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CPEB phosphorylation and cytoplasmic polyadenylation are catalyzed by the kinase IAK1/Eg2 in maturing mouse oocytes

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SUMMARY

In both vertebrates and invertebrates, the expression of several maternal mRNAs is regulated by cytoplasmic polyadenylation. In *Xenopus* oocytes, where most of the biochemical details of this process have been examined, polyadenylation is controlled by CPEB, a sequence-specific RNA binding protein. The activity of CPEB, which is to recruit cleavage and polyadenylation specificity factor (CPSF) and poly(A) polymerase (PAP) into an active cytoplasmic polyadenylation complex, is controlled by Eg2-catalyzed phosphorylation. Soon after CPEB phosphorylation and resulting polyadenylation take place, the interaction between maskin, a CPEB-associated factor, and eIF4E, the cap-binding protein, is destroyed, which results in the recruitment of mRNA into polysomes. Polyadenylation also occurs in maturing mouse oocytes, although the biochemical events that govern the reaction in these cells are not known. In this study, we have examined the phosphorylation of CPEB and have assessed the necessity of this protein for polyadenylation in maturing mouse oocytes. Immunohistochemistry has revealed that all the factors that control polyadenylation and translation in *Xenopus* oocytes (CPEB, CPSF, PAP, maskin, and IAK1, the murine homologue of Eg2) are also present in the cytoplasm of mouse oocytes. After the induction of maturation, a kinase is activated that phosphorylates CPEB on a critical regulatory residue, an event that is essential for CPEB activity. A peptide that competitively inhibits the activity of IAK1/Eg2 blocks the progression of meiosis in injected oocytes. Finally, a CPEB protein that acts as a dominant negative mutation because it cannot be phosphorylated by IAK1/Eg2, prevents cytoplasmic polyadenylation. These data indicate that cytoplasmic polyadenylation in mouse oocytes is mediated by IAK1/Eg2-catalyzed phosphorylation of CPEB.

Key words: Polyadenylation, Oocyte maturation, CPEB, Mouse, IAK1

INTRODUCTION

In early animal development, the temporal and spatial expression of maternally inherited mRNA is necessary for processes as diverse as oocyte maturation (Sheets et al., 1994; Stebbins-Boaz et al., 1996; Mendez et al., 2000a), embryonic cleavage (Groisman et al., 2000; Uto and Sagata, 2000) and establishment of the body plan (Simon et al., 1996; Wu et al., 1997; Schroeder et al., 1999; Carrera et al., 2000). One mechanism responsible for the translational activation of several maternal messages is cytoplasmic polyadenylation, which has been studied most extensively in *Xenopus* oocytes. In this species, several mRNAs that are dormant in oocytes have short poly(A) tails, usually ~20 nucleotides in length. After the induction of oocyte maturation by progesterone, some of these mRNAs undergo cytoplasmic polyadenylation-induced translation (McGrew et al., 1989; Sheets et al., 1995; Stebbins-Boaz et al., 1996). Two *cis* elements in the 3′ untranslated region of responding mRNAs direct cytoplasmic polyadenylation, the cytoplasmic polyadenylation element (CPE) and the polyadenylation hexanucleotide AAUAAA (Fox et al., 1989; McGrew et al., 1989). The CPE is bound by CPEB, a zinc finger and a RNA recognition motif (RRM)-containing protein (Paris et al., 1991; Hake and Richter, 1994; Hake et al., 1998), while the hexanucleotide is bound by a cytoplasmic form of cleavage and polyadenylation specificity factor (CPSF) (Bilger et al., 1994; Dickson et al., 1999). The stimulation of polyadenylation is most probably due to the kinase Eg2, which is activated soon after oocytes are exposed to progesterone (Andresson and Ruderman, 1998). Eg2 phosphorylates CPEB Ser174, which resides within an LDS/TR motif that is conserved among other vertebrate CPEB proteins (Mendez et al., 2000a). Once phosphorylated on this residue, CPEB recruits CPSF and poly(A) polymerase (PAP) into an active polyadenylation complex (Mendez et al., 2000b).

While the core features of cytoplasmic polyadenylation have been elucidated, they do not indicate a priori how polyadenylation stimulates translation. The mechanism of translational activation was suggested by initial studies demonstrating that the CPE, which is necessary for polyadenylation during maturation, also mediates translational repression (masking) prior to maturation (de Moor and Richter, 1999). The studies implied that CPEB, the only factor identified that could bind the CPE, activated and repressed

translation. How CPEB could act both positively and negatively was indicated when maskin, a CPEB-interacting factor, was identified (Stebbins-Boaz et al., 1999). In oocytes, maskin not only binds CPEB, but also the cap-binding protein eIF4E. This configuration of factors excludes the interaction between eIF4E and eIF4G, which is essential for cap-dependent translation (for a discussion of initiation factors, see Gingras et al., 1999). During oocyte maturation at a time commensurate with polyadenylation, the interaction between maskin and eIF4E is disrupted, which presumably then allows eIF4G to bind eIF4E and promote initiation (Stebbins-Boaz et al., 1999). It seems plausible that polyadenylation could be involved in the dissociation of maskin from eIF4E. It should also be borne in mind that polyadenylation might also stimulate translation in a maskin-independent manner, for example, by inducing cap-specific 2′-*o*-methylation (Kuge and Richter, 1995).

