenylation, initially of B4 mRNA (Hake & Richter, 1994) and subsequently of c-mos, cdk-2, cyclins, and G10 mRNAs. This protein binds the CPEs of these mRNAs, and its presence in egg lysates is necessary to support their polyadenylation (Stebbins-Boaz et al., 1996). Injection of CPEB antibody into oocytes not only blocks polyadenylation in vivo, but also blocks progesterone-induced maturation, suggesting that CPEB is critical for early development (Stebbins-Boaz et al., 1996). Though the actual part that CPEB plays in polyadenylation is not yet clear, it may recruit or stabilize factors such as cleavage and polyadenylation specificity factors and poly(A) polymerase to the 3′ terminus of CPE-containing mRNAs (Bilger et al., 1994).

Here we show that the clam masking protein, p82, is related to the *Xenopus* oocyte CPEB protein. Cloning of p82 cDNAs revealed that the clam protein is highly similar to frog CPEB and related proteins from other organisms. Upon oocyte maturation p82 undergoes rapid phosphorylation, prior to its regulated proteolysis, and this modification correlates with the translational activation of the maternal cyclin A mRNA. We also show that phosphorylation of p82 is downstream of cdc2 kinase.

RESULTS

Clam oocyte p82 is related to *Xenopus* CPEB

Prompted by some of the properties shared by clam p82 and the *Xenopus* CPEB (XCPEB) we tested whether these proteins are related. Both proteins UV crosslink to U-rich motifs in the 3′ UTRs of translationally regulated maternal mRNAs and both are phosphorylated upon oocyte activation (Paris & Richter, 1990; Paris et al., 1991; Hake & Richter, 1994; Walker et al., 1996). The clam protein undergoes an apparent size shift to 92 kDa, whereas the frog protein alters from 62 kDa to ~64 kDa in SDS-PAGE. These altered mobilities were shown to be due to phosphorylation (Paris & Richter, 1990; Walker et al., 1996). A rabbit polyclonal serum raised against recombinant XCPEB (Hake & Richter, 1994) was used to probe Western blots of clam and *Xenopus* oocyte and egg proteins. The immune serum detected a number of clam proteins, the most

prominent bands being 82 and 92 kDa in oocyte and egg extracts respectively, whereas the preimmune serum did not detect either of these proteins (Fig. 1A, lanes 2,3 and 5,6). Although equal amounts of clam and frog proteins were used in this Western blot, XCPEB was barely detectable in the egg lysate (Fig. 1A, lane 1), presumably due to its reduced levels at this stage (Hake & Richter, 1994).

To test whether the 82- and 92-kDa clam proteins detected by the anti-XCPEB serum were the same polypeptides as those detected previously in UV crosslinking experiments, immunoprecipitations were carried out of oocyte and egg proteins labeled by virtue of covalent crosslinking to their RNA substrates. Two RNA probes were transcribed in vitro in the presence of [^{32}P]-UTP, the ribonucleotide reductase masking element RNA (Walker et al., 1996) and as a control, the terminal 44 nt of *Xenopus* B4 maternal mRNA, containing the CPE and hexanucleotide motifs (Paris & Richter, 1990) and used in crosslinking experiments. Various clam and frog oocyte and egg polypeptides acquired covalently linked stubbs of labeled RNAs upon UV irradiation and subsequent RNAse A treatment (Fig. 1A, lanes 1–3; Hake & Richter, 1994; Walker et al., 1996). The XCPEB protein of 62 kDa crosslinked to the B4 RNA probe was indeed detected by immunoprecipitation (Fig. 1B, lane 12). As shown in Figure 1B (lanes 7 and 8), radiolabeled clam p82 and p92 were both specifically precipitated using the anti-XCPEB serum from the mix of crosslinked oocyte and egg proteins. The preimmune serum failed to precipitate any labeled proteins (data not shown). Interestingly, this experiment indicates that both p82 and XCPEB crosslink more efficiently to their cognate RNA probes than to the heterologous probes (Fig. 1B, compare lanes 7,8 with 10,11, and 9 with 12), implying that each protein binds RNA with considerable sequence specificity. The Western blot and immunoprecipitation data clearly demonstrate that clam p82 and its phosphorylated form p92 are antigenically related to frog CPEB.

Finally, the anti-XCPEB serum was used to investigate clam p82 localization in the oocyte. Nuclear and cytoplasmic extracts prepared from clam oocytes were analyzed on Western blots and showed p82 to be essentially confined to the cytoplasm (Fig. 1A, lanes 7 and 8). In contrast, the minor proteins of 42, 64, 138 and 165 kDa recognised by the anti-XCPEB serum appeared to be predominantly nuclear.

