Cytoplasmic poly(A) elongation is widely utilized during the early development of many organisms as a mechanism for translational activation. Targeting of mRNAs for this mechanism requires the presence of a U-rich element, the cytoplasmic polyadenylation element (CPE), and its binding protein, CPEB. Blocking cytoplasmic polyadenylation by interfering with the CPE or CPEB prevents the translational activation of mRNAs that are crucial for oocyte maturation. The CPE sequence and CPEB are also important for translational repression of mRNAs stored in the Xenopus oocyte during oogenesis. To understand the contribution of protein metabolism to these two roles for CPEB, we have examined the mechanisms influencing the expression of CPEB during oogenesis and oocyte maturation. Through a comparison of CPEB mRNA levels, protein synthesis, and accumulation, we find that CPEB is synthesized during oogenesis and stockpiled in the oocyte. Minimal synthesis of CPEB, < 3.6%, occurs during oocyte maturation. In late oocyte maturation, 75% of CPEB is degraded coincident with germinal vesicle breakdown. Using proteasome and ubiquitination inhibitors, we demonstrate that CPEB degradation occurs via the proteasome pathway, most likely through ubiquitin-conjugated intermediates. In addition, we demonstrate that degradation requires a 14 amino acid PEST domain.

Key Words: cytoplasmic polyadenylation; RNA binding protein; early development; oocyte maturation; PEST domain.
in both Xenopus (de Moor and Richter, 1999) and mouse (Tay et al., 2000). Reporter mRNAs containing a CPE upstream of a nuclear polyadenylation hexanucleotide are translationally masked when injected into immature oocytes in comparison to the same mRNAs lacking CPEs. During oocyte maturation, only the CPE-containing mRNAs are cytoplasmically polyadenylated and translationally activated (de Moor and Richter, 1999). In immature oocytes, CPEB may mediate translational repression through an interaction with Maskin, which is an eIF4E binding protein that may interfere with recruitment of eIF4G to the translation initiation complex (Stebbins-Boaz et al., 1999). The CPE and CPEB thus have a dual function in early Xenopus development: the masking of maternal mRNAs from the translational apparatus (de Moor and Richter, 1999; Stebbins-Boaz et al., 1999) and the cytoplasmic polyadenylation of these messages (Hake and Richter, 1994; Stebbins-Boaz et al., 1996).

The dual function of CPEB may be regulated by alterations in its phosphorylation state. During oocyte maturation, CPEB is sequentially phosphorylated on multiple residues (Paris et al., 1993; Mendez et al., 2000a). Phosphorylation on serine 174 by Eg2 kinase recruits cleavage and polyadenylation specificity factor (CPSF) into an active cytoplasmic polyadenylation complex (Mendez et al., 2000b). In vivo, Eg2 phosphorylation of CPEB within 2 h of induction of maturation activates CPEB’s ability to mediate c-mos mRNA cytoplasmic polyadenylation (Mendez et al., 2000a). Later in maturation (~4 h after induction), CPEB is phosphorylated in a cdc2-dependent manner and ~75% is degraded, coincident with the onset of germinal vesicle breakdown (GVBD; de Moor and Richter, 1997), the first visible indication that meiosis has reinitiated. These late phosphorylation events correlate with the switch from the masked to the translationally activated state of additional important maternal mRNAs such as cyclin B1 and histone B4 (de Moor and Richter, 1997). The pool of CPEB that remains is also phosphorylated and is present in mature oocytes and eggs, where cytoplasmic polyadenylation is robust (Hake and Richter, 1994). It is currently unknown, however, whether there is a functional link between CPEB degradation and release from translational repression of the CPE containing mRNA in the maturing oocyte. In addition, the functional differences which allow one population of CPEB to be targeted for degradation and yet another to be protected from degradation are unknown.

We are interested in how the fate of an individual CPEB molecule is determined within the context of its transition from a translational repressor to a translational activator. A necessary component of this line of inquiry is knowledge of the principal factors that regulate the quantity of CPEB during oogenesis and oocyte maturation. In the current study, we have found that CPEB mRNA levels remain constant during early oogenesis. After a 20% increase in overall CPEB protein levels during late oogenesis, no significant synthesis of CPEB occurs during oocyte maturation. CPEB protein thus appears to be stockpiled and stable until GVBD, when 75% is degraded. We demonstrate that degradation of CPEB requires the proteasome and a 14 amino acid PEST domain that is conserved in vertebrate and clam CPEB. Targeting to the proteasome may also involve CPEB ubiquitination.

**MATERIALS AND METHODS**

**Plasmid Constructs**

The construction of HA-CPEB was identical to that of Myc-CPEB (Hake and Richter, 1994), except that the 5' primer sequence for PCR was 5' GTCCTTAGAGAATCCATCATGATGTC- CAGATACTGTGGACTGGGCCTCACCACGAAATG 3'. ∆PESTs and ∆PESTL were constructed by using the polymerase chain reaction with oligonucleotides flanking the regions to be deleted (codons 182–196 or 182–209, respectively), but designed so that amplification proceeded away from that region and around the entire plasmid. The 3' antisense primer was identical for generation of both deletion mutants, began on the 5' side of the nucleotides encoding codon 182, and was 5' TCTGAGATTCAAAATA- GAGCC 3'. For generation of ∆PESTs, the 5' sense primer began 3' of the nucleotides encoding codon 196 and was 5' TCTGACCCAC- CTTTACAGCCTA 3'. The ∆PESTL sense 5' primer began 3' of the nucleotides encoding codon 209 and was 5' TCTTCCTGCGCTG- CATTTTC 3'. Silent mutations were created at codons 181 (CGC 224 AGA) and 197 (TCA 224 TCT) to generate a unique BglII site that was used for ligation of the resultant PCR products and to screen for the deletion mutants. Double-stranded sequencing of the coding region of both mutants confirmed that the appropriate deletions were obtained and that no additional mutations were introduced.