Cytoplasmic polyadenylation also takes place in maturing mouse oocytes, where at least some of the key features are similar to those described in *Xenopus*. For example, the CPE and AAUAAA drive polyadenylation-induced translation (Vassalli et al., 1989; Huarte et al., 1992, Gebauer et al., 1994; Tay et al., 2000; Oh et al., 2000). In addition, the CPE (also referred to as the ACE, or adenylation control element) also represses translation before maturation (Stutz et al., 1997; Stutz et al., 1998; Tay et al., 2000), as is the case in *Xenopus*. However, aside from the fact that mouse oocytes contain CPEB (Gebauer and Richter, 1996; Tay et al., 2000), and that polyadenylation is important for meiotic progression (Gebauer et al., 1994; Tay et al., 2000), little is known about the biochemical features of the process in these species.

In this study, we have analyzed the molecules that mediate cytoplasmic polyadenylation in maturing mouse oocytes. All the factors that are involved in this process in *Xenopus* (CPEB, CPSF, PAP, maskin, IAK1/Eg2) are also present in the cytoplasm of mouse oocytes. After the induction of oocyte maturation, a kinase becomes active that phosphorylates CPEB Ser174, an event that is crucial for cytoplasmic polyadenylation. The injection of a peptide that is known to block the activity of IAK1/Eg2 impedes meiotic progression. Furthermore, the injection of an mRNA encoding a truncated form of CPEB, which acts as a dominant negative mutation because it cannot be phosphorylated by IAK1/Eg2, inhibits cytoplasmic polyadenylation. These data indicate that IAK1/Eg2 and CPEB are essential components for cytoplasmic polyadenylation in mouse oocytes.

MATERIALS AND METHODS

Collection and culture of oocytes

Germinal vesicle (GV) stage cumulus-enclosed oocytes from 19-21 day CD-1 mice (Harlan Sprague-Dawley) were isolated in phosphate-buffered saline containing isobutylmethylxanthine (IBMX, 0.15 mM) and polyvinylpyrrolidone (3 mg/ml; M_r, 40,000), and the cumulus cells removed by mechanical shearing. After collection, the oocytes were washed with M16 medium supplemented with 10% fetal calf serum and 0.3% bovine serum albumin.

Analysis of polyadenylation

[^{32}P]UTP-labeled murine cyclin 3′ UTR (plus or minus the CPE), radioinert *Xenopus* full-length wild-type CPEB and ΔN CPEB, which lacks residues 1-146 (Mendez et al., 2000a), were synthesized in vitro by T3 or T7 RNA polymerase. Depending on the experiment, the labeled RNA (~6×10^6 cpm/µl) alone was injected into GV stage oocytes, or was mixed with the unlabeled RNAs (0.7 mg/ml, equal volumes) before injection (~10 pl per injection). The oocytes were cultured in the presence of isobutylmethylxanthine (IBMX) for 6 hours, after which time they were washed in IBMX-free medium and cultured for an additional 16-17 hours. RNA was then extracted from the oocytes according to Tay et al. (Tay et al., 2000), and the RNA was resolved on a 6% polyacrylamide gel containing 8 M urea and phosphorimaged.

Analysis of polyadenylation factors

Whole-mount immunohistochemistry was performed as described by Messinger and Albertini (Messinger and Albertini, 1991). Briefly, oocytes were fixed in 100 mM Pipes, 5 mM MgCl, 2.5 mM EGTA, 0.1% aprotinin, 1 mM DTT, 50% D_2O, 1 µM taxol, 0.1% Triton X-100 and 2% formaldehyde for 20 minutes at 37°C, and then blocked in phosphate-buffered saline containing 2% BSA, 2% powdered milk, 2% goat serum and 0.1% Triton X-100 for 30 minutes at room temperature. The antibodies (1:100 dilution) directed against murine CPEB (Tay et al., 2000), *Xenopus* maskin (Stebbins-Boaz et al., 1999), CPSF100 (Mendez et al., 2000b), IAK1 (Transduction Laboratories) and *Xenopus* PAP (Gebauer and Richter, 1995) were added to the blocking buffer and the oocytes were incubated at 37°C for 1 hour with gentle rotation. After washing with the blocking solution, the secondary fluorophore-labeled antibody (1:1000), which was either Alexa 488 goat anti-mouse IgG or Alexa 594 goat anti-rabbit IgG (Molecular probes) was added, again in blocking buffer. The oocytes were also simultaneously stained with DAPI. Western blots were probed with the antibodies noted above; for CPEB, 110 GV oocytes were analyzed, 250 oocytes for maskin, 535 oocytes for CPSF100, 180 oocytes for IAK1 and 200 oocytes for poly(A) polymerase.