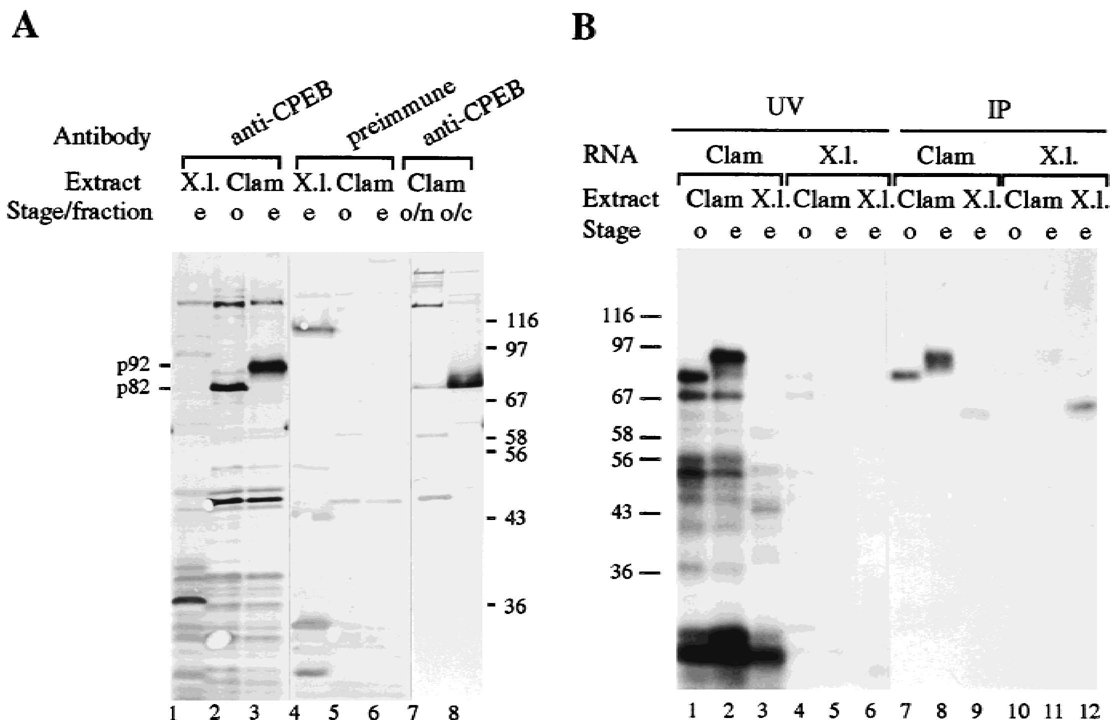

FIGURE 1. Clam p82 and p92 are recognised by anti-*Xenopus* CPEB serum. **A**: Immunoblot of *Xenopus* oocyte, clam oocyte (o), and egg extracts (e) using equal amounts of protein. Clam oocyte nuclear (n) and cytoplasmic (c) fractions were loaded in equivalent cell amounts. The blots were probed with either anti-*Xenopus* CPEB serum or preimmune serum. Immunoreactive proteins of 82 and 92 kDa in clam oocyte and extracts respectively are indicated. **B**: UV-crosslinked clam p82 and p92 can be immunoprecipitated with anti-XCPEB serum. Clam oocyte and egg extracts and *Xenopus* egg extract were UV crosslinked to [^{32}P]-labeled RNAs corresponding to the clam ribonucleotide reductase 3′ UTR masking element (lanes 1–3 and 7–9) and the CPE and hexanucleotide signal of the *Xenopus* B4 mRNA (lanes 4–6 and 10–12). Several UV reactions were pooled, incubated with rabbit anti-XCPEB serum and protein A-Sepharose beads, and bound material released with SDS sample buffer.

p82 cDNA from clam oocytes

cDNAs encoding p82 were isolated from a clam ovary cDNA library. Degenerate oligonucleotides, designed according to the conserved portions surrounding the second RNA recognition motif (RRM) region of XCPEB and its related *Drosophila* and *Caenorhabditis elegans* polypeptides (Hake & Richter, 1994), were synthesized as primers for RT-PCR using clam oocyte RNA as a template (see Materials and Methods for details). The PCR product of the expected size of 418 nt, confirmed by sequencing to contain nucleotide sequence highly homologous to the second RRM region of CPEB, was used to screen a clam ovary λgt22 library. This led to the isolation of 22 cDNAs of various sizes that were shown by restriction digests and partial sequencing to all contain the same mRNA, differing only in their 5′ termini.

The longest cDNA obtained was 2,878 nt in length and contained a single long open reading frame (ORF). The first AUG codon in this ORF, located at nucleotides 163–165, gives a predicted protein-coding region of 1,950 nt. Although this AUG is in a relatively poor Kozak context for initiation codons ($^{-3}$AUCAUGU^{+4} compared to the consensus among vertebrate mRNAs of $^{-3}$ACCAUGG^{+4}; Kozak, 1989), the three upstream in-frame stop codons ensure that this AUG serves as the initiation codon. As Northern analysis subsequently showed (Fig. 5B), p82 mRNA is an astounding 9 kb-long message. Thus the cloned p82 cDNAs, with 762 nt of 3′ UTR terminating in primer-derived 18 As, only contained a partial 5′ and 3′ noncoding region. We noted that this portion of the 3′ UTR contains five copies of a motif with the consensus sequence GGAAC(A/C)AG(G/A)GAGGGT(A/C)NACAATT. However, we can only speculate about the significance of these sequences at present.

p82 is a member of the CPEB family

Comparison of the predicted p82 amino acid sequence with the GenBank database revealed that p82 has extensive homology to the XCPEB protein (41% identity overall; Hake & Richter, 1994), mouse CPEB (44%; Gebauer & Richter, 1996) and goldfish CPEB (40%; Y. Katsu & Y. Nagahama, pers. comm.; Figs. 2 and 3). Goldfish CPEB was obtained by library screening with an RT-PCR product of oocyte mRNA using degenerate oligonucleotides different from those in this study (Y. Katsu & Y. Nagahama, pers. comm.). Considerable sequence conservation is also seen between clam p82 and *Drosophila* orb, a protein implicated in mRNA localization (34%; Christerson & McKearin, 1994; Lantz et al., 1994), as well as an unidentified, considerably shorter *C. elegans* protein (32%; Fig. 3). Homology between p82, the CPEB proteins, and the fly and worm polypeptides is principally confined to their C-terminal

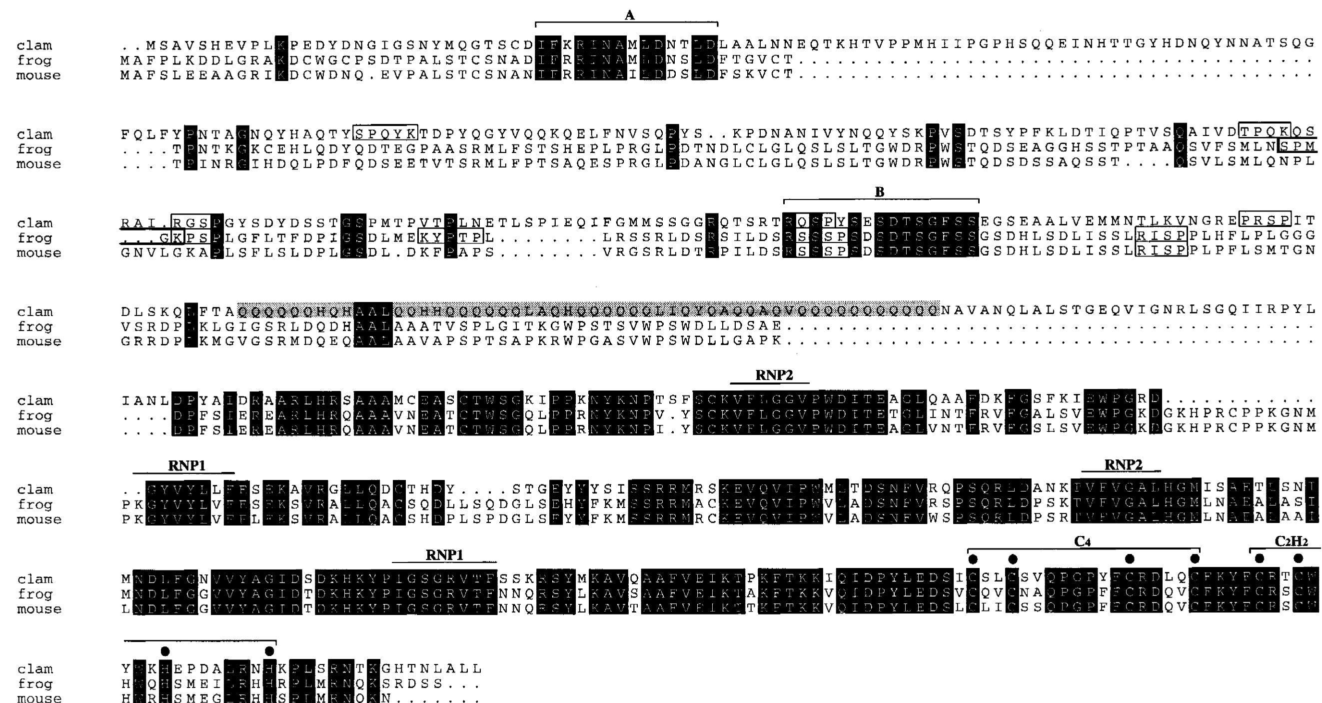

FIGURE 2. Alignment of the predicted p82 protein with homologs from frog and mouse. Identical residues present in p82 and *Xenopus* and mouse CPEB are shaded black. Highlighted are the conserved A and B motifs; the ribonucleoprotein-type RNA recognition motifs RNP 2 and 1 motifs; and the C_4, C_2H_2 zinc finger region (solid circles denote conserved cysteine and histidine residues) and consensus cdc2 phosphorylation sites (boxed). The polyglutamine tract in p82 is shaded grey.