**Oocyte Isolation and Culture**

For maturation experiments, Xenopus laevis females were primed with 50 IU pregnant mare serum gonadotropin 3–6 days before removal of ovarian lobes. These were digested with collagenase and dispase to release the oocytes (Kuge and Richter, 1995), which were incubated in 1× Barth's for at least 3 h prior to use. Oocytes corresponding to Dumont Stages I–VI (Dumont, 1972) were grouped based on size, rinsed with 1× XB (Hake and Richter, 1994), and stored at −80°C until use.

**RNA Transcription and Injections**

pHA-CPEB and deletion mutants were linearized with XhoI, and in vitro transcription was performed with the M message machine kit from Ambion (Austin, TX), following the manufacturer’s procedure. RNA was extracted with phenol/chloroform and then ethanol precipitated twice. Sets of oocytes were injected at the equatorial band with 50 nl of a 2 ng/μl solution of HA-tagged CPEB mRNA for a final concentration of ~100 ng per oocyte. As a control for injection-mediated activation, additional oocytes were injected with buffer alone. Injected oocytes were incubated in 1× Barth's saline supplemented with streptomycin for a minimum of 15 h to allow protein synthesis, before induction of maturation with progesterone.
Northern Blot Analysis

RNA was isolated from oocytes at the indicated stages of development and analyzed by Northern blotting as described (Hake et al., 1990). The PstI fragment of p12.5 (xCPEB; Hake and Richter, 1994) was random-prime labeled with a 32P-dCTP using the Decaprime kit (Ambion) following the manufacturer’s instructions. Membranes were hybridized and washed following the procedure of Church and Gilbert (1984) and visualized by autoradiography.

35S-Methionine Incorporation Assays

Stage VI oocytes were placed in a 10 × 35 mm Petri dish and induced to mature with 10 μg/ml progesterone. Immediately following the addition of the hormone, 30 oocytes were injected with 46 nl of 1104 Ci/mmol 35S-methionine (New England Nuclear). After 1 h, 25 of the 30 maturing oocytes were frozen on dry ice. Directly following freezing of the first set, a second set of 30 oocytes was removed from the pool of maturing oocytes, injected with 35S-methionine, incubated for 1 h, and frozen. This pattern was repeated every hour until 50% of the remaining oocytes had undergone maturation, which was scored by the presence of a white spot in the animal pole, indicative of germinal vesicle (nuclear) breakdown (GVBD) and the resumption of meiosis I. At this point a final hour of injections was completed. The final percentage of maturation varied from 75–90%. All oocytes were stored at −80°C. Extracts of the frozen oocytes were prepared by homogenization in 1× XB with protease inhibitors followed by centrifugation (Hake and Richter, 1994). To compensate for variability in the amount of mature or immature oocytes was measured using slight modifications of the procedures of Glaas et al. (1998) by adding 2 μl extract and 1 μl of a 10 mM stock of the fluorogenic peptide, N-succinyl-leu-leu-val-tyrosine-7-amido-methylcoumarine (Suc-LLVY-MCA; Sigma, St Louis, MI) to 97 μl buffer I (50 mM Tris pH 7.4, 2 mM DTT, 5 mM MgCl2, 2 mM ATP). The mixture was incubated at room temperature with time points removed at 15, 30, 45, and 60 min. The reaction was stopped by addition of 3 ml 1% SDS. Peptidase activity results in release of the fluorescent MCA moiety, which was monitored by excitation at 350 nm and fluorescent emission at 440 nm (Shimadzu RF5000). To study the influence of MG132 on Suc-LLVY-MCA hydrolytic activity in the extract, 1 μl of a 4 mM stock solution (in 40% DMSO) of MG132 was preincubated with the extract at room temperature for 20 min before addition of the fluorogenic peptide.

RESULTS

CPEB Is Synthesized during Oogenesis and Stockpiled in the Oocyte

As a basis for our investigations into the role of CPEB in translational repression and cytoplasmic polyadenylation, we examined the mechanisms regulating CPEB protein levels during oogenesis and oocyte maturation. First, CPEB mRNA and protein accumulation during oogenesis and
oocyte maturation were compared to determine whether significant alterations in mRNA levels could account for the 75% decrease in CPEB protein levels observed during oocyte maturation (Hake and Richter, 1994; de Moor and Richter, 1997). On a per oocyte basis, CPEB mRNA accumulates during early oogenesis until Stage III, remains constant through Stage V oocytes, decreases slightly in Stage VI oocytes, and then remains relatively constant throughout oocyte maturation (Fig. 1A). CPEB mRNA levels were normalized by comparison to those of cyclin B1 mRNA (panel 2) and ribosomal RNA (panel 3). Kobayashi et al. (1991) demonstrated, using RNAase protection, that cyclin B1 mRNA is present in equivalent amounts per oocyte throughout oogenesis. Normalized values for CPEB mRNA levels were obtained by dividing the CPEB mRNA band density by the cyclin B1 mRNA band density (values indicated by CPEB/B1). CPEB mRNA levels during oocyte maturation were compared to those for rRNA, as rRNA levels remain constant throughout oocyte maturation (Busby and Reeder, 1982).

