Cytoplasmic polyadenylation elements mediate masking and unmasking of cyclin B1 mRNA

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During oocyte maturation, cyclin B1 mRNA is translationally activated by cytoplasmic polyadenylation. This process is dependent on cytoplasmic polyadenylation elements (CPEs) in the 3' untranslated region (UTR) of the mRNA. To determine whether a titratable factor might be involved in the initial translational repression (masking) of this mRNA, high levels of cyclin B1 3' UTR were injected into oocytes. While this treatment had no effect on the poly(A) tail length of endogenous cyclin B1 mRNA, it induced cyclin B1 synthesis. A mutational analysis revealed that the most efficient unmasking element in the cyclin 3' UTR was the CPE. However, other U-rich sequences that resemble the CPE in structure, but which do not bind the CPE-binding polyadenylation factor CPEB, failed to induce unmasking. When fused to the chloramphenicol acetyl transferase (CAT) coding region, the cyclin B1 3' UTR inhibited CAT translation in injected oocytes. In addition, a synthetic 3' UTR containing multiple copies of the CPE also inhibited translation, and did so in a dose-dependent manner. Furthermore, efficient CPE-mediated masking required cap-dependent translation. During the normal course of progesterone-induced maturation, cytoplasmic polyadenylation was necessary for mRNA unmasking. A model to explain how cyclin B1 mRNA masking and unmasking could be regulated by the CPE is presented.

Keywords: cyclins/cytoplasmic polyadenylation/meiotic maturation/translational control/*Xenopus*

Introduction

Translational control of maternally inherited mRNA plays an important role in the oogenesis and early development of both vertebrates and invertebrates (Cooperstock and Lipshitz, 1997; Goodwin and Evans, 1997; Hake and Richter, 1997; Stebbins-Boaz and Richter, 1997). These dormant mRNAs, which are synthesized and stored in the growing oocyte, become translationally activated during the completion of meiosis (oocyte maturation), or during early embryogenesis. For example, the polyadenylation-induced translation of *bicoid* mRNA in *Drosophila* and *c-mos* mRNA in the mouse and *Xenopus* are essential for the early development of these organisms (Gebauer *et al.*, 1994; Salles *et al.*, 1994; Sheets *et al.*, 1995). While the sequences conferring cytoplasmic polyadenylation in *Drosophila* are unknown, they have been extensively

characterized in maturing *Xenopus* and mouse oocytes. Two elements in the 3' untranslated regions (3' UTRs) of responding mRNAs are required, the cytoplasmic polyadenylation element (CPE, consensus structure of UUUUA₁₋₂U) and the polyadenylation hexanucleotide (AAUAAA) (Fox *et al.*, 1989; McGrew *et al.*, 1989; Paris and Richter, 1990; Sheets *et al.*, 1994; Stebbins-Boaz and Richter, 1994; Stebbins-Boaz *et al.*, 1996). The CPE is bound by the cytoplasmic polyadenylation element-binding protein CPEB, which is essential for polyadenylation (Hake and Richter, 1994; Stebbins-Boaz *et al.*, 1996). While the mechanism of polyadenylation-induced translation is unclear, at least some mRNAs undergo an obligate cap-specific 2'-O-methylation (Kuge and Richter, 1995; Gillian-Daniel *et al.*, 1998; Kuge *et al.*, 1998). Alternatively, or perhaps in addition to the cap methylation, the translation activation of mRNA may be mediated by poly(A)-binding protein–translation initiation factor interactions (Hentze, 1997; Le *et al.*, 1997; Craig *et al.*, 1998; Preiss and Hentze, 1998).