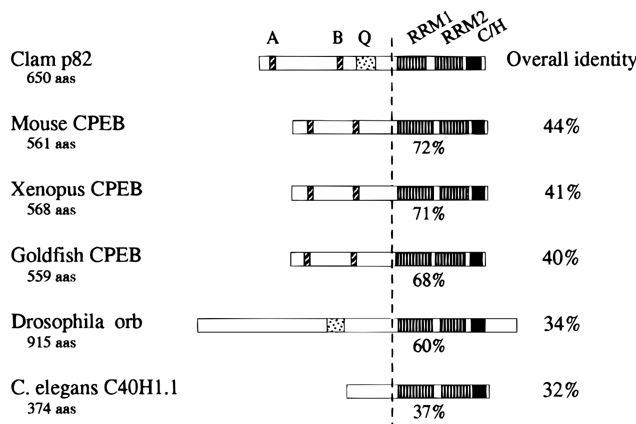

FIGURE 3. The CPEB family of RNA-binding proteins. Schematic representation of a comparison between p82 and related proteins from other organisms. Clam p82, *Xenopus*, mouse and golfish CPEBs, and related proteins are represented to scale. The RNA-recognition motifs (RRMs) and the cysteine/histidine-rich regions (C/H) are shown as boxed regions. The glutamine-rich region (Q-rich region) present in *Drosophila* orb and clam p82 is denoted by a stippled box. A and B represent small islands of homology between p82 and the CPEB proteins. The numbers indicate the percentage of amino acid identity of the various proteins/domains with respect to p82 obtained using BESTFIT and GAP programs. The number of amino acids predicted for each protein is indicated on the left.

halves, which contain the two tandem RRMs and, further downstream, an invariant set of eight cysteine and histidine residues (Figs. 2 and 3). The RRM, one of the best characterized RNA-binding motifs, is composed of 80–90 amino acids, and is present in one or more nonidentical copies in proteins that bind pre-mRNA, mRNA, pre-rRNA and snRNA (Burd & Dreyfuss, 1994; Nagai et al., 1995; Siomi & Dreyfuss, 1997). The RRM is specified by two short, conserved peptides, RNP2 and RNP1, with their characteristic array of aromatic and hydrophobic residues and separated by ~30 amino acids, and these are present in each of the RNA-binding domains of the predicted p82 protein. Comparison of the amino acid sequences also reveals that p82, in common with the CPEBs, orb, and the *C. elegans* protein, contains motifs reminiscent of C_4, C_2H_2 zinc finger domains present in a variety of RNA-binding proteins. Indeed, XCPEB requires zinc for RNA binding (Hake et al., 1998).

Although there is little similarity overall between the clam and the vertebrate CPEB proteins outside of the RRM and C/H regions, two short N-terminal motifs denoted A and B are conserved (see Figs. 2 and 3). Motif A, shared by *Xenopus*, mouse, goldfish CPEBs, and clam p82 is FK/RRINAM/ILDN/DT/SLD. The second N-terminal region of homology between the CPEB proteins and p82 is motif B, which includes the conserved sequence SDTSGFSS. The homology between the *Xenopus*, mouse, and goldfish proteins extends over much of their N-terminal halves; for example, mouse and *Xenopus* CPEBs share 68% identical residues in this portion of protein (Gebauer & Richter, 1996). Neither motif A nor B is present in orb or the related *C. elegans* protein. At present we have no firm evidence for the role of either motif in clam p82/CPEB (see Discussion).

In the central portion of p82, adjacent to the RRMs, there is a stretch of 54 amino acids of which 38 (70%) are glutamine residues. A glutamine-rich region of similar size is also found in the orb protein, although this motif is absent from the *Xenopus*, mouse, and goldfish CPEBs (Fig. 3, highlighted in grey).

Studies in vitro and in vivo have implicated $p34^{cdc2}$ kinase as a p82 kinase, functional during oocyte maturation (Walker et al., 1996; also see Figs. 6 and 7). The $p34^{cdc2}$ kinase consensus sequence was originally proposed to be K/R-S/T-P-(X)-K, based on the six histone H1 sites, and this consensus holds for most other cyclin-cdks. A more extensive comparison of cyclin-cdk substrates has revealed that their common aspects reduce to having a proline on the C-terminal site adjacent to the S/T residue and a nearby basic amino acid, although of these 2 factors, the most important appears to be the adjacent proline (Pines, 1995). Since p82 undergoes such a large shift in apparent molecular weight during meiotic maturation, often seen to take place in multiple steps (see Fig. 5), it is likely that more than one amino acid residue is phosphorylated. There are several possible cdc2 phosphorylation sites in p82, although few correspond exactly to the consensus sequence. These putative phosphorylation sites are all in the N-terminal half of p82 (Fig. 2, boxed motifs).

The cloned gene encodes p82

The 1,950-nt-long ORF predicts a protein of 650 amino acids with a calculated molecular weight of 73.4 kDa. This differs markedly from the apparent molecular weight of the p82 polypeptide on SDS-PAGE. To determine the apparent size of the protein encoded by the cDNA, it was transcribed in vitro with T7 RNA polymerase and translated in the nuclease-treated reticulocyte lysate cell-free system. The capped p82 mRNA synthesized a protein migrating with an apparent molecular weight of ~81 kDa (Fig. 4A, lane 1). This [^{35}S]-Met labeled polypeptide migrated approximately 1–2 kDa faster than the lysate p82 protein UV crosslinked to ^{32}P-labeled masking element RNA run alongside, presumably because the latter is covalently tagged with a stub of RNA, thus retarding its migration (data not shown). The in vitro translated p82 polypeptide is phosphorylated in egg extracts, but is not modified by incubation in oocyte lysate. Reticulocyte lysate containing labeled p82 (to which cycloheximide was added to prevent further synthesis) was added to an equal volume of clam oocyte or egg extract and the mixed lysates were incubated at 18 °C for 2 h (Fig. 4A, lanes 2 and 3). A decreased mobility of the 82-kDa protein was only observed in egg/retic lysates, similar to the size change