Analysis of CPEB phosphorylation

Peptides spanning a region from murine CPEB that contains the LDS/TR motif that is recognized by IAK1/Eg2 (RGSRLDTRPILDSRSSC), or a mutant sequence with alanine for serine substitutions (RGSRLDARPILDARSSC), which cannot be phosphorylated by this kinase (Mendez et al., 2000a) were coupled to maleimide-activated ovalbumin (Pierce). The conjugates were added to extracts prepared as follows. 50 GV, GVBD (MI) or MII stage oocytes were frozen and thawed three times in 4.25 µl of buffer (Hampl and Eppig, 1995a) that contained 125 mM Mops, pH 7.2, 300 mM β-glycerophosphate, 2 mg p-nitrophenylphosphate, 7.5 mM EGTA, 0.5 mM Na_3VO_4, 75 mM MgCl, 5 mM dithiothreitol, 10 µg/ml each of aprotinin, soybean trypsin inhibitor, pepstatin A and leupeptin, and 25 µg BSA). After brief centrifugation, 10 µl of wild-type or mutant peptide conjugated to ovalbumin was to the supernatant, together with 1 µCi [γ-^{32}P]ATP 1 mM phenylmethylsulfonyl fluoride, and 0.025 µg protein kinase A inhibitor (final reaction volume of 30 µl). The solution was incubated for 1 hr at 30°C and then analyzed by 12% PAGE.

For the phosphopeptide mapping, purified baculovirus-expressed *Xenopus* CPEB was added to extracts made as described above, but in this case from 600 MI stage oocytes. The entire extract was then resolved by 8% SDS PAGE and CPEB was electroblotted onto nylon membrane and digested with trypsin. The 2D phosphopeptide mapping procedure has been described (Boyle et al., 1991; Mendez et al., 2000a). In addition, CPEB was phosphorylated in vitro with purified baculovirus expressed *Xenopus* Eg2, and the phosphopeptide map was similarly derived.

Some oocytes were injected with ovalbumin-coupled wild-type and mutant peptides (as noted above). In this case, the peptides were brought up to a concentration of ~5 mg/ml, and ~10 pl was injected per oocyte. The oocytes were incubated for up to 10 hours, and were scored for the presence or absence of the first polar body.

RESULTS

Polyadenylation and meiotic progression

Cytoplasmic polyadenylation of cyclin B1 mRNA takes place during mouse oocyte maturation and is detectable as early as metaphase I, which occurs within 6 hours of culture (Tay et al., 2000). While the assay used to detect this polyadenylation is sensitive (the PCR-based PAT assay, Salles et al., 1994), it can miss some poly(A) lengthening if the existing poly(A) tail on the substrate mRNA is under ~20 nucleotides, which is too short to anneal with oligo (dT) and thus would not be reverse transcribed. Consequently, we injected ^{32}P-labeled cyclin 3′ UTR that was devoid of an existing tail into oocytes, and directly determined polyadenylation by gel electrophoresis and phosphorimaging. Fig. 1 shows that polyadenylation of the CPE-containing RNA was detectable as early as three hours post-injection. An identical RNA that had the CPE deleted did not undergo cytoplasmic polyadenylation. Therefore, cytoplasmic polyadenylation occurs before germinal vesicle breakdown, and implies that all the factors that are necessary for this process are stored in the cytoplasm of oocytes.

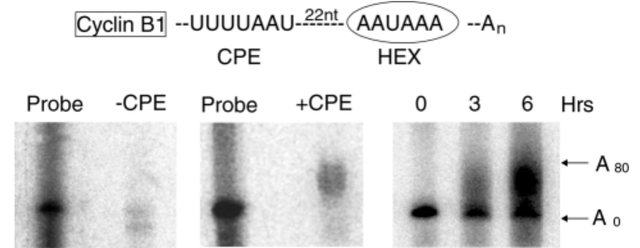

Fig. 1. Polyadenylation in maturing mouse oocytes. Radiolabeled 3′ UTR of cyclin B1 mRNA, which contains a CPE and polyadenylation hexanucleotide (top) was injected into GV stage oocytes that were then allowed to mature for 16.5 hours (center), or for 3 (GV intact) and 6 (GVBD) hours (right). The RNA was extracted and assessed by denaturing PAGE and phosphorimaging. The approximate size of the newly acquired poly(A) tail is indicted. As a control, a mutated cyclin B1 3′ UTR that did not contain a CPE was also injected and the oocytes were analyzed after 16.5 hours of culture. For this experiment, the left panel represents 21 oocytes (–CPE), the middle panel 87 oocytes (+CPE), and the right panel 31 (0 hours), 27 (3 hours) and 25 (6 hours) oocytes. All analyses were performed twice.