In a large number of cases, translational repression in oocytes and early embryos is correlated with a short poly(A) tail, generally 30–50 nucleotides. In mouse oocytes, the short poly(A) tail of tissue-type plasminogen activator (tPA) mRNA was shown to be due to the cytoplasmic deadenylation of transcripts which were appended with the usual long (~200 nt) poly(A) tail in the nucleus. This deadenylation, which occurs in growing oocytes, is dependent on the same sequence element that conveys cytoplasmic polyadenylation upon oocyte maturation, i.e., the CPE, also referred to as the adenylation control element or ACE (Huarte *et al.*, 1992). In this same vein, some mRNAs with long poly(A) tails that are translated in mature *Xenopus* oocytes undergo a specific *cis* element-directed deadenylation following fertilization, which results in translational repression (Bouvet *et al.*, 1994; Stebbins-Boaz and Richter, 1994; Paillard *et al.*, 1998). Finally, other mRNAs which have long poly(A) tails and are translated in the immature oocyte, such as the ribosomal protein mRNAs, undergo deadenylation and translational repression during oocyte maturation. This deadenylation is a default mechanism that does not require any specific sequences in the mRNA (Varnum and Wormington, 1990).

While a short poly(A) tail may explain inefficient translation of some maternal mRNAs such as the one encoding *Xenopus* histone B4, it should be borne in mind that low, but detectable levels of this message are found on polysomes in immature oocytes. These cells also contain a substantial amount of B4 protein, presumably accumulated during the long period of oogenesis (Dworkin *et al.*, 1985; Smith *et al.*, 1988; De Moor and Richter, 1997). However, other maternal mRNAs encoding such proteins as the vertebrate regulator of meiosis, Mos, need

to be repressed more strongly in oocytes to prevent untimely accumulation of the protein. Even low levels of Mos could induce oocyte maturation (Ferrell and Machleder, 1998) and, if this occurs precociously, it would abort normal development.

Like Mos, the cyclins have a crucial role in the initiation and completion of meiosis (Taieb *et al.*, 1997). The mRNAs for cyclin B are translationally regulated in the oocytes and/or early embryos of a large number of animals (Kobayashi *et al.*, 1991; Dalby and Glover, 1992; Galas *et al.*, 1993; Sheets *et al.*, 1994; De Moor and Richter, 1997). Vertebrate oocytes complete the S phase of meiosis I early in their development and are arrested in prophase during the growth phase of oogenesis. In *Xenopus*, while significant levels of cyclin B2 accumulate during this time, the levels of cyclin B1 are very low and the mRNA is absent from the polysomal fraction (Gautier *et al.*, 1990; Kobayashi *et al.*, 1991; De Moor and Richter, 1997). Progesterone induces the resumption of meiosis by activating the cdc2/cyclin B kinase complex, also called maturation-promoting factor (MPF). If levels of the cdc2-binding cyclins are elevated by oocyte injection of protein or mRNA, meiosis I occurs independently of progesterone treatment, indicating that a strict control of cyclin levels is important for the hormone dependence of maturation (Swenson *et al.*, 1986; Westendorf *et al.*, 1989; Roy *et al.*, 1990). After germinal vesicle breakdown at the end of meiosis I, most of the cyclin B2 is destroyed while a greatly enhanced translation of cyclin B1 mRNA occurs concomitant with its polyadenylation (Kobayashi *et al.*, 1991; De Moor and Richter, 1997). This increased synthesis of cyclin B1 prevents a complete shutdown of cdc2/cyclin B activity between meiosis I and II, which is essential to prevent the formation of a nuclear envelope and the initiation of DNA replication. Further accumulation of cyclin B induces full cdc2/cyclin B activity and initiates meiosis II, which is followed by an anaphase arrest until fertilization (Taieb *et al.*, 1997).

In a previous study, we showed that the polyadenylation-induced translation of cyclin B1 mRNA required the activation of cdc2/cyclin B and depended upon the arrangement of the CPEs in the 3' UTR (De Moor and Richter, 1997). During the course of these experiments, we noticed that oocytes injected with cyclin B1 3' UTR RNA fragments occasionally matured faster than uninjected controls. Here, we follow up on this observation and show that the injection of large amounts of cyclin B1 3' UTR RNA into oocytes induces cyclin B1 accumulation and cdc2/cyclin B activation in the absence of progesterone. Moreover, this translational activation of cyclin B1 mRNA occurs in the absence of polyadenylation. Mutational analysis of the injected RNA reveals that the CPE is sufficient for the unmasking activity. When fused to a reporter mRNA, the cyclin B1 3' UTR represses translation 10-fold in injected oocytes. The same effect is obtained when the reporter is appended with a 3' UTR containing multiple CPEs. Translation of the reporter-cyclin B 3' UTR mRNA is increased by 25-fold when the oocytes are induced to mature by progesterone. However, this translational activation (unmasking) is dependent upon cytoplasmic polyadenylation. We propose that, in immature oocytes, translational repression is maintained by a protein complex that binds the CPE. This repression can be alleviated experimentally