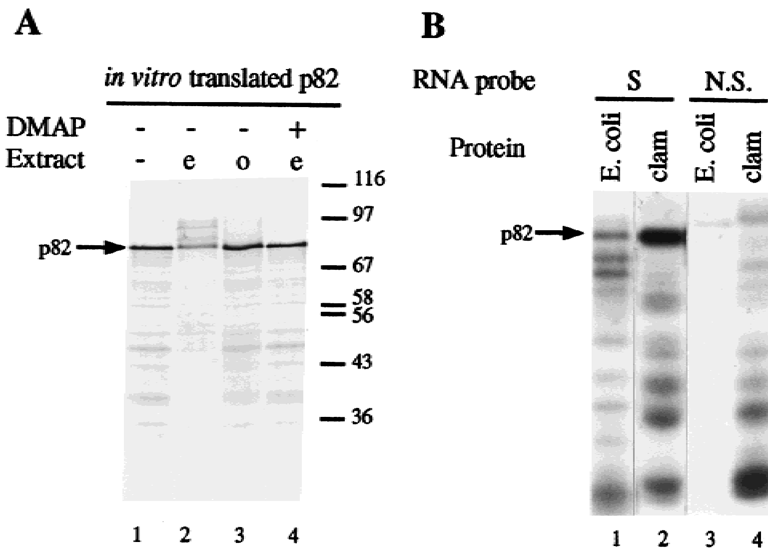

FIGURE 4. The cloned gene encodes p82. **A**: p82 cDNA was transcribed in vitro and the resulting capped mRNA translated in the reticulocyte lysate cell-free system (lane 1). Following addition of cycloheximide to 0.1 mg/mL, an equal volume of oocyte (lane 2) or activated egg clam lysate (lanes 3 and 4) were added for a further 2 h at 18 °C. Sample in lane 4 also contained 1 mM 6-DMAP. All samples were analyzed on 15% SDS-PAGE alongside molecular weight markers as indicated, followed by autoradiography. **B**: Expression of His-tagged p82 was induced with IPTG, and total bacterial lysates (lanes 1 and 3), and clam oocyte S10 proteins (lanes 2 and 4) were UV crosslinked to RR 3′ UTR masking element RNA (S, lanes 1 and 2) and a negative control RNA (N.S., lanes 3 and 4). All samples were analyzed on 15% SDS-PAGE, followed by autoradiography.

seen in UV crosslinking and Western blot analysis of egg extracts, previously demonstrated to be due to phosphorylation (Walker et al., 1996; also see Fig. 1, lanes 2 and 3). However, the mixed lysate assay resulted in only partial modification of the in vitro translated protein. Addition of the kinase inhibitor 6-DMAP to the mixed lysate abolished this modification (Fig. 4A, lane 4), supporting our proposition that it is because of the physiologically relevant cdc2 kinase (Walker et al., 1996, and see below).

To confirm that the cloned cDNA did in fact encode clam p82 of predicted RNA-binding specificity, a recombinant His-tagged p82 was expressed in *Escherichia coli* with IPTG induction (see Materials and Methods for details), and the bacterial protein was subjected to UV crosslinking. We found that at least in *E. coli,* rp82 was poorly expressed and the minor soluble fraction did not bind well to Ni^{+}-NTA columns, whether the tag was N- or C-terminal, in native buffers (data not shown). Nevertheless, as shown in Figure 4B, we were able to examine RNA crosslinking of total bacterial lysate, alongside clam lysate, with both a ribonucleotide reductase masking element probe and a ribonucleotide reductase 3′ UTR probe that had p82 binding sites deleted (see Minshall et al., this issue). An *E. coli* protein of the size of p82 is indeed present in induced cultures when bacterial protein is crosslinked to positive (Fig. 4B, lane 1), but not to negative control RNA (Fig. 4B, lane 3). No protein of this size was present in uninduced cells (data not shown). The correct size of the in vitro translation product, its ability to be phosphorylated on incubation in activated clam egg extract, and its predicted RNA-binding specificity strongly suggest that the cDNA obtained from screening the library encodes the full-length masking element-binding protein p82.

Developmental expression of p82 polypeptide and mRNA

To examine the modification and regulation of p82 in vivo, samples of fertilized clam oocytes, taken at time intervals, were probed on Western blots with the anti-XCPEB serum. Figure 5A shows that p82 is fully phosphorylated by 30 min postfertilization at the time of meiosis I, and the phosphorylation state and the level of the protein is unchanged at 1 h, in the interphase I egg. However, by the first mitotic division at around 1.5 h, the level of p92 drops considerably and by 2 h postfertilization, no immune-reactive protein is present in the embryos. Neither p82 or p92 reappeared later in embryonic development. We detected neither polypeptide in somatic tissues from adult clams, such as the abductor muscle, the main foot, or gill, nor was it detected in clam sperm (data not shown). These results suggest that p82/p92 protein is only required during the very early development of the clam oocyte, up until the first mitotic cell division.

The size and the level of p82 mRNA during development were examined on a Northern blot of RNAs purified from the same staged cells as previously probed with a [^{32}P]-labeled DNA corresponding to part of the coding region of p82. Labeled DNA markers run in the same denaturing gel revealed that p82 mRNA is approximately 9 kb in length (Fig. 5B). As the mRNA encoding the small subunit of ribonucleotide reductase (known to be just over 1.7 kb long) migrated according to its size in this gel system (not shown) it seems unlikely that the astonishing size prediction for p82 mRNA is a result of incorrect gel running. In contrast to its encoded protein, p82 mRNA levels remain constant up until 4–5 h postfertilization. After this time, the RNA starts to be degraded and is not detected at 16 h postfertilization (Fig. 5B). This seems to be a characteristic

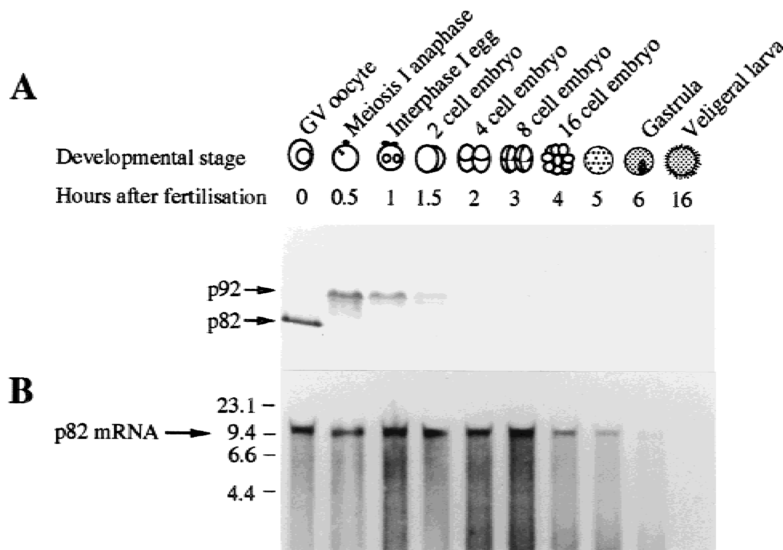

FIGURE 5. Developmental expression of clam p82 and mRNA. **A**: Western blot of extracts prepared from fertilized clam oocytes between 0–16 h, probed with rabbit anti-*Xenopus* CPEB serum. Equivalent numbers of oocytes and embryos were loaded (~2,000) for each time point. Positions of p82 and p92 and the approximate stage of development is indicated. **B**: Northern blot analysis of p82 mRNA during clam development. Total RNA was isolated from clam oocytes and developing embryos between 0–16 h, electrophoresed in a 1.5% agarose gel, blotted to nitrocellulose membrane, and hybridized with [^{32}P]-labeled p82 cDNA.