Multiple polyadenylation factors are cytoplasmic in mouse oocytes

Polyadenylation-induced translation in *Xenopus* oocytes requires CPEB, CPSF, PAP, maskin and the kinase Eg2, whose murine homolog is IAK1 (Stk6 – Mouse Genome Informatics). In full grown oocytes, CPEB and maskin are localized to the animal pole while in embryos these factors, as well as Eg2, are found on spindles and/or centrosomes (Groisman et al., 2000; Giet and Prigent, 1999). Of these factors, only CPEB has been linked with cytoplasmic polyadenylation in mouse oocytes, and none have been examined for localization (Gebauer and Richter, 1996; Tay et al., 2000). To assess whether these factors are present in the cytoplasm of mouse oocytes and, if so, whether they are localized in patterns similar to those observed

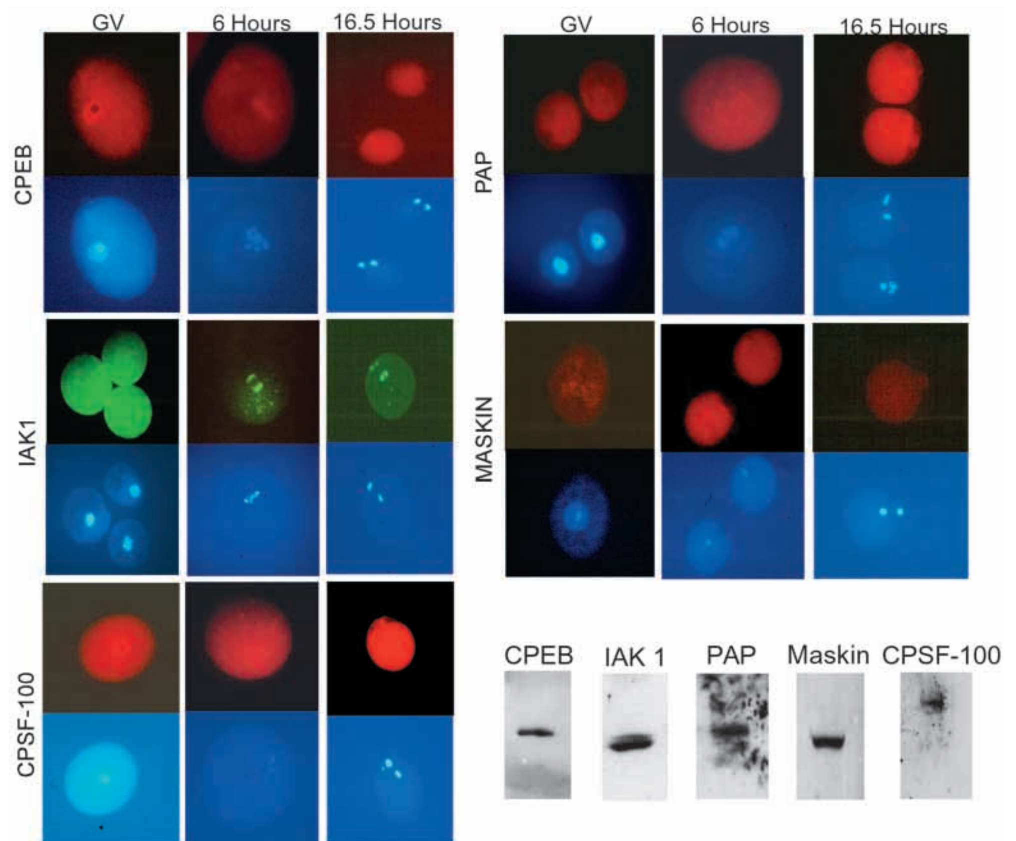

Fig. 2. Immunohistochemistry of polyadenylation/translation factors in maturing mouse oocytes. Oocytes with an intact GV, or those cultured for 6 (usually at MI), or 16.5 (MII) hours were immunostained with antibodies directed against CPEB, IAK1 (Eg2), the 100 kDa subunit of CPSF, PAP and maskin (the signals for these proteins are red; IAKI is in green). The oocytes were also counterstained with DAPI (blue). Each immunostaining procedure was performed on at least 30 oocytes. Western blots of GV stage oocyte protein were probed with the same antibodies noted above; in each case, only a single immunoreactive species was observed. No signal was observed in either the immunofluorescence experiment or in the western blot experiment when preimmune serum was used as the source for the first antibody (data not shown).

in *Xenopus* oocytes, whole-mount immunohistochemistry was performed. Fig. 2 shows that CPEB immunoreactivity was detectable at all stages of oogenesis throughout the oocyte. IAK1/Eg2 was also detected throughout the oocyte at the GV stage, but at MI and MII, it was virtually exclusively localized to spindles. This observation is not unexpected because members of this family of kinases are often spindle-associated (Nigg, 2001). CPSF100, PAP and maskin immunoreactivities were observed thought the oocyte at all stages of maturation (Fig. 2). Western blots of GV stage extracts probed with the antibodies noted above demonstrate that only a single species of protein was detected in each case. With one exception, the sizes of these immunoreactive species were those predicted based on experiments performed with *Xenopus* material; the size of the maskin immunoreactive band, however, was somewhat smaller in the mouse, 120 kDa versus 150 kDa in *Xenopus*. The data in Fig. 2 show that all the factors that are thought to play key roles in polyadenylation-induced translation are stored in the cytoplasm of oocytes before maturation. However, in contrast to the situation in *Xenopus* oocytes (Groisman et al., 2000), these results demonstrate that there is little subcellular localization of these factors in mouse oocytes.