Fig. 1. Injection of the cyclin B1 3' UTR induces cyclin B1 synthesis. (A) Sequence of the cyclin B1 3' UTR fragment used in this study. Two CPEs (A and C), and a potential CPE (B), are indicated by ovals. The polyadenylation signal (HEX) is highlighted. (B) Time course of events after the injection of the cyclin B1 3' UTR RNA or a control polylinker RNA into immature *Xenopus* oocytes. Shown are, from top to bottom, a Western blot stained with anti-cyclin B1 antibody, a Northern blot probed with cyclin B1 cDNA, a histone H1 kinase assay, and Western blots stained with anti-Mos antibody and anti-CPEB antibody.

by injected CPEs, or normally by cytoplasmic polyadenylation. The role of CPEB in the masking/unmasking process is discussed.

Results

Injection of cyclin B1 3' UTR induces cyclin B1 translation

While assessing the polyadenylation of cyclin B1 mRNA in oocytes, we observed that the injection of its 3' UTR occasionally enhanced the rate of progesterone-induced maturation. To explore this further, we performed injection experiments with high concentrations of the cyclin B1 3' UTR fragment depicted in Figure 1A. It contains two consensus CPEs ('A' and 'C') and a third one ('B') that is suspected to function weakly (Stebbins-Boaz *et al.*, 1996; Hake *et al.*, 1998). This RNA fragment also includes the hexanucleotide AAUAAA (HEX). When 1.5 pmole of this RNA was injected into oocytes, cyclin B1 protein was synthesized and accumulated to a detectable level within 10 h as determined by Western blotting (Figure 1B). The injection of polylinker RNA did not have any effect on cyclin B1 protein levels. Even though the cyclin B1 3' UTR induced the synthesis of cyclin B1, it did not stimulate polyadenylation of the cyclin B1 mRNA (Figure 1B). This suggests that an inhibitory factor bound to the cyclin 3' UTR can be competed *in trans*, and that translation of cyclin B1 can be activated in the absence of polyadenylation.

As stated in the Introduction, elevated levels of cyclin B1 would be expected to induce maturation in the absence

of progesterone. Indeed, germinal vesicle breakdown (GVBD) occurred in 85% of the oocytes 20 h after 3' UTR injection, which was indicated by the appearance of a white spot at the animal pole and the activation of MPF as measured by the histone H1 kinase assay (Figure 1B). Furthermore, the rate and final percentage of maturation increased with increasing amounts of injected RNA (results not shown). This demonstrates that the translational masking of cyclin B1 mRNA is essential for the maintenance of the immature state of the oocyte.

To determine whether the injection of cyclin B1 3' UTR unmasks other mRNAs in oocytes, we examined Mos accumulation. In contrast to cyclin B1, Mos was not detected until 25 h post-injection, and even then, only weakly (Figure 1B). This accumulation is most likely to be due to a feedback loop from active MPF that enhances *c-mos* mRNA translation (Gotoh *et al.*, 1995; De Moor and Richter, 1997). These results indicate that the unmasking mediated by the cyclin B1 3' UTR is not a general phenomenon.