The amount of CPEB protein per oocyte increases between Stages I and II of oogenesis (compare lanes 1 and 2, Fig. 1B) and remains relatively constant until the transition from Stage V to Stage VI oocytes (lanes 5 and 6), where there is an approximately 20% increase in amount. During early oocyte maturation, CPEB protein levels are stable and then decline drastically during late maturation, decreasing to 25% of the level found earlier, as previously observed (Hake and Richter, 1994; de Moor and Richter, 1997, 1999). Alterations in the levels of CPEB protein are apparent when normalized against the amount of the Xenopus helicase, Xp54. Ladomery et al. (1997) demonstrated that Xp54 is maintained at a constant level per oocyte throughout oogenesis and oocyte maturation. The ratio of CPEB band density divided by Xp54 band density is indicated (CPEB/Xp54, Fig. 1B). The doublet observed in lane 4 for Xp54 was not reproducible.

In summary, CPEB protein appears to be stockpiled early during oogenesis, with either increased stabilization or increased protein synthetic activity contributing to an increase in amount between Stages V and VI of oogenesis. Parallel expression of CPEB mRNA and protein until Stage III suggests that CPEB mRNA is readily available for translation during this time. The decrease in CPEB protein level in late oocyte maturation (M, lane 9, Fig. 1B) does not correlate with the stable level of CPEB mRNA (M, lane 9, Fig. 1A), indicating that the decrease in CPEB protein is not due to an increase in CPEB mRNA instability.

CPEB Synthesis Is Minimal during Oocyte Maturation

Although the previous analyses indicate that CPEB protein accumulates and that overall levels remain relatively constant until GVBD, they do not reveal the dynamics of protein synthesis and degradation that could contribute to alterations in the CPEB pool. More specifically, it is unclear whether the CPEB present in mature oocytes, which is necessary for cytoplasmic polyadenylation, arises from new protein synthesis during oocyte maturation, or by protection from the degradation that removes over 75% of CPEB from the oocyte.

To examine the potential contribution of new CPEB synthesis to the CPEB pool during oocyte maturation, we analyzed the rates of CPEB synthesis in Stage VI oocytes and at 1-h intervals during oocyte maturation (Fig. 2). To analyze CPEB synthesis during oocyte maturation, oocytes were induced to mature with progesterone and then in-
some pathway inhibitors on the metabolism of endogenous CPEB in vivo. CPEB is degraded coincident with GVBD, which occurs ~4 h into the ~6-h maturation process. To avoid perturbing early events in maturation that may require proteasome activity (Sawada et al., 1997), we attempted to block CPEB degradation by injecting inhibitors into oocytes in which maturation had been initiated but had not yet advanced to GVBD. We found that non-GVBD oocytes taken from a batch of maturing oocytes where 50% had achieved GVBD (GVBD_{50}) were suitable for these studies.

MG132 is a membrane-permeant peptide aldehyde widely used to block the chymotryptic activity of the proteasome (reviewed by Lee and Goldberg, 1998). Because its effects last for several hours without obvious toxicity to the cells (Rock et al., 1994), we used this inhibitor for our in vivo assays. We analyzed the influence of MG132 on CPEB degradation during progesterone-induced maturation by injecting nonwhite spot oocytes at GVBD_{50} with either MG132 or buffer with 10% DMSO (Fig. 3A). Time points were taken immediately after addition of progesterone (Immature; Im), at GVBD_{50} (G_{50}), and 10 and 120 min after 100% GVBD (M_{10} and M_{120}). The pattern of CPEB expression in control buffer-injected immature oocytes (lane 1), and the decrease in CPEB level apparent when the oocytes were fully mature (M_{120}, compare lanes 2 and 4), is identical to that observed during maturation of nonperturbed oocytes (data not shown; de Moor and Richter, 1997). The injection of MG132 into oocytes at GVBD_{50} successfully blocked the degradation of CPEB that occurs as oocytes progress through GVBD (MG132/M_{120}, lane 5), whereas injection of 10% DMSO alone did not (compare lanes 4 and 5). The rate and percentage of GVBD in the MG132-injected oocytes were similar to that observed for buffer injected oocytes (Fig. 3B). The calpain inhibitor E-64 did not affect CPEB degradation or proteasomal activity (data not shown).

As a control for proteasomal activity in all of these samples, quick cytoplasmic extracts were prepared from immature and mature oocytes and tested for their ability to degrade the fluorogenic peptide, Suc-Leu-Leu-Val-MCA, in a spectrofluorometric assay (Fig. 3C). The proteasome was active in extracts from both immature and mature oocytes with nearly identical activities at 79.4 ± 16.1 and 82.6 ± 13.2 Functional Units/(milligrams of extract protein × minutes), respectively. Addition of MG132 to extracts effectively inhibited the proteasome and reduced the proteasome activity in immature and mature oocyte extracts to 10.8 ± 2.9 and 8.05 ± 2.4 FU/(mg × minutes), respectively.