Phosphorylation of CPEB

The Eg2 catalyzed phosphorylation of *Xenopus* CPEB Ser174 is necessary for cytoplasmic polyadenylation (Mendez et al., 2000a). All known vertebrate CPEB proteins contain two copies of the highly conserved LDS/T*R motif (Fig. 3A), the phosphorylation sequence for IAK1/Eg2 (Mendez et al., 2000a). Having demonstrated that mouse oocytes contain IAK1/Eg2, we sought to determine whether murine CPEB is also phosphorylated on this critical residue and, if so, whether it is necessary for cytoplasmic polyadenylation. However, because of the practical restriction in obtaining sufficient quantities of maturing mouse oocytes for 2D phosphopeptide mapping of endogenous CPEB, we have taken an alternative approach. That is, while extracts of maturing oocytes have detectable levels of kinase activity (see below), the putative endogenous substrate (i.e. CPEB) is present in too limited an amount for biochemical analysis. Consequently, we have supplemented extracts with either a peptide derived from CPEB, or *Escherichia coli*-expressed CPEB, which would serve as substrates for the phosphorylation reaction. In *Xenopus* oocytes, this approach has shown that exogenous and endogenous CPEB substrate proteins are phosphorylated in an identical manner (Mendez et al., 2000a).

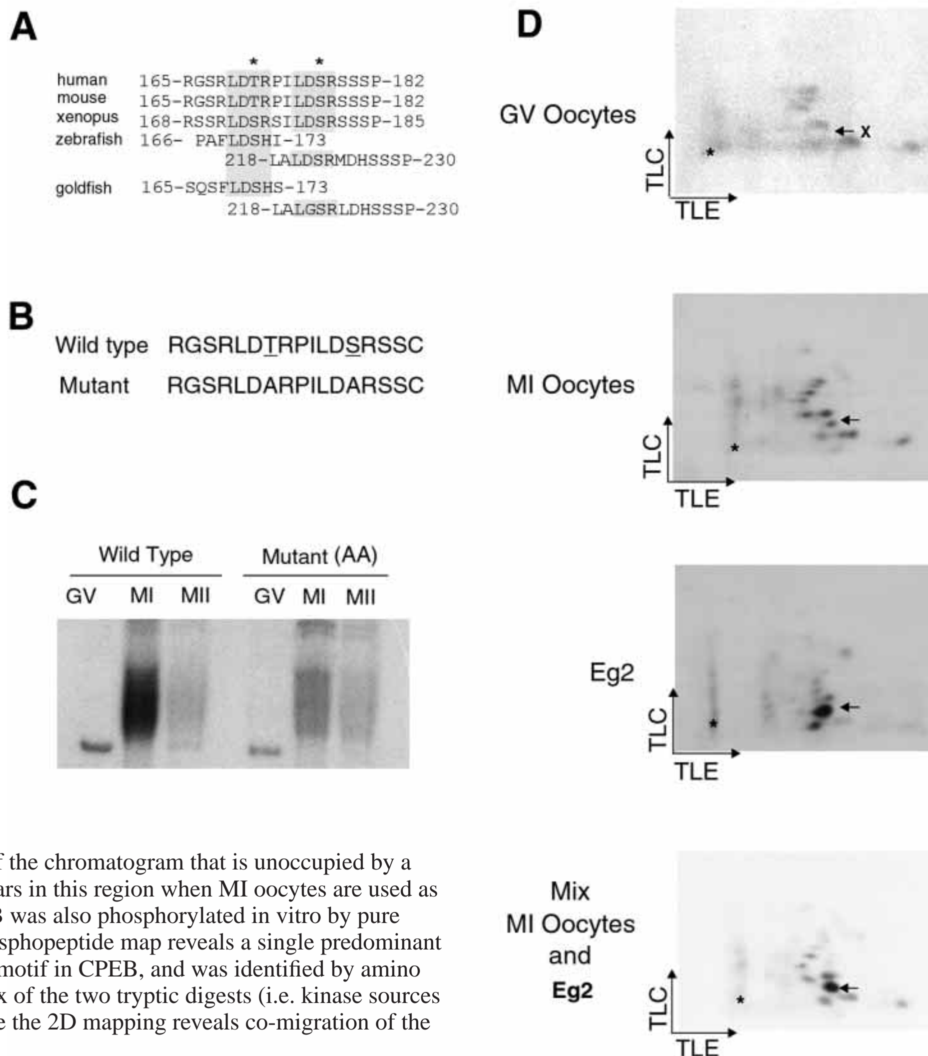

Fig. 3. Phosphorylation of CPEB. (A) A selected alignment of vertebrate CPEB proteins containing the two conserved LDS/TR motifs. Asterisks denote consensus IAK1/Eg2 phosphorylation sites. (B) Sequences of wild-type and mutant IAK1/Eg2 substrate peptides derived from mouse CPEB. The C-terminal cysteine is not found in CPEB, but was added to the peptide so it could be coupled to ovalbumin. (C) Phosphorylation of substrate peptide during mouse oocyte maturation. The peptides noted above were coupled to ovalbumin and added to extracts prepared from GV, MI and MII stage mouse oocytes that were supplemented with [γ-^{32}P]ATP. After incubation, the radioactive products were analyzed by SDS-PAGE. (D) 2D phosphopeptide mapping of CPEB. *Escherichia coli*-expressed *Xenopus* CPEB was added to extracts prepared from GV or MI stage mouse oocytes that were supplemented with [γ-^{32}P]ATP. After incubation, the products were resolved by SDS-PAGE and CPEB was electroblotted onto PVDF membrane. CPEB was then digested with TPCK-treated trypsin and the resulting phosphopeptides were resolved in two dimensions. The asterisk denotes the origin. In the top panel, 'X' denotes an area of the chromatogram that is unoccupied by a phosphopeptide. A new phosphopeptide appears in this region when MI oocytes are used as the kinase source. Purified recombinant CPEB was also phosphorylated in vitro by pure baculovirus-expressed Eg2. The resulting phosphopeptide map reveals a single predominant spot (arrow) corresponding to the first LDSR motif in CPEB, and was identified by amino acid sequencing (Mendez et al., 2000a). A mix of the two tryptic digests (i.e. kinase sources were MI oocytes and recombinant Eg2) before the 2D mapping reveals co-migration of the LDSR phosphopeptides (arrow).