Because the cyclin B 3' UTR contains several binding sites (CPEs) for the cytoplasmic polyadenylation factor CPEB, we investigated whether the injection of this RNA influenced the phosphorylation or abundance of this factor. Phosphorylation of CPEB occurs at GVBD in maturing oocytes and is manifested by a mobility shift when examined by SDS gel electrophoresis. Commensurate with phosphorylation, most of the protein is degraded (Hake and Richter, 1994; De Moor and Richter, 1997). As shown in the last panel of Figure 1B, CPEB was stable until it was phosphorylated ~10 h after cyclin B1 was synthesized. This suggests that the modification or degradation of CPEB is not involved in the unmasking of cyclin B1 mRNA by 3' UTR injection.

Translation of cyclin B mRNA is induced by injection of the CPE

To precisely define the regions of the cyclin B1 3' UTR that induces cyclin B1 synthesis, we injected several additional RNAs and harvested the oocytes after 12 h. First, as negative and positive controls, we again demonstrate that while injected polylinker RNA had no effect, injected cyclin B1 3' UTR (sB1) induced the synthesis of cyclin B1 protein. A mutated cyclin B1 3' UTR (sB1-CPE0), which contains U to G transversions in all three CPEs, had an only slightly reduced capacity to induce cyclin B1 translation. However, the cyclin B1 levels induced by this RNA were inconsistent, since in about one-half of our experiments this RNA induced very little cyclin B1 synthesis (experiments 2 and 3 in Figure 2A). Moreover, the injection of this RNA never induced GVBD. The histone B4 mRNA 3' UTR, which contains a CPE and a hexanucleotide (AAUAAA), but otherwise bears no resemblance to the cyclin B1 3' UTR, induced cyclin B1 synthesis and GVBD. Similarly, a polylinker RNA containing a single CPE (CPE1) induced cyclin B1 mRNA translation as well. Finally, polylinker RNA containing only the AAUAAA hexanucleotide was unable to stimulate cyclin B1 synthesis. Taken together, these results demonstrate that a CPE is necessary and sufficient to reproducibly induce translation of endogenous cyclin B1 mRNA in injected into oocytes.

We have constructed two point mutations of CPE1

Fig. 2. Injection of the CPE is sufficient to induce cyclin B1 synthesis. Various RNAs were injected into immature oocytes, 1.5 pmol of RNA/oocyte and incubated for 12 h. Extracts were analyzed by Western blotting for cyclin B1 (cycB1) and MAP kinase as a loading control (MAPK). (A) C-P refers to no RNA injection, immature oocyte and C+P refers to no RNA injection but the oocytes were matured with progesterone. For the subsequent lanes, immature oocytes were injected with control RNA (polylinker), cyclin B1 3' UTR (sB1), cyclin B1 3' UTR with mutated CPEs (sB1-CPE0), histone B4 3' UTR (B4 3' UTR), a single CPE in polylinker sequence (CPE1) and a hexanucleotide (AAUAAA) in polylinker sequence (Hex). (B) Oocytes were injected with CPE1-containing RNA and other RNAs containing the denoted mutations in this sequence. (C) Sequences of RNAs containing U-rich elements. The black boxes indicate polylinker sequences and open boxes indicate other 3' UTR sequences. (D) Western blot for cyclin B1 from oocytes injected with the RNAs depicted in (C).

RNA by substituting U for G residues at critical positions. These mutations, which were expected to render these sequences non-functional for cytoplasmic polyadenylation, did not induce cyclin B1 synthesis following oocyte injection (Figure 2B). However, another mutation of CPE1, UUUUUAAU to UUUUUAAU, which can promote cytoplasmic polyadenylation (Stebbins-Boaz *et al.*, 1996), did activate cyclin B1 mRNA translation. These results demonstrate that a functional CPE is necessary for the

induction of cyclin B1 synthesis by CPE-like sequences *in trans*.