**Deletion of the PEST Domain Prevents Degradation of CPEB**

Regions rich in proline, glutamate, serine, and tyrosine (PEST sequences) target proteins for rapid degradation (Rechsteiner and Rogers, 1996). Our comparison of CPEB homologs from frog, mouse, and clam (Walker et al., 1999) revealed a highly conserved 15 amino acid domain that
The addition of 40 mg functional units/milligrams of protein extract to trofluorimetry. Proteasomal activity in the extracts is expressed as the ability to degrade a fluorogenic peptide as monitored by spectrofluorimetry and this did not change after overnight (18 h) incubation. Oocytes from frog 2 were 65% mature after 12 h injected with MG132 was virtually identical to that of buffer.

GVBD50 (G50) 50 nl of buffer plus 10% DMSO alone or with 1 mM MG132 were injected into non-GVBD oocytes and a sample was immediately taken (lane 2). The remaining oocytes were incubated until 10 or 120 min after 100% GVBD (M10 and M120, lanes 3–5). Crude protein extracts were then analyzed by SDS–PAGE, immunoblotting with anti-CPEB antiserum and detection by enhanced chemiluminescence. The open arrow indicates the position of phosphorylated eCPEB. (A) Immature oocytes (Im) were induced to mature by addition of progesterone. At GVBD (G50) 50 nl of buffer plus 10% DMSO alone or with 1 mM MG132 were injected into non-GVBD oocytes and a sample was immediately taken (lane 2). The remaining oocytes were incubated until 10 or 120 min after 100% GVBD (M10 and M120, lanes 3–5). Crude protein extracts were then analyzed by SDS–PAGE, immunoblotting with anti-CPEB antiserum and detection by enhanced chemiluminescence. The open arrow indicates the position of phosphorylated eCPEB. (B) The percentage GVBD of oocytes from frog 2 were 65% mature after 12 h of observation, and this did not change after overnight (18 h) incubation. (C) Spectrofluorimetric proteasome activity assay. Extracts from immature and mature oocytes were assayed for their ability to degrade a fluorogenic peptide as monitored by spectrofluorimetry. Proteasomal activity in the extracts is expressed as functional units/milligrams of protein extract × minutes (FU/mg × min). The effect of MG132 on this activity was assessed by addition of 40 μM MG132 to the reaction (lanes indicated with “MG132”). Control lanes (“Buffer”) received 1 μl of 40% DMSO vehicle.

FIG. 3. Degradation of endogenous CPEB during maturation is blocked by the proteasome inhibitor MG132. (A) Immature oocytes (Im) were induced to mature by addition of progesterone. At GVBD (G50) 50 nl of buffer plus 10% DMSO alone or with 1 mM MG132 were injected into non-GVBD oocytes and a sample was immediately taken (lane 2). The remaining oocytes were incubated until 10 or 120 min after 100% GVBD (M10 and M120, lanes 3–5). Crude protein extracts were then analyzed by SDS–PAGE, immunoblotting with anti-CPEB antiserum and detection by enhanced chemiluminescence. The open arrow indicates the position of phosphorylated eCPEB. (B) The percentage GVBD of oocytes from frog 2 were 65% mature after 12 h of observation, and this did not change after overnight (18 h) incubation. (C) Spectrofluorimetric proteasome activity assay. Extracts from immature and mature oocytes were assayed for their ability to degrade a fluorogenic peptide as monitored by spectrofluorimetry. Proteasomal activity in the extracts is expressed as functional units/milligrams of protein extract × minutes (FU/mg × min). The effect of MG132 on this activity was assessed by addition of 40 μM MG132 to the reaction (lanes indicated with “MG132”). Control lanes (“Buffer”) received 1 μl of 40% DMSO vehicle.

scored 19.18 with the PESTFind algorithm (lightly shaded box, Fig. 4A; Rogers et al., 1986; http://www.icnet.uk/cgi-bin/runpest.pl). Further comparison of this region with CPEB homologs from human and zebrafish revealed that significant, high PEST homology extends for an additional 13 amino acids (Fig. 4A; entire shaded region). This extended PEST domain scores 17.75 with PESTFind, a value higher than PEST sequences functionally proven to be proteolytic signals in a number of short-lived proteins (reviewed in Rechsteiner and Rogers, 1996). The 28 amino acid PEST domain is interrupted by a histidine and hence is bipartite because positively charged amino acids are not generally found within PEST sequences. Outside of the PEST domain, the amino terminal halves of vertebrate and clam CPEB are not highly conserved. In contrast, the carboxy-terminal domains containing the RNA recognition motifs and the zinc finger domain are highly conserved.

Based on the conserved nature of the PEST domain in the CPEB protein family and the high score that this region has with the PEST-find program, we tested the hypothesis that this domain might be required for targeting CPEB for degradation. A deletion mutant of HA-tagged CPEB was constructed that removed the extended PEST domain (ΔPESTLong, Fig. 4B). The metabolism of wild-type HA-CPEB and the ΔPESTΔ mutant during oocyte maturation was assessed by in vivo translation of the wild-type and mutant mRNAs, followed by HA-immunoblotting (see Materials and Methods). The processing of wild-type HA-CPEB during progesterone-induced oocyte maturation was identical to that observed for endogenous CPEB (compare Fig. 4C, lanes 1–4 with Fig. 3A). HA-CPEB is present in immature oocytes. Ten minutes after the appearance of GVBD (M10), the electrophoretic mobility of HA-CPEB is reduced; then at 120 min after GVBD (M120), over 80% of the protein is degraded. This degradation of HA-CPEB is also mediated by the proteasome, as shown by the prevention of degradation by injection of oocytes with MG132 (Fig. 4C, lane 4).