Extracts prepared from GV, MI and MII stage oocytes were supplemented with [γ-^{32}P]ATP and primed with two CPEB-derived peptides covalently linked to ovalbumin. The first peptide encompassed the two potential sites of phosphorylation by IAK1/Eg2, while the second peptide contained mutations in the highly conserved S and T residues (Fig. 3B). Although neither peptide was phosphorylated in GV stage extracts, the wild-type peptide was phosphorylated at MI, but only slightly at MII (Fig. 3C). The mutant peptide, however, was phosphorylated much less than wild type in MI stage extracts, and again was only slightly phosphorylated at MII. These data suggest that the kinase activity that is most prevalent at MI preferentially phosphorylates the T and S residues present in the LDS/TR motifs.

In order to define more precisely the target sites on CPEB for the MI kinase activity noted above, 2D phosphopeptide mapping was performed. In this case, the substrate was purified recombinant *Xenopus* CPEB, because mouse CPEB is insoluble after bacterial expression. At the GV stage, there were relatively few sites of CPEB phosphorylation, and even these were close to background levels (Fig. 3D). At MI, however, several sites were phosphorylated, including one that corresponded to the main LDS/TR phosphorylation site, which is clearly shown by the position of this spot compared with the position of the main site of CPEB phosphorylation when purified baculovirus expressed Eg2 was used as the kinase source (Fig. 3, arrows). To confirm that this phosphopeptide is indeed the same one as that detected when recombinant Eg2 was the kinase source, aliquots of the two reactions were mixed before the 2D mapping (Fig. 3D). Co-migration of the two spots demonstrates that CPEB is in fact phosphorylated on Ser174 by a mouse oocyte kinase. Therefore, mouse oocytes contain a developmentally regulated kinase activity that closely matches that predicted for IAK1/Eg2 in which CPEB is phosphorylated on the critical residue that is necessary for cytoplasmic polyadenylation. In this regard, it is worth noting that in *Xenopus* oocytes, Eg2 is the only kinase that phosphorylates CPEB Ser 174.

An IAK1/Eg2 blocking peptide inhibits polar body formation

We next examined whether the IAK1/Eg2 kinase activity is involved in oocyte maturation in the mouse. GV stage oocytes were injected with a CPEB-derived peptide (Fig. 3B) that specifically blocks Eg2 kinase activity and thereby inhibits *Xenopus* oocyte maturation (Mendez et al., 2000a). Fig. 4 shows that this peptide only minimally slowed the rate of first polar body formation compared with uninjected controls. In *Xenopus*, this wild-type peptide also only slowed, but did not block, meiotic progression (Mendez et al., 2000a). However, in *Xenopus*, the mutant alanine-containing peptide (Fig. 3B) dramatically inhibited maturation in injected oocytes. The interpretation of these results is that while Eg2 binds both the wild-type and mutant peptides, once the wild-type peptide is phosphorylated, these molecules then dissociate and the kinase is free to phosphorylate another substrate peptide (i.e. the wild-type peptide is a competitive inhibitor). However, because Eg2 cannot phosphorylate the mutant peptide, the molecules remain attached, thereby irreversibly lowering the effective concentration of the kinase (i.e. the mutant peptide is a dominant inhibitor). Therefore, the mutant peptide would be expected to be the most efficacious in abrogating Eg2-dependent meiotic progression, which is the case observed in injected mouse oocytes (Fig. 4).