AU-rich elements (AREs) have been implicated in translational repression, most notably those present in several cytokine mRNAs (Kruys *et al.*, 1989; Jacob and Tashman, 1993; Crawford *et al.*, 1997). To test whether these elements can induce cyclin B1 mRNA translation, we synthesized RNAs containing the AREs of the granulocyte-macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor- α (TNF- α) 3' UTRs and injected them into oocytes. As depicted in Figure 2C, these RNAs contain large numbers of A and U residues, but no known CPEs (UUUUAU or UUUUAU). These RNAs failed to induce cyclin B1 synthesis in injected oocytes (Figure 2D). In addition, we tested a synthetic ARE (SYN) and a different type of cytoplasmic polyadenylation element, the embryonic CPE of Cl2, which is very U-rich. These RNAs also did not activate cyclin B1 mRNA translation. As is usual for capped RNAs in the *Xenopus* oocyte, all RNAs tested were similarly stable, with 45–35% remaining after this incubation time (results not shown). These results demonstrate that the unmasking of cyclin B1 mRNA by the injected RNA is specific for the maturation-type CPE.

Multiple CPEs are sufficient for masking

While the experiments described above demonstrate that the injection of CPE RNA is sufficient to unmask endogenous cyclin B1 mRNA, they do not address the question of what sequences are sufficient for masking *in cis*. To investigate this, we constructed a series of chloramphenicol acetyl transferase (CAT) mRNAs containing the cyclin B1 3' UTR and mutated forms of this 3' UTR. As a control and a point of reference, we used a CAT mRNA without any cyclin B1 3'UTR insert (T3CAT). To avoid possible competition for putative masking factors, only 0.5 fmole of CAT mRNA was injected, 3000-fold less RNA (in moles) than was injected in the previous set of experiments. As shown in Figure 3A, the cyclin B1 3' UTR repressed the translation of T3CAT ~10-fold (T3CAT-sB1) in immature oocytes from two different frogs. CAT mRNA containing the cyclin B1 3' UTR with a mutated hexanucleotide also was strongly repressed. However, the translation of a chimeric RNA containing mutations in the CPEs (T3CAT-sB1-CPE0) was only partially repressed, by only ~2.5-fold compared with T3CAT.

To investigate whether the CPE alone is sufficient for translational repression, we constructed an additional set of CAT mRNAs with multiple copies of CPE1 (Figure 2B) inserted in their 3' polylinker sequences. As shown in Figure 3B, increasing numbers of CPEs repressed translation in a dose-dependent manner. One CPE repressed translation slightly in the oocytes of two out of three frogs; two and three CPEs repressed translation progressively in the oocytes of all three frogs. We therefore conclude that the CPE is a masking element, capable of acting *in cis* as an inhibitor of translation.

Polyadenylation is essential for mRNA unmasking during oocyte maturation

During progesterone-induced maturation, cyclin B1 mRNA undergoes CPE-dependent polyadenylation and translational activation (Sheets *et al.*, 1994; Stebbins-Boaz

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Fig. 3. CPE-mediated masking of a reporter mRNA. (A) CAT assays were performed on batches of 4 or 5 oocytes injected with the denoted CAT mRNAs that contained various cyclin B1 3' UTRs. sB1 RNA contained both CPEs and polyadenylation hexanucleotide, sB1-CPE0 RNA contained no CPE, and sB1dHex RNA contained no hexanucleotide. The patterned bars in all panels indicate experiments using oocytes from different frogs. (B) CAT assays were performed on oocytes injected with CAT mRNAs containing increasing numbers of CPEs in their 3' polylinker sequence. The nomenclature of the CAT mRNAs reflects the difference between the two CAT vectors used: pT3CAT (T3CAT) and pCT3 (CAT) differ in their 5' polylinker regions by 15 nucleotides.

and Richter, 1994; De Moor and Richter, 1997). Yet, the results presented above indicate that the CPEs in cyclin B1 mRNA have a second function, that of translational repression in the immature oocyte. To explore the relationship between polyadenylation and translational activation (unmasking), we injected the CAT mRNAs containing the various cyclin B1 3' UTRs into oocytes and examined their polyadenylation and translation following progesterone treatment. Figure 4A shows Northern blots of CAT mRNA from injected oocytes, either frozen immediately after injection or incubated with and without progesterone. It is evident that all mRNAs were similarly stable in mature and immature oocytes. The CAT mRNA containing the full cyclin B1 3' UTR (T3CAT-sB1) was polyadenylated upon progesterone-induced maturation, which was abolished by mutation of the CPEs (T3CAT-sB1-CPE0) or the hexanucleotide (T3CAT-sB1-dHex). None of the CAT mRNAs containing the synthetic CPEs (only CAT-3CPE1 is shown) were polyadenylated, which was expected because these constructs lack the polyadenylation hexanucleotide.