HA-CPEB containing a deletion of the PEST domain (ΔPESTL, Fig. 4B) is not degraded during oocyte maturation: at M120, the level of ΔPESTL is similar to that present in immature oocytes (Fig. 4C compare lane 10 with 8). In addition, only half of the ΔPESTL shifts to a lower mobility form (lanes 9 and 10), whereas all of the wild-type HA-CPEB (Fig. 4C, lanes 2–4) and endogenous CPEB (Fig. 3, lanes 3–5) migrate more slowly in SDS–PAGE.

To examine the possibility that there may be a smaller region within the 28 amino acid PESTL domain that is the signal for degradation, we deleted 14 amino acids of the ΔPESTL (Fig. 4C, lane 5) and examined its metabolism during oocyte maturation (Fig. 4C, lanes 5–7). Deletion of PESTS also prevents CPEB degradation during oocyte maturation (lanes 5–7). Although it appears from this immunoblot that ΔPESTS is not stabilized to the same extent as ΔPESTL, this appears to be an artifact of the variable levels of recombinant protein expression in individual oocytes. Further analysis of multiple sets of oocytes expressing each of the three recombinant proteins indicates that, whereas HA-CPEB is always degraded, in comparison, ΔPESTS and ΔPESTL are each 80–100% stabilized (data not shown). Thus, a se-
Deletion of the PEST domain prevents CPEB degradation. (A) Comparison of the PEST domain (shaded) and flanking amino acids in vertebrate and clam CPEB. Light shading: PEST domain conserved in clam. Dark shading: extended PEST domain conserved in vertebrates. The initial PEST domain amino acid and accession numbers are frog (Arg181, gb AAA80483.1), mouse (Arg178, NP_031961.1), human (Arg104, dbj BAB14496.1), Z-fish 1 (Zor1, Ser173, dbj BAA75637.1), Z-fish 2 (Zor2, Ser144, dbj BAA75638.1), and clam p82 (Arg230, gb AFO91712). Only amino acids that are identical among the various proteins are shown. A plus sign (+) indicates the position of conservative amino acid differences in comparison with Xenopus CPEB. Dashes indicate where gaps were introduced to aid in alignment. Amino acids in bold are positively charged. (B) Schematic diagram of wild-type HA-tagged CPEB and PEST deletion mutants. Gaps indicate the position of the amino acids removed from wild-type CPEB to generate ΔPESTS and ΔPESTL. The RNA binding domain is indicated by R1 (RNA recognition motif 1), R2 (RNA recognition motif 2), and ZF (zinc-finger domain). The asterisk indicates the position of the Eg2 phosphorylation site. (C) RNA encoding HA-tagged CPEB or the indicated deletion mutant was injected into immature oocytes, which were subsequently incubated overnight. In the morning, immature oocytes (Im) were induced to mature with progesterone. For HA-CPEB, 50 nl of buffer plus 10% DMSO alone or with 1 mM MG132 were injected into non-GVBD oocytes at GVBD50 (M10 and M120, lanes 2–4). For the deletion mutants, no injections of DMSO or MG132 were performed, and samples were taken at the indicated times after appearance of GVBD. Protein extracts from 2.5 oocytes for each sample were analyzed by SDS-PAGE, immunoblotting with anti-CPEB antiseraum, and chemiluminescence. The immunoblot was then probed with anti-Xp54 as a loading control.

FIG. 4. Deletion of the PEST domain prevents CPEB degradation. (A) Comparison of the PEST domain (shaded) and flanking amino acids in vertebrate and clam CPEB. Light shading: PEST domain conserved in clam. Dark shading: extended PEST domain conserved in vertebrates. The initial PEST domain amino acid and accession numbers are frog (Arg181, gb AAA80483.1), mouse (Arg178, NP_031961.1), human (Arg104, dbj BAB14496.1), Z-fish 1 (Zor1, Ser173, dbj BAA75637.1), Z-fish 2 (Zor2, Ser144, dbj BAA75638.1), and clam p82 (Arg230, gb AFO91712). Only amino acids that are identical among the various proteins are shown. A plus sign (+) indicates the position of conservative amino acid differences in comparison with Xenopus CPEB. Dashes indicate where gaps were introduced to aid in alignment. Amino acids in bold are positively charged. (B) Schematic diagram of wild-type HA-tagged CPEB and PEST deletion mutants. Gaps indicate the position of the amino acids removed from wild-type CPEB to generate ΔPESTS and ΔPESTL. The RNA binding domain is indicated by R1 (RNA recognition motif 1), R2 (RNA recognition motif 2), and ZF (zinc-finger domain). The asterisk indicates the position of the Eg2 phosphorylation site. (C) RNA encoding HA-tagged CPEB or the indicated deletion mutant was injected into immature oocytes, which were subsequently incubated overnight. In the morning, immature oocytes (Im) were induced to mature with progesterone. For HA-CPEB, 50 nl of buffer plus 10% DMSO alone or with 1 mM MG132 were injected into non-GVBD oocytes at GVBD50 (M10 and M120, lanes 2–4). For the deletion mutants, no injections of DMSO or MG132 were performed, and samples were taken at the indicated times after appearance of GVBD. Protein extracts from 2.5 oocytes for each sample were analyzed by SDS-PAGE, immunoblotting with anti-CPEB antiseraum, and chemiluminescence. The immunoblot was then probed with anti-Xp54 as a loading control.