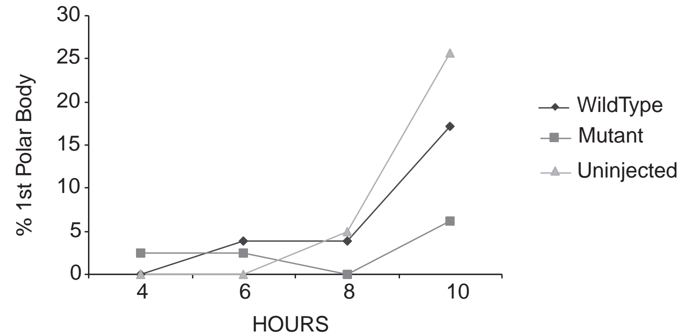

Fig. 4. IAK1/Eg2 blocking peptide inhibits first polar body extrusion. Oocytes were injected with the peptides noted in Fig. 3B, except that in this case they were not coupled to ovalbumin. The rate of polar body extrusion during maturation was assessed. While the wild-type peptide only moderately inhibited polar body extrusion, the mutant, alanine-containing peptide was more effective in doing so. Although both peptides bind Eg2, the binding to the mutant peptide is essentially irreversible, and thus it is a more effective competitive inhibitor of kinase activity. In this analysis, 29 oocytes were assessed for the wild-type peptide, 18 oocytes for the mutant peptide and 47 oocytes that were not injected. The experiment was performed twice.

A truncated CPEB that is not phosphorylated by IAK1/Eg2 acts as dominant negative protein in mouse oocytes

We have shown that two mutated CPEBs act as dominant negative proteins and inhibit polyadenylation and meiotic progression in *Xenopus* oocytes. The first mutant has had both serines in the LDS/TR motif mutated to alanine. This protein cannot be phosphorylated by IAK1/Eg2, and inhibits polyadenylation-induced translation. The second mutant CPEB has both LDS/TR motifs intact, but lacks the N-terminal 146 residues that are involved in the CPEB-IAK1/Eg2 interaction (CPEB ΔN). Hence, this protein is also not phosphorylated by IAK1/Eg2 (Mendez et al., 2000a). To determine whether a truncated CPEB acts as a dominant negative protein in mouse oocytes, we mixed mRNA encoding wild-type or mutant truncated CPEB ΔN (Fig. 5) together with radiolabeled cyclin B1 3′ UTR and injected them into oocytes. The oocytes were held in the immature stage with IBMX for ~6 hours, to allow for sufficient synthesis of the exogenous CPEB, which was followed by incubation in IBMX-free medium to allow maturation to proceed. The RNA was then extracted and polyadenylation was examined. Fig. 5 demonstrates that while injected wild-type CPEB had no effect on cyclin RNA polyadenylation, CPEB ΔN almost completely inhibited this process (less that ~10% polyadenylation at MII compared with CPEB wild-type injected, which was greater than 60% polyadenylation at the same stage). However, this reduced polyadenylation by CPEB ΔN somewhat surprisingly had little effect on meiotic progression. We conclude that not only is CPEB necessary for cytoplasmic polyadenylation in mouse oocytes, but that it

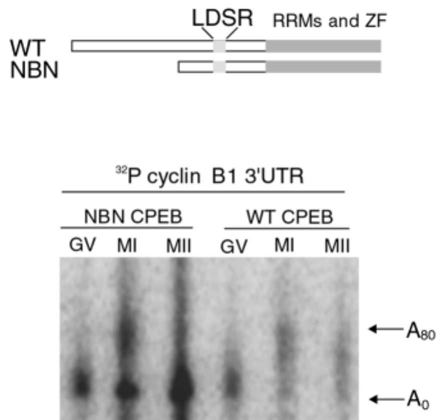

Fig. 5. CPEB is required for cytoplasmic polyadenylation. GV stage oocytes were injected with a mixture of radiolabeled cyclin B1 3′ UTR and mRNA encoding either wild-type CPEB or a mutant CPEB (ΔN) that lacks the N-terminal 146 amino acids. Both proteins contain the two LDSR motifs, as well as the RNA recognition motifs (RRM) and zinc finger (ZF) that are necessary for RNA binding (Hake et al., 1998). The RNA was then extracted from maturing oocytes and the polyadenylation of the cyclin B1 3′ UTR was analyzed by denaturing PAGE and phosphorimaging.

must also be phosphorylated at the LDS/TR motif by IAK1/Eg2 for this process to occur.

DISCUSSION

The results presented in this study show that (1) all the key polyadenylation factors are stored in the cytoplasm of mouse oocytes; (2) the kinase IAK1/Eg2 is most likely activated upon the induction of oocyte maturation; (3) IAK1/Eg2 phosphorylates CPEB serine 174; and (4) this phosphorylation event is necessary for cytoplasmic polyadenylation during meiotic maturation. These observations indicate that the main functions of CPEB in mouse oocytes, to mask and activate mRNA translation, are similar to those in *Xenopus* oocytes. For example, the masking function of the CPE (ACE) in mouse oocytes has been documented for tissue plasminogen activator (tPA) (Stutz et al., 1997; Stutz et al., 1998) and cyclin B1 (Tay et al., 2000) mRNAs. While the protein responsible for the tPA mRNA repression is unknown, that which mediates cyclin mRNA repression is clearly CPEB (Tay et al., 2000). The demonstration in this report that maskin (or at least specific maskin immunoreactivity) is present in the mouse oocyte cytoplasm implies that this protein is the most proximal arbiter of translation, via its interaction with CPEB and eIF4E (Stebbins-Boaz et al., 1999). In addition, the findings that the AAUAAA sequence is necessary for polyadenylation (Vassalli et al., 1989) and that CPSF 100 kDa is cytoplasmic indicates that, as in *Xenopus* oocytes, the multisubunit CPSF, which is recruited to mRNA by CPEB, attracts poly(A) polymerase to the end of the mRNA (Mendez et al., 2000b). Finally, because IAK1/Eg2 activity is stimulated upon the induction of maturation and because this kinase phosphorylates CPEB Ser174, the signaling events that link an initial decrease in cAMP (Eppig, 1991) to the polyadenylation machinery are at least partly conserved between *Xenopus* and mouse.