The histogram in Figure 4A depicts the ratio of CAT activity in progesterone-treated (mature) oocytes relative to that present in immature oocytes. Maturation resulted in an increase in CAT activity by ~25-fold in the two experiments, demonstrating that the cyclin B1 3' UTR is sufficient to mask as well as unmask mRNA. However, deletion of the hexanucleotide abolished the unmasking.

Fig. 4. mRNA unmasking requires cytoplasmic polyadenylation. (A) CAT assay from oocytes injected with various chimeric mRNAs and incubated in the presence or absence of progesterone. The graph shows the CAT activity in progesterone-matured oocytes relative to the activity in immature oocytes. The dotted line indicates the level of no stimulation. Also shown are Northern blots of CAT mRNA from oocytes injected with the noted chimeric mRNAs. The oocytes were immediately frozen after injection (0), or incubated in the absence (-) or presence (+) of progesterone. (B) CAT assay from oocytes injected with polyadenylated mRNA. Three CAT mRNAs were injected with and without a 30 nucleotide poly(A) tail and incubated in the presence or absence of progesterone. The CAT activity was then determined and normalized for the amount of mRNA injected.

Similarly, the CAT mRNA with three synthetic CPEs in the 3' UTR (CAT-3CPE1) was not translationally activated upon maturation. The residual repression observed when the CPEs of sB1 were mutated (T3CAT-sB1-CPE0) also was not relieved by maturation. These data show that during the normal course of oocyte maturation, mRNA unmasking requires cytoplasmic polyadenylation.

To determine whether a poly(A) tail is sufficient to unmask mRNA, we injected oocytes with various CAT mRNAs containing a short poly(A) tail. This 30 nucleotide appendage stimulated the translation of a control CAT mRNA (T3CAT) ~3-fold in the immature oocyte, but failed to unmask CPE containing mRNAs lacking a hexanucleotide (T3CAT-sB1-dHEX, CAT-3CPE1). Progesterone-induced maturation also did not enhance translation of these constructs. This is in contrast to the situation in maturing mouse oocytes, where a short poly(A) tail is

sufficient to unmask mRNAs containing CPE sequences, even in the absence of a hexanucleotide (Stutz *et al.*, 1998). We conclude that a short poly(A) tail is insufficient to induce unmasking, both in the immature and the mature *Xenopus* oocyte.

CPEB binds unmasking sequences

The simplest explanation for the ability of the CPE to induce translation *in trans* and inhibit it *in cis* is that it binds a repressor protein. The only factor known to bind the maturation-type CPE is CPEB (Hake and Richter, 1994). Thus, either CPEB is a masking protein as well as a cytoplasmic polyadenylation-inducing protein, or there is another factor that also binds the CPE and can inhibit translation. To characterize the proteins that bind the CPE, we first used UV crosslinking to the CPE1 RNA, which contains a CPE in a polylinker context and an identical polylinker RNA lacking this insert (Figure 2). As shown in Figure 5A, the only protein that crosslinked specifically to the CPE was CPEB. Secondly, we performed gel shifts with oocyte extracts using CPE1 as a probe. A single prominent shifted band was observed, which contained CPEB as evidenced by the fact that it was supershifted by CPEB antibody (Figure 5B). A minor, slower migrating complex probably also contained CPEB because it was supershifted by CPEB antibody, but this was observed in only one batch of extract.