Methylated Ubiquitin and Ubiquitin Aldehyde Stabilize Several Larger Forms of CPEB in Immature Oocytes

The majority of proteins degraded by the proteasome are targeted to this multi-subunit protease through the attachment of ubiquitin to lysine residues by isopeptide bonds. Polyubiquitination can occur by the attachment of additional ubiquitin molecules to lysines in the host protein, to lysines in the attached ubiquitins, or a combination of these activities. These multiubiquitinated protein species are excellent substrates for degradation by the proteasome. Analysis of mono- and polyubiquitinated protein species is difficult, however, because the ubiquitinated protein is such a good substrate for the proteasome (reviewed by Hershko and Ciechanover, 1998; Koegl et al., 1999). There can also be significant isopeptidase activity that acts to deubiquitinate proteins. Several modified forms of ubiquitin can be used to interfere with the metabolism of ubiquitinated proteins and thus help preserve ubiquitinated protein intermediates. Methylated ubiquitin blocks polyubiquitination of proteins because it cannot serve as a substrate for further ubiquitination (Hershko and Heller, 1985). However, methylated ubiquitin can be a substrate of ubiquitin-protein lyases that normally recycle ubiquitin from ubiquitin conjugates. Ubiquitin aldehyde efficiently inhibits these lyases and other isopeptidases (Dang et al., 1998), causing an increase in ubiquitin conjugates and a significantly decreased rate of conjugate breakdown (Hershko and Rose, 1987; Saheffer and Cohen, 1996). Thus, we used a combination of both ubiquitin species to try to perturb the degradation of CPEB and, at the same time, enhance the retention of potentially ubiquitinated CPEB intermediates.

We first analyzed the effect of methylated ubiquitin and ubiquitin aldehyde on CPEB by injection of these derivatives into immature oocytes, incubating the oocytes for 60 min, and then analyzing CPEB by immunoblotting (Fig. 5A). By the 60-min time point (lane 3), the accumulation of a 74 kDa CPEB reactive band is evident, presumably due to the retention of ubiquitinated CPEB in the immature oocyte. Uninjected oocytes incubated in parallel did not accumulate this species (as seen in Fig. 3, lane 1). The modified form is larger, at 74 kDa, than the typical phosphorylated species observed in mature oocytes of 64 kDa (Paris et al., 1991). To compare these two size variants of CPEB directly, the sample electrophoresed in Fig. 5A, lane 3, was reelectrophoresed on a longer gel (Fig. 5B, lane 1) and compared with a time course of CPEB expression during normal maturation (Fig. 5B, lanes 2–4). The 74-kDa form of CPEB is clearly larger than the 64-kDa phosphorylated form of CPEB present in mature oocytes (Fig. 5B, compare lane 1 with lane 4 and refer to lighter exposure below).

Intriguingly, in sets of nonperturbed, synchronous, and...
rapidly maturing oocytes, CPEB caught just prior to degra-
dation, at GVBD 50, has a heterogeneous size distribution
that is not observed in either immature or mature nonper-
turbed oocytes (compare Fig. 5B, lane 3, with Fig. 5A lane 1,
and Fig. 5B lane 4): There is a cluster of bands from 64–68
kDa and a larger band of 74 kDa (lane 3). The cluster of
CPEB reactive bands over 90 kDa does not appear reproduc-
ibly, even when the same sample is analyzed on subsequent
gels and is thus not considered to be CPEB. The shift
upward of the 62 kDa band to 64 kDa is most apparent in
the shorter exposure and upon comparison to the position
of Xp54 on the same immunoblot. We suspect that this
upward shift is due, at least in part, to hyperphosphoryla-
tion, which occurs during oocyte maturation (Paris et al.,
1991). Notably, the 74-kDa band (lane 3, Fig. 5B) has
the same mobility as the potentially ubiquitinated 74-kDa band
in immature oocytes treated with methylated ubiquitin and
ubiquitin aldehyde (lane 1). This observation suggests that
the 74-kDa band in lane 3, which has risen through the
course of normal CPEB metabolism, may be ubiquitinated
CPEB.

In addition, recombinant HA-CPEB observed at GVBD 50
and when the proteasome is blocked also has a very heter-
ogeneous size distribution (Fig. 5C, lanes 2 and 4). The
74-kDa band appears at GVBD 50 and then, in mature oo-
cytes, the amount of HA-CPEB is greatly reduced, and only
the 64-kDa band is evident. Although not observed for the
endogenous protein example shown in Fig. 2A, this same
array of size variants has been observed for endogenous
CPEB at GVBD 50 in other experiments (data not shown).