expression pattern for maternal mRNA (cyclin A, cyclin B, small subunit of ribonucleotide reductase) in this organism, in contrast to zygotic mRNAs (histone H3, tubulin) whose levels increase and remain relatively stable over this period of development (Westendorf, 1988). Although not seen particularly clearly in this blot, we noted on comparing independent samples of oocyte and egg RNAs a small shift indicative of shortening of p82 mRNA after fertilization, most probably because of deadenylation.

p82 phosphorylation precedes unmasking of maternal mRNA and temporally correlates with p34^{cdc2}/cyclin B kinase activation

p82 is phosphorylated in meiotically mature clam oocytes (Figs. 1, 5). To determine the timing of this modification more precisely, samples were withdrawn at frequent intervals from a fertilized oocyte culture for immunoblot analysis. Alterations in electrophoretic mobility indicative of phosphorylation first appear at the time the nucleus breaks down (germinal vesicle breakdown (GVBD), at 8 min postactivation) and are essentially complete by 16 min after fertilization (Fig. 6). As noted previously, the increase in size from p82 to p92 is step-wise and this may imply that more than one amino acid residue of p82 is being phosphorylated during oocyte activation. The total amount of p82 does not change significantly in the first 70–80-min postfertilization period (Fig. 6B and data not shown).

To examine the temporal correlation between p82 phosphorylation and the activation of translation of masked maternal mRNAs, the levels of cyclin A protein in this fertilized culture were determined by Western blotting. Detectable levels of newly synthesized cyclin A were first observed at ~16–18 min after fertilization (Fig. 6B, second panel), in agreement with previous observations (Swenson et al., 1986; Westendorf et al., 1989).

The precise timing of p34^{cdc2}/cyclin B kinase activation in this culture was assessed by incubating small-scale extracts with [γ^{32}P]-ATP and histone H1 (a preferred substrate for p34^{cdc2}/cyclin B kinase). H1 kinase activity is very low in unactivated oocytes, increases dramatically at ~6–8 min postfertilization, and remains at these high levels during the course of the experiment (Fig. 6B, third panel). In fertilized clam oocytes, p42^{MAPK} undergoes a transient, one-time activation, and this is coincident with its phosphorylation resulting in an apparent increase in size from 42 to 44 kDa (Shibuya et al., 1992). Immunoblotting of the same samples with anti-MAP kinase antibodies showed that p42^{MAPK} undergoes activation earlier than H1 kinase, at ~2 min postfertilization, and appears to be fully phosphorylated by 4 min (Fig. 6B, fourth panel), in agreement with previous studies (Shibuya et al., 1992). In comparing the two kinase activation time courses it seems most probable that a cyclin-dependent kinase is responsible for p82 phosphorylation.

Olomoucine, a cdc2 kinase inhibitor, delays p82 phosphorylation and translational activation of masked mRNA

Previously we showed that inhibitors of serine/threonine kinases prevent p82 phosphorylation in in vitro activated oocyte extracts. We also provided evidence, using recombinant p27^{Kip1}, a specific inhibitor of cyclin-dependent kinases (cdks), implicating cdc2 kinase (Walker et al., 1996). The timing of the activation of H1 kinase on oocyte activation (Fig. 6) also suggests that a cdk may be responsible for p82 phosphorylation in vivo.

The ATP analog olomoucine is one of the most specific cdk inhibitors known (Vesely et al., 1994). Olomou-

cine inhibits M-phase promoting factor activity in metaphase-arrested *Xenopus* egg extracts and the starfish oocyte G2/M transition in vivo and blocks plant cells at the G1/S and G2/M cell cycle transitions as well as various human cell lines at G1/S (Glab et al., 1994; Vesely et al., 1994). Because olomoucine is permeable to cells (Vesely et al., 1994), it was tested as a possible p82 kinase inhibitor to investigate the effects of this inhibition on translational activation.

Clam oocytes were preincubated for 5 min in seawater with olomoucine or the negative control compound dimethyl-olomoucine before being parthenogenetically activated. Control cells activated in the absence of inhibitors or in the presence of dimethyl-olomoucine completed nuclear breakdown by 8 min as normal. GVBD, however, was delayed by olomoucine in a dose-dependent manner. In the presence of the highest dose of olomoucine (200 μM), oocytes took ~30 min to complete GVBD (Fig. 7A).

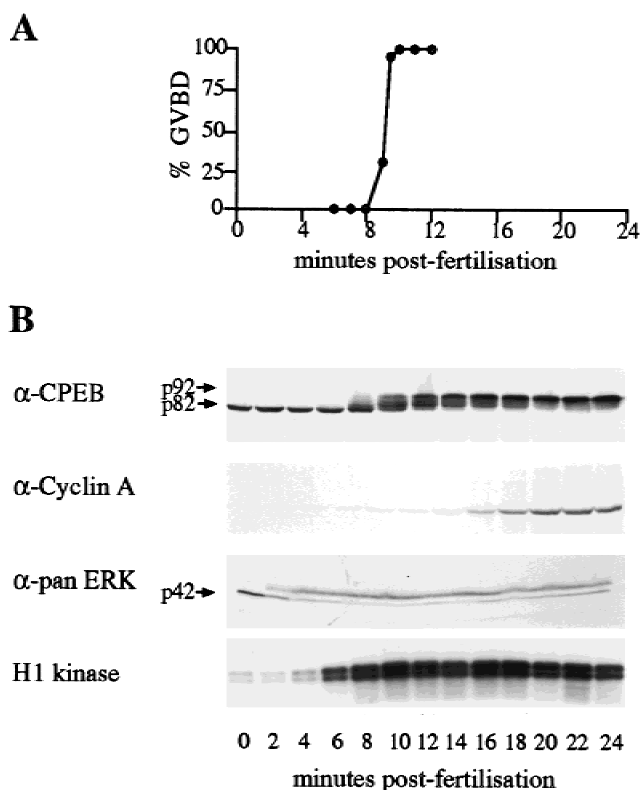

FIGURE 6. p82 is rapidly phosphorylated during meiotic maturation. A clam oocyte culture was fertilized and maintained at 19°C with constant stirring. **A**: Oocytes from the culture were monitored by light microscopy to assess the number of oocytes that had undergone germinal vesicle breakdown (GVBD), shown as percentage of total oocytes. **B**: At 2-min intervals after sperm addition, duplicate samples were withdrawn and prepared for Western blotting or H1 kinase assays. Aliquots containing ~2,000 cells were run on 10% polyacrylamide gels and transferred to nitrocellulose. The immunoblots were probed with antibodies directed against *Xenopus* CPEB, cyclin A, and ERK/MAP kinase and detected with alkaline phosphatase-linked secondary antibodies. H1 kinase assays were analyzed by 15% polyacrylamide gel electrophoresis and autoradiography.