To test the binding of CPEB to the RNAs used in the unmasking assays depicted in Figure 2, we performed gel-shift competitions, again using CPE1 as the probe. The cyclin B1 3' UTR (sB1), as expected, competed efficiently, as did CPE1 RNA itself (Figure 5C). Mutation in the CPEs in the cyclin B1 3' UTR abolished the competition for CPEB binding (sB1-CPE0), as reported previously (Stebbins-Boaz *et al.*, 1996; Hake *et al.*, 1998). Polylinker RNA (PL) did not compete for binding, nor did the GM-CSF ARE. This last result indicates that CPEB has a very high degree of specificity, because many CPE-like sequences appear in this RNA (Figure 2D). These data show that there is a strong correlation between native CPEB binding and cyclin B1 unmasking by RNAs *in trans*.

Finally, we produced CPEB in a coupled transcription-translation system and used this protein in gel-shift experiments. Wild-type CPEB produced in this manner again shifted CPE1 RNA as described for the oocyte extracts, confirming that this band represents an RNA-CPEB complex (Figure 5D, CPE1, WT). This shift was completely dependent upon the presence of a functional CPEB, because a mutant form of the protein which is incapable of RNA binding, due to two point mutations in its zinc finger (Hake *et al.*, 1998), did not produce a shifted band (Figure 5D, CPE1, A₃A₇). The two mutated CPEs that were incapable of inducing cyclin B1 mRNA translation in Figure 2B, were not shifted by CPEB (CPE1 M1, M2). CPE2, which does unmask cyclin B1 mRNA, formed a shifted complex with wild-type CPEB which was indistinguishable from that formed with CPE1. We therefore conclude that the CPE masking elements in the cyclin B1 mRNA are bound by CPEB with high specificity. Closely related sequences that do not bind CPEB do not unmask *in trans*. These results suggest that CPEB is involved in the masking process.

Fig. 5. CPEB is the only detectable CPE-binding protein. **(A)** Proteins binding to CPE1 and polylinker RNA (CPE1, PL, see Figure 2) were analyzed by UV crosslinking. The sizes of the protein markers (in kDa) are indicated, as is CPEB. **(B)** CPE1 RNA was incubated with (+) or without (–) extract, in the presence or absence of CPEB antiserum, or normal rabbit serum (control). **(C)** CPE1 RNA was incubated with (+) or without (–) oocyte extract and the denoted competitor RNAs at a 20–2000-fold molar excess. **(D)** A coupled transcription/translation lysate (TNT lysate) was incubated with (+) or without (–) a wild-type (WT) or RNA-binding deficient (A_3A_7) CPEB encoding plasmid. The lysates were then used for gel mobility shift assays with the indicated RNAs indicated as probes.

Efficient CPE-mediated masking requires cap-dependent translation

Translation initiation normally occurs at the 5' end of an mRNA and requires the recruitment of eIF-4G to the eIF-4E-cap complex. One possible mode of CPE-mediated masking could be an interference with this early step of translation. To test this hypothesis we employed the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV), an RNA sequence that can bind eIF-4G independently of eIF-4E and thus initiate translation in a cap-independent manner (Pestova *et al.*, 1996; Kolupaeva *et al.*, 1998). In addition to conferring cap-independent initiation, the strong secondary structure of this 5' UTR also precludes cap-mediated translation. We appended a 3' UTR containing 3 CPEs (ECAT-3CPE1) to the end of CAT mRNA that included the EMCV IRES at

its 5' end (ECAT, (Jang *et al.* 1988). While the ECAT mRNA was translated less efficiently than T3CAT, this was not influenced by the co-injection of cap analog. This is in contrast to the translation of T3CAT, which was repressed by >500-fold when the cap analog was co-injected (Figure 6A). Thus, the EMCV internal initiation site is functional in *Xenopus* oocytes.

As shown in Figure 6B, the translation of the CAT-3CPE1 construct was repressed an average of 20-fold when compared with T3CAT. In contrast, the translation of ECAT-3CPE1 mRNA was repressed by only 2.5-fold compared with ECAT. No differences in stability were detected for the control and CPE containing mRNAs (results not shown). Thus, we infer that CPE-mediated masking interferes predominantly with the early, cap-dependent step of translation initiation.