CPEB functions during two distinct phases of meiosis

Although mouse oocytes do not require protein synthesis to enter or complete maturation, polyadenylation-induced translation potentiates the rate of this process. In maturing mouse oocytes, there are two phases of M-phase promoting factor (MPF, a heterodimer of cdc2 and cyclin B) activity that drive the MI and MII transitions. The increase in this activity is regulated at the translational level (Hampl and Eppig, 1995b), which is, at least in part, due to the cytoplasmic polyadenylation of cyclin B1 mRNA (Tay et al., 2000). The findings presented in this study demonstrate that CPEB is responsible for the translation of this mRNA. Another mRNA whose polyadenylation-induced translation is essential for proper meiotic progress encodes Mos, a serine/threonine kinase. In mouse oocytes, Mos is a component of cytostatic factor (CSF), a protein complex that arrests meiosis at MII. The destruction of CSF activity by disruption of the *Mos* gene results in a high incidence of parthenogenetic activation. In these cases, the now mitotically dividing oocytes occasionally fail to be ovulated and give rise to ovarian cysts and tumors (Colledge et al., 1994; Hashimoto et al., 1994; Choi et al., 1996). While mouse oocytes contain some Mos protein, they also contain dormant CPE-containing *Mos* mRNA that, upon the induction of maturation, undergoes polyadenylation-induced translation. The destruction of *Mos* mRNA (O'Keefe et al., 1989) or the abrogation of *Mos* mRNA polyadenylation (Gebauer et al., 1994) prevents Mos synthesis, inhibits proper MII arrest and can occasionally lead to parthenogenesis. Therefore, the translational activation of *Mos* mRNA, by CPEB-mediated polyadenylation, is necessary for the late phase of oocyte meiosis.

CPEB function is also crucial at the pachytene stage of prophase I. CPEB-deficient female mice are sterile because they lack ovaries altogether, or contain only malformed ovaries. Late stage embryos (18.5 dpc) contain ovaries, but they are devoid of oocytes. Earlier embryonic ovaries (16.5 dpc) do contain oocytes, but their chromatin is fragmented and/or dispersed, suggesting a defect in synapsis or chromosome pairing. While such oocytes contain wild-type levels of mRNAs that encode two key components of the synaptonemal complex (synaptonemal complex proteins 1 and 3), they are not translated. These observations, plus the fact that these mRNAs contain CPEs in their 3′ UTRs that are bound by CPEB both in vitro and in vivo, demonstrate that the regulated translation of synaptonemal complex protein mRNAs by CPEB is essential for progression through first meiotic prophase (J. T. and J. D. R., unpublished).

Oh et al. have shown that cytoplasmic polyadenylation of CPE-containing mRNAs occurs after fertilization in the mouse, which suggests that maternally inherited sequences may play an important, but heretofore unknown role in the embryo of this species (Oh et al., 2000; see also Tong et al., 2000). However, whether embryonic polyadenylation is CPEB dependent is unclear because Tay et al. have been unable to detect this protein in the fertilized egg (Tay et al., 2000). Furthermore, if only a small amount of CPEB remains stable after fertilization (e.g. ~10%), as is the case in *Xenopus* (Groisman et al., 2000), then it would not necessarily have been detected, and thus could promote cytoplasmic polyadenylation in the embryo (see below). Clearly, additional experiments are

required to determine whether CPEB is involved embryonic polyadenylation.

A conserved signaling pathway promotes oocyte maturation

In *Xenopus* oocytes exposed to progesterone, the natural inducer of maturation, a transient but essential decrease in cAMP is followed by the activation of Eg2 (Andresson and Ruderman, 1998). Decreased cAMP is also necessary for mouse oocyte maturation, which in this case is followed by the activation of IAK1. It seems likely that IAK1/Eg2 is itself activated by phosphorylation, and the kinase responsible is almost certainly the same in mouse and *Xenopus*. IAK1/Eg2 then becomes localized at spindles and centrosomes, where it may be involved in the assembly of these structures (Nigg, 2001). However, as noted above, no CPEB or maskin can be detected after fertilization. Therefore, while the signaling events leading to translational activation during oocyte maturation are conserved among vertebrates, the events that control translation after fertilization may be quite different, and could reflect the relative importance of maternally inherited mRNA.

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