The appearance of H1 kinase activity was delayed by olomoucine in a dose-dependent fashion to the same extent as the timing of GVBD (Fig. 7B). Western blotting of these samples with anti-CPEB antibodies showed that olomoucine delayed p82 phosphorylation in activated oocytes, also in a dose-dependent manner (Fig. 7C and data not shown). This delay in phosphorylation was slightly longer than the delay of GVBD, implying that a common factor is required, presumably $p34^{cdc2}$ kinase. The negative control compound, dimethyl-olomoucine, did not affect the appearance of H1 kinase or p82 phosphorylation (Fig. 7C, top panel). In contrast to its effect on H1 kinase, the activation of MAP kinase was largely unaffected by olomoucine, even at the highest concentration used (Fig. 7C, bottom panel).

To examine the effects of the delay of p82 phosphorylation on the translational activation of the masked maternal mRNAs, aliquots of oocytes from the time courses were blotted for analysis with anti-cyclin A serum. Translational activation of cyclin A was found to be delayed by olomoucine in a dose-dependent manner (Fig. 7C), with cyclin A protein appearing ~10 min after the phosphorylation of p82. These results are consistent with the proposal that p82 phosphorylation is upstream of, and is required for, the activation of cyclin A translation.

To confirm that the effect of olomoucine on the appearance of cyclin A protein during meiotic maturation was because of its inhibitory kinase action rather than on protein synthesis itself, olomoucine (200 μM) was added to mitoticly dividing clam embryos in the presence of [^{35}S]-methionine. Determination of the rates of protein synthesis showed that the kinase inhibitor did not effect incorporation in cells that had already undergone activation (data not shown). The effect of olomoucine on the activation of the translation of the masked mRNAs during meiotic maturation can therefore be attributed to its inhibition of cdk kinase.

DISCUSSION

The studies reported here lead to the following main conclusions. We show that clam p82 is a member of the CPEB family—the 82 kDa and the phosphorylated 92 kDa proteins cross-react with anti-XCPEB antibodies in both Western and immunoprecipitation assays. Furthermore, nucleic acid hybridization probes based on conserved domains of CPEB proteins led to the cloning of a cDNA that encoded a 82 kDa protein. The encoded protein behaved according to its identity in two independent assays—it was phosphorylated by clam egg kinase(s) in vitro and, more importantly, the recombinant polypeptide expressed in *E. coli* specifically bound the masking element of clam ribonucleotide reductase mRNA. Based on inhibitor studies, we conclude that clam p82 is subject to phosphorylation

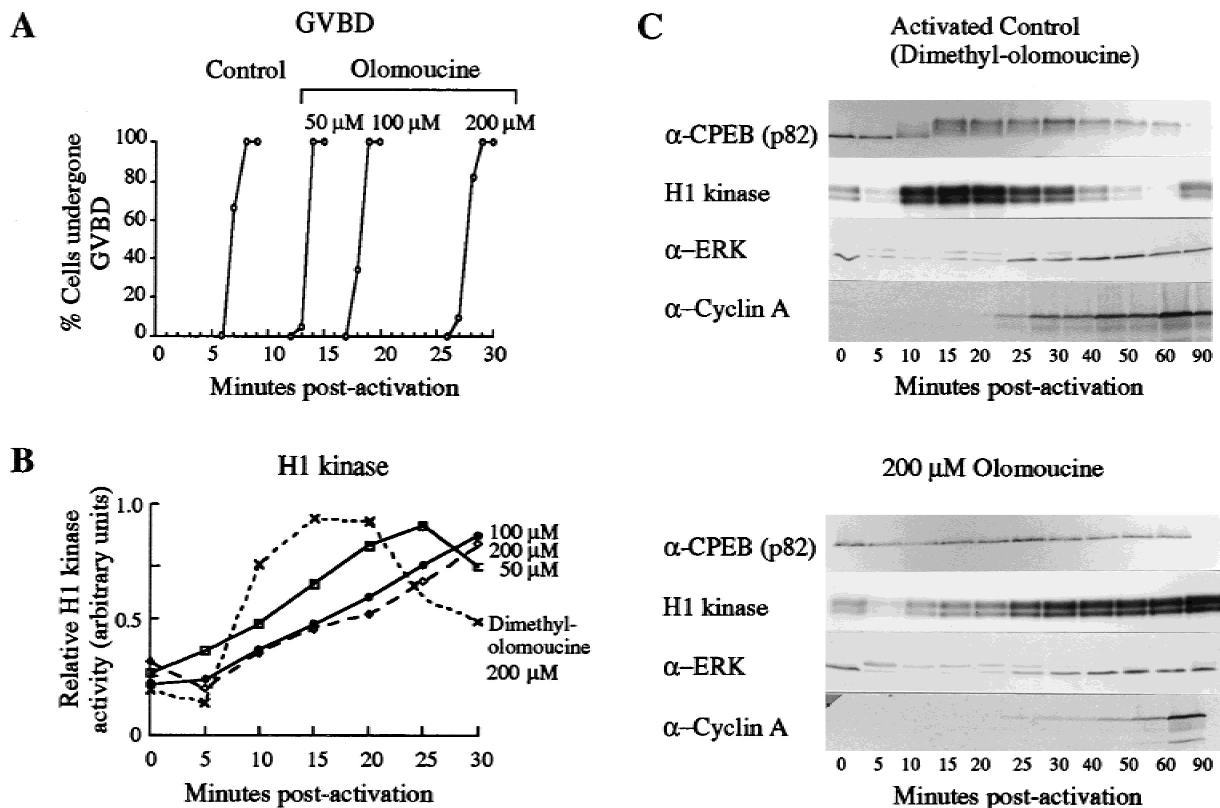

FIGURE 7. Olomoucine delays the phosphorylation of p82, activation of H1 kinase, and translation of cyclin A mRNA. Olomoucine and its negative control dimethyl olomoucine were added up to 200 μM final to two separate cultures that were parthenogenetically activated 5 min later by addition of 40 mM KCl. **A**: Oocytes were monitored microscopically to determine timing of GVBD. **B**: At indicated times, samples were withdrawn and analyzed for H1 kinase activity as in Figure 6 and subsequent densitometry. **C**: At indicated times, samples were withdrawn and analyzed by Western blotting with anti-XCPEB, cyclin A, and ERK antibodies as in Figure 6.

either directly or indirectly by cdc2 kinase in meiotically maturing oocytes. Last, we noted that clam p82 undergoes regulated proteolysis, following its phosphorylation, at about the time of the first mitotic division.