Further evidence that ubiquitination of CPEB is required
for targeting it for degradation was obtained by monitoring
the effects of methylated ubiquitin and ubiquitin aldehyde
on CPEB degradation during oocyte maturation. Non-
GVBD oocytes at GVBD 50 were injected with these modi-
fied forms of ubiquitin, and subsequently incubated until
60 min past 100% GVBD (M 60). As seen in Fig. 5B, lanes 5
and 6, CPEB cross-reactive bands show the same size

FIG. 5. Methylated ubiquitin and ubiquitin aldehyde stabilize unique, high molecular weight forms of CPEB in vivo. (A) Stage VI oocytes
were injected with 50 nl of inhibitor cocktail containing 400 μM methylated ubiquitin, 20 μM ubiquitin aldehyde, and 10% DMSO, or
control buffer containing 10% DMSO. Time points were taken 0, 30, and 60 min postinjection. Protein extracts were analyzed by
SDS-PAGE, immunoblotting with anti-CPEB antibody, and chemiluminescence. Each lane contains two oocyte equivalents of protein. All
immunoblots were reprobed with anti-Xp54 antibody as a loading control (lower panels). (B) The sample from Fig. 4A, lane 3, was
reelectrophoresed on a longer gel (lane 1) with the following additional protein samples. Fully grown Stage VI oocytes were induced to
mature with progesterone. Time points were taken 2 h after induction (E), at GVBD 50 (G 50), and 60 min after 100% GVBD, i.e., completion
of maturation (M 60). For lanes 5 and 6, methylated ubiquitin and ubiquitin aldehyde were injected into oocytes at GVBD 50. Samples were
analyzed as in part A, above. A lighter exposure is presented in the middle panel. Sizes of the heterogeneous CPEB forms are indicated
between parts A and B, and to the right of the light exposure. (C) HA-tagged CPEB is very heterogeneous at GVBD 50 and when the
proteasome is blocked by MG132. Oocytes were primed with 100 ng of HA-CPEB mRNA overnight and induced to mature with
progesterone. Time points were removed at GVBD 50, M 60, and at M 60 in MG132 injected oocytes (as described above). Samples were
analyzed as above, except with anti-HA antibody. The positions of endogenous CPEB (eCPEB) and HA-tagged CPEB (HA-CPEB) in the
appropriate immunoblots are indicated.
heterogeneity in samples taken immediately after injection (lane 5), as in control-injected oocytes (lane 3). In oocytes that complete GVBD, CPEB degradation is inhibited by methylated ubiquitin and ubiquitin aldehyde: 70% is degraded in the presence of these inhibitors compared to 90% degradation in control, buffer-injected oocytes (Fig. 5B, compare lane 6 with lane 4). Additional experiments to confirm that CPEB is ubiquitinated by immunoprecipitation and immunoblotting with antiubiquitin antibodies were unsuccessful (data not shown).

DISCUSSION

We are interested in how the fate of an individual CPEB molecule is determined within the context of its transition from translational repressor to translational activator. We have begun to approach this by examining the principal factors regulating the levels of CPEB during oogenesis and oocyte maturation. In the current study, we have demonstrated that CPEB is stockpiled during oogenesis and degraded by the proteasome pathway during oocyte maturation. Deletion of a 14 amino acid PEST domain that is highly conserved in vertebrates and clam completely stabilizes CPEB, thus indicating that this domain is necessary for targeting CPEB for degradation.

CPEB mRNA and protein amounts increase in parallel until stage III of oogenesis, suggesting that existing CPEB mRNA is directly translated into protein. Between stages V and VI, CPEB mRNA levels decrease slightly and protein levels increase slightly. CPEB mRNA levels may be adjusting to an equilibrium between a low level of turnover and the decrease in overall transcription that occurs late in oogenesis (reviewed in Davidson, 1976), whereas the slight increase in CPEB protein levels may be due to the overall increase in protein synthetic rates that occurs during late oogenesis (Taylor and Smith, 1985).

CPEB protein is synthesized primarily during oogenesis and stockpiled in the oocyte. New CPEB synthesis during oocyte maturation contributes 36 pg to the existing CPEB pool during oocyte maturation. We have previously shown that CPEB is an abundant protein present at 3 ng per stage VI oocyte and 1 ng per mature oocyte or egg (Hake and Richter, 1994). Thus, new CPEB accumulation during oocyte maturation accounts for 3.6% of the CPEB pool present in mature oocytes. This is clearly insufficient to account for the 25% that persists after oocyte maturation. However, since we do not know whether CPE functions catalytically or stoichiometrically, we cannot determine whether this is a functionally significant contribution to the CPEB pool.

We believe it is unlikely that the slight increase in translation of CPEB observed during oocyte maturation is due to translational activation mediated by cytoplasmic polyadenylation. To date, functional CPEs have been mapped to within 100 nt 5′ of the nuclear polyadenylation hexanucleotide (Richter, 1999 and references therein). Analysis of the CPEB mRNA 3′UTR (Genbank No. U14169) reveals a putative CPE; however, it is located 230 nt 5′ of the hexanucleotide. Additional analyses would be required to determine if this CPE is functional, and, given the low level of CPEB translation during oocyte maturation, translational activation of CPEB during this period does not appear to be a significant aspect of its regulation.

Protein degradation has a significant impact on the levels of CPEB: 75% is degraded shortly after germinal vesicle breakdown, the first visible sign that meiosis has been reinitiated (de Moor and Richter 1997; this study). We have demonstrated that the decrease in CPEB protein concentration is not related to alterations in CPE mRNA levels, as these remain stable throughout oocyte maturation. Indeed, we find that CPEB degradation is dependent upon the proteasome pathway.

In an effort to map the region within CPEB required for degradation, we focused on a 28 amino acid PEST domain that is highly conserved in the vertebrate CPEB family (this study) and contains a smaller domain that is also conserved in clam p82/CPEB (Walker et al., 1999). PEST domains are required for the degradation of many different classes of proteins by several different degradation pathways (Rechsteiner and Rogers, 1996). There are several examples in which phosphorylation on specific serine and threonine residues within the PEST domain target the protein for the ubiquitin-proteasome degradation pathway (Baeuerle, 1998; Fleming and Wang, 2000; also see references in Rechsteiner and Rogers, 1996). Two lines of evidence suggest that this is a possible scenario for the involvement of the PEST domain in CPEB degradation. First, the ΔPEST mutants of CPEB are not phosphorylated to the same extent as the wild-type protein (Fig. 4C). Only 50% of the ΔPEST deletion mutant proteins shift in mobility during oocyte maturation, and, in addition, the size shift observed is not as great as it is for the wild-type protein. Deletion of the PEST domain may remove residues that are usually phosphorylated or a signal necessary for phosphorylation outside of the PEST domain. The absence of these residues, and perhaps their nonphosphorylated neighbors, may preclude recognition of CPEB by E3 ligases or other proteins that aid in targeting CPEB for degradation. Further analysis of this possibility will require analysis of the metabolism of CPEB containing point mutations of physiologically phosphorylated positions, in combination with an examination of the proteins that may recognize this domain and mediate targeting for degradation.

Second, we provide data suggesting that CPEB requires ubiquitination for degradation. By using modified forms of ubiquitin that perturb multiubiquitination and deubiquitination, we were able to stabilize a 74-kDa form of CPEB in immature oocytes that most likely contains one or two ubiquitin conjugates (Fig. 5A, lane 3). Methylated ubiquitin typically leads to the accumulation of low molecular weight protein–ubiquitin conjugates (Ben-Shahar et al., 1997; Pickart and Vella, 1988), whereas ubiquitin aldehyde prevents the breakdown of mult ubiquitin conjugates (Dang et al., 1998). The presence of these potential ubiquitin-
CPEB conjugates in immature oocytes suggests that the ubiquitination machinery is functioning. This does not result in the degradation of a significant fraction of CPEB in immature oocytes, because treatment of immature oocytes with inhibitors of the proteasome had no effect on the accumulation of CPEB (data not shown).

The similarity of this immature oocyte form of CPEB with the 74-kDa form observed at GVBD50 in the absence of inhibitors leads us to speculate that the latter form may represent CPEB that is mono- or diubiquitinated and in the process of being targeted for degradation (Shaefler, 1994). These types of intermediates would be predicted to be very transient. Indeed, although we have observed these modified forms of CPEB in oocytes from four different females at GVBD50, we only see them when the oocytes are maturing synchronously and achieve 100% GVBD within 6 h.

Additional support for the requirement of ubiquitin in targeting CPEB for degradation comes from our studies demonstrating that injection of methylated ubiquitin and ubiquitin aldehyde into maturing oocytes inhibits CPEB degradation. Hershko et al. (1991) were similarly able to delay cyclin degradation in clam oocyte extracts with methylated ubiquitin. They were unsuccessful, however, in observing the predicted monoubiquitinated species, presumably due to the action of isopeptidases. The inclusion of ubiquitin aldehyde in our experiments may have aided our visualization of the higher molecular weight, potentially ubiquitinated intermediates by inhibiting these isopeptidases.

It has recently been shown that the PEST domain of CPEB is necessary for microtubule binding (Groisman et al., 2000). In oocytes, CPEB is enriched in the animal pole, presumably through its association with microtubules. In cleavage stage embryos, CPEB is associated with the mitotic apparatus and centrosome. This ensures that cyclin B1 protein is concentrated near the mitotic apparatus, which appears to be a requirement for normal cell division (Groisman et al., 2000). The tethering of CPEB to microtubules via the PEST domain may block accessibility of this domain to other proteins that would modify and thus target it for ubiquitination and proteasomal degradation. Protein interactions mediated via the PEST domain have been shown to impede target protein degradation in other systems: the PEST domains within lxBα andlxBβ denote two Ras-like guanine triphosphatases that interfere with recognition of these domains by the degradative machinery (Fenwick et al., 2000). Although the domain required for CPEB degradation has been identified, and a compelling duality in function of this domain has been elucidated, the nature of the functional differences which allow one population of CPEB to be targeted for degradation and yet another to be protected are still unknown.

In this study, we have shown that CPEB is stockpiled during oogenesis and degraded by the proteasome pathway during GVBD through recognition of the PEST domain. Negligible synthesis of CPEB occurs during oocyte maturation, suggesting that the segregation of CPEB into two pools, one that is degraded, and one that is protected and persists for the promotion of polyadenylation, occurs through the functional sequestration of CPEB. This could be achieved perhaps by the interaction of CPEB, through its PEST domain, with various cellular components. Further experiments are in progress to examine this possibility.

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