The CPEB family of proteins, several of which have been documented to bind CPE U-rich motifs, $U_{4-6}A_{1-2}U$ (Paris & Richter, 1990; Gebauer & Richter, 1996; Hake et al., 1998; Minshall et al., this issue; Fig. 4), share at their C-termini two tandem RRM domains followed by a set of eight conserved cysteine and histidines (Figs. 2 and 3). The order and conserved spacing of these cysteine and histidine residues in CPEB polypeptides, along with the established role for many zinc-coordinating proteins in binding to nucleic acids, suggests that this highly conserved protein domain forms two zinc-binding sites that mediate RNA binding in addition to that furnished by the RRMs. Indeed recent data indicate that both the RRMs, in particular RRM1, in addition to the nonstandard C_6H_2 zinc region, are necessary for specific RNA binding (Hake et al., 1998).

Although we have not undertaken a systematic study of the RNA-binding domains of p82 as yet, our initial data support those of Hake et al. (1998). We find that specific RNA binding requires the two RRMs and zinc finger region; a truncated p82 containing RRM1 alone is insufficient for RNA binding (G. Thom & N. Standart, unpubl.). Multiple-domain RNA-binding proteins are prevalent, whether containing several copies of one type (e.g., RRM or KH domains) or a mix of two types of domains (e.g., KH and RGG or RRM and KH; Burd & Dreyfuss, 1994; Siomi & Dreyfuss, 1997). While such multiplicity contributes to the specific RNA-binding characteristics of each protein, it is also worth noting that an RRM alone can mediate protein–protein binding (Scherly et al., 1990).

At present it is not known whether *Drosophila* orb or the CPEB-related *C. elegans* proteins bind RNA, though it is a fair assumption given their sequence homology in the RNA-binding region to documented RNA-binding proteins. In this case, it would be interesting to identify their RNA targets, to aid our understanding of the role of CPEB proteins in regulating RNA expression (see below).

p82/CPEB is subject to regulated proteolysis, principally during first mitosis (Fig. 5 and data not shown), at about the time that clam cyclins are destroyed (Hunt et al., 1992). The N-terminal halves of p82 and CPEBs share two short motifs, A and B (Figs. 2 and 3). We

note that motif A bears a loose homology to the cyclin destruction box, which is necessary for proteolysis by the ubiquitin-mediated pathway and whose conserved features are RXALGXIXN (Funabiki et al., 1996; Yamano et al., 1996). Although originally defined in cyclins, the destruction box has been identified in other proteins, such as fission yeast Cut2, whose degradation is required for sister chromatid separation (Funabiki et al., 1996). While the location and overall sequence of the conserved CPEB A motif is reminiscent of the cyclin destruction box, including the absolutely invariant R residue and the terminal N residue, the second most conserved residue L is not present in p82/CPEB, which instead has an A. The one known deviation among the cyclins is B3 which has an F in place of L (Yamano et al., 1996). Motif B and its flanking regions resemble PEST sequences that are commonly found in proteins that are unstable. These polypeptide sequences are enriched in proline, glutamic acid, serine, and threonine, which target proteins for rapid destruction (Rechsteiner & Rogers, 1996). Indeed, in the *Xenopus*, mouse, and goldfish proteins, this region scored very highly using a PEST-FIND computer program (Rechsteiner & Rogers, 1996).

Experiments in vitro (Walker et al., 1996) and in vivo (this article and data not shown) strongly suggest that p82 is a major cdc2 kinase substrate, with several phosphorylation sites. Recent experiments provide additional evidence that both cdc2 and MAP kinase activity is required for full p82 phosphorylation; with MAP kinase modification preceding and "priming" protein for cdc2 phosphorylation (Y. Katsu, N. Minshall, Y. Nagahama, and N. Standart, submitted). The precise number and identity of phosphorylation sites in p82 is under mutagenesis investigation; our current state of knowledge indicates that the five sites referred to in Figure 2, while playing a part in p82 phosphorylation, do not account for complete modification (G. Thom & N. Standart, unpubl.). We speculate that, as in the case of poly(A) polymerase, we may need to extend our identification to include nonconsensus cdc2 sites (Colgan et al., 1998). Our interest in identifying these residues is to generate mutant proteins that cannot be modified, for example in injected maturing *Xenopus* oocytes, and that may reveal a role for this modification, for example in protein degradation and/or RNA-binding.

On progesterone activation of *Xenopus* oocytes, CPEB is phosphorylated directly or indirectly by a cdc2-type kinase at the time of GVBD, presumably at a smaller number of sites than p82, as the apparent size increase is only 2 kDa or so (Paris et al., 1991; Hake & Richter, 1994; de Moor & Richter, 1997). The levels of both CPEB protein and its mRNA decrease by about 75% in the activated egg (Hake & Richter, 1994). Indeed, CPEB persists in embryos up to the gastrula stage and this is presumed to be functionally significant for polyadenylation (Hake & Richter, 1994). In frog oocytes there is strong evidence that cdc2 activation requires active MAPK, with MAPK appearing to act upstream of cdc2 in a mos-dependent way (Nebreda & Hunt, 1993; Posada et al., 1993; Huang & Ferrell, 1996). In contrast, mos does not appear to play a role during clam meiotic maturation and if a mos homolog does exist in the clam oocyte, it possibly plays a role in DNA replication (J. Ruderman, pers. comm.). The role of CPEB phosphorylation in *Xenopus* is not known, although it has been ruled out as a significant factor in the polyadenylation of class I (i.e., mos-independent) mRNAs (Ballantyne et al., 1997; de Moor & Richter, 1997).

In Minshall et al. (this issue) we show that clam p82 is a functional homolog of *Xenopus* CPEB, but also that in addition to its role in cytoplasmic polyadenylation in the activated egg, it functions as a translational repressor in the oocyte. Future studies addressing the stage-specific modification of p82 by phosphorylation and subsequent proteolysis may help our understanding of the dual function of clam p82/CPEB in regulating maternal mRNA expression in early development.

MATERIALS AND METHODS

6-DMAP was obtained from Sigma. Olomoucine (2-(2-hydroxyethylamino-6-benzylamino-9-methylpurine) and the negative control compound dimethylamino-olomoucine (2-dimethylaminoethylamino-6-benzylamino-9-methylpurine) were from LC Laboratories.

Preparation of clam and *Xenopus* lysates and collection of clam tissues

Surf clams (*Spisula solidissima*) were collected at the Marine Biological Laboratory in Woods Hole, Massachusetts, USA. In vitro fertilization and monitoring development of the eggs/embryos by staining with lacto-orcein was described previously (Hunt et al., 1992). The detailed preparation of postmitochondrial extracts from clam oocytes and from oocytes parthenogenetically activated by 40 mM KCl was according to Walker et al. (1996). Small-scale extracts were prepared by homogenizing cells on ice in T buffer (pH 6.8) by repeatedly pipetting 10 times through a $27\frac{1}{2}$-gauge needle attached to a 1 mL syringe and then centrifuging at 14,000 rpm for 5 min at 4 °C. The procedure of Dessev et al. (1989) was used to separate clam oocyte nuclear and cytoplasmic fractions. *Xenopus* CSF-arrested egg lysates were made as described (Murray, 1991).

Adult tissues were obtained from clams by dissection and washed thoroughly in millipore-filtered sea water to ensure that no gametes were present before being homogenized with an Omni mixer in T buffer (pH 6.8). Protein content of S10 lysates was determined by the Bradford assay (Bradford, 1976).

Cdc2 kinase assays using calf thymus histone H1 (Boehringer Mannheim) were performed as previously described

(Walker et al., 1996). Autoradiographs were analyzed on an ImageQuant for quantitation of bands.

Immunoblotting and immunoprecipitation

Protein samples were separated on 15% polyacrylamide-SDS gels and processed for Western blotting (Walker et al., 1996). Antibody dilutions were as follows: rabbit polyclonal anti-XCPEB and preimmune sera (1:10,000), mouse monoclonal antibody α-pan ERK (Transduction Laboratories) was used at 1:200, affinity purified anti-cyclin A (generous gift of K. Swenson; 1:10,000); and goat anti-rabbit or anti-mouse alkaline phosphatase-conjugated antibodies (both Sigma, dilution 1:6,000). The blots were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (Harlow & Lane, 1988).

UV-crosslinked, ^{32}P-labeled proteins were immunoprecipitated essentially according to Borman et al. (1993). UV-crosslinking reactions were performed as described below in 3 wells of an Eliza plate (with the omission of heparin), pooled, and diluted in 100 μL NETS buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.05% NP 40). Following the addition of 2 μL of anti-XCPEB or preimmune sera, the tubes were incubated for 1 h at 4 °C with constant agitation. Ten microliters of protein A-Sepharose 6MB beads (Pharmacia) were then added and incubation continued at 4 °C for 1 h. The beads were washed three times with NETS buffer before being resuspended in 2 $\times$ SDS sample buffer and boiled for 5 min. The samples were analyzed by 15% polyacrylamide-SDS electrophoresis and autoradiography.

UV crosslinking

Transcription and UV crosslinking was carried out essentially as in Walker et al. (1996). The cDNA subclones of the RR 3′ UTR containing the masking element (nt 83–216) region and the 3′ terminal region negative for p82 binding (nt 305–454, Δf) are described in detail in Minshall et al. (this issue). The selected region of the *Xenopus* B4 RNA 3′ UTR (nt 1128–1158) was described previously (Paris & Richter, 1990).

Cloning and sequencing p82 cDNA

Degenerate primers were based on the conserved motifs flanking the second RRM region of the *Xenopus* CPEB, *Drosophila* orb, and the *C. elegans* C40H1.1 cDNA sequences. *Eco*R I and *Bam*H I restriction sites (underlined) were included in the upstream and downstream oligonucleotide primers, respectively, to aid subsequent subcloning into pGEM1. The sequences of the oligonucleotides with degenerate positions enclosed in parentheses were upstream oligo 5′GC GGAATTCGT(N)(G/C)A(R)GT(N)AT(C/T/A)CC(N)TGG3′ and downstream oligo 5′GCGGGATCCTG(P)TGCCA(N)T(GC) CCA(R)CA3′. These primers were used in PCR reactions with a temperature profile of 95 °C (30 s), 45 °C (30 s), and 72 °C (60 s) for 30 cycles. Template DNA was produced by random hexamer-primed reverse transcription of total clam oocyte RNA using the First Strand cDNA Synthesis Kit (Boehringer Mannheim).

p82 cDNAs were isolated from a *Spisula* ovarian cDNA library constructed in λgt22, generously provided by R.E. Palazzo and G. Peng (University of Kansas), in which the cDNA inserts are tailed at the 5′ end with *Sal* I and with *Not* I at the 3′ end. A DNA probe was obtained from the subcloned RT-PCR product by labeling with [α-^{32}P] dCTP using the PrimeIt II Random Primer Kit (Stratagene). Plating and screening of the library and the isolation of plaque pure phage DNA were according to standard techniques (Ausubel et al., 1995). The 2.8 kbp cDNA (GenBank accession number AF091712) was sequenced on both strands by John Lester of Cambridge University's Department of Biochemistry using an Applied Biosystems 373 DNA Sequencer, following ligation of the insert into *Sal* I, *Not* I-restricted pSport 1 (Gibco-BRL). *Not* I-linearized pSportp82 cDNA was transcribed in vitro with T7 RNA polymerase and the capped mRNA translated in the rabbit reticulocyte lysate cell-free system (Kaminski & Standart, 1996).

Recombinant His-tagged p82

The p82 expression plasmid was constructed by inserting the open reading frame of p82 as an *Nde*I–*Xho*I fragment into pET21b (Novagen). The cDNA was produced by PCR from pSportp82, using oligonucleotides 5′ GCG CGC CCA TAT GTC AGC TGT G 3′ and 5′ TGA GAA AGC TCG AGT AAG GCC A 3′. The amino acid sequence of the bacterially expressed p82 differed from the clam expressed protein by the addition of a C-terminal extension with the sequence EHH HHHH. A culture of transformed BL21(DE3) was grown to an A_{600} of ~0.3 and induced for 4 h at 37 °C and 300 rpm by addition of IPTG to 0.4 mM. Cells were harvested by centrifugation at 5,000 rpm for 5 min and resuspended in 0.01–0.02 vol. 300 mM NaCl, 50 mM sodium phosphate buffer, pH 7.8. Cells were lysed using a french pressure cell and subsequent addition of 1% v/v Triton-X100.

Northern blotting

Phenol-extracted RNA samples were treated with glyoxal and DMSO and separated by electrophoresis through a vertical 1.4% agarose gel in recirculating 10 mM sodium phosphate buffer (pH 6.9), 90 V for 1 h. Following transfer to Hybond-N, RNA was fixed by UV crosslinking. Hybridization with random primed labeled probe (p82 ORF 673-nt-long *Bgl* II fragment) was carried out at 65 °C for 16 h. Blots were washed twice for 45 min at 60 °C in 0.1% w/v SDS, 0.1 $\times$ SSC prior to detection by autoradiography. To ensure that equivalent amounts of RNA were loaded per lane, the same RNA samples were run on a gel that was then stained with ethidium bromide to visualize the ribosomal RNA.

NOTE ADDED IN PROOF

While this paper was under review, a study of the cloning and characterization of the zebrafish homolog of *Xenopus* CPEB was published (Bally-Cuif et al., 1998, *Mech Dev 77*:31–47). Zebrafish CPEB is very homologous to vertebrate CPEBs and contains motifs A and B as well as the highly conserved RNA-binding domains.

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