Discussion

In this report, we show that the CPEs of the cyclin B1 3' UTR (sB1) are translational masking elements in the immature oocyte. The endogenous cyclin B1 mRNA can be activated by the injection of either sB1 RNA or an RNA containing a synthetic CPE (CPE1), indicating that these RNAs compete for a translational repressor. This effect is highly specific for CPE sequences and cannot be mimicked by other AREs (Figure 2). Competition for this repressor does not induce the polyadenylation of the cyclin B1 mRNA, since none is observed in the sB1 injected oocytes (Figure 1). In addition, the cyclin B1 3' UTR can repress the translation of a reporter mRNA *in cis* (Figure 3). Insertion of increasing numbers of CPEs in the 3' UTR of the reporter results in increasing repression, indicating that CPEs alone are sufficient for masking. Because the unmasking of repressed reporter mRNAs during oocyte maturation requires the presence of an AAUAAA hexanucleotide (Figure 4), we propose that cytoplasmic polyadenylation is essential to activate the translation of mRNAs containing multiple CPEs. These results demonstrate that the CPE is a bifunctional translational control element; it activates translation by way of cytoplasmic polyadenylation during oocyte maturation and inhibits translation in the immature oocyte.

The only protein known to bind the CPE is CPEB, which is essential for cytoplasmic polyadenylation (Hake and Richter, 1994; Stebbins-Boaz *et al.*, 1996). Although indirect, our data suggest that CPEB is also the protein mediating the CPE-induced translational repression. This is based on the fact that in UV crosslinking assays and RNA gel retardations the only CPE-binding protein we could detect was CPEB (Figure 4). Moreover, CPEB binding to the CPE is strongly correlated with translational repression. We have conducted a number of experiments to assess directly whether CPEB inhibits translation in a CPE-dependent manner. These include the addition of recombinant CPEB to *in vitro* translation systems, transfection of CPEB expression constructs into tissue culture cells, and overexpression of CPEB in oocytes by mRNA injection. In no case did we definitively observe specific translational inhibition (results not shown). This leads us to speculate that, while CPEB is involved in the masking process, it is not the only factor that is required. When bound to the CPE, CPEB could possibly recruit other

Fig. 6. Efficient CPE-mediated masking requires cap-dependent translation. (A) CAT activity in oocytes injected with mRNAs lacking (T3CAT) or containing (ECAT) the EMCV sequences in the presence (+) or absence (-) of cap analog. (B) CAT activity in oocytes injected with chimeric mRNAs containing (3CPE1) or lacking (T3CAT) CPEs in the presence (ECAT, ECAT-3CPE1) or absence (T3CAT, CAT-3CPE1) of an IRES.

Fig. 7. A model for masking and unmasking of cyclin B1 mRNA. The 3' UTR of cyclin B1 mRNA contains several CPEs that are bound by CPEB. The CPEB molecules recruit a hypothetical cap-binding protein complex (?), possibly including eIF-4E, which renders the cap inaccessible to (other) translation initiation factors. Excess injected CPE-containing RNA binds CPEB, thereby removing the masking complex from the endogenous mRNA. This allows the cap to bind a full complement of translation initiation factors. During normal, progesterone-induced oocyte maturation, the hexanucleotide-binding factor [HBF, possibly CPSF (compare with Bilger *et al.*, 1994)], which, in turn recruits poly(A) polymerase (PAP), resulting in polyadenylation of the mRNA. PAP may be complexed with a 2'-O-methyltransferase, which methylates the cap (CH_3). This modified mRNA has a higher affinity for translation initiation factors. These events displace or inactivate the CPEB-bound cap-binding complex and release the cap for the binding of initiation factors.

factors to the mRNA that are ultimately responsible for preventing translation initiation.

How can the presence of CPEs in the 3' UTR mediate the repression of translation initiation at the 5' end of the mRNA? In the case described here, translation inhibition is independent of a poly(A) tail, and thus cannot involve the sequestration of the poly(A)-binding protein or the poly(A) tail. Consequently, the masking by CPEs must somehow inhibit the formation of initiation complexes at the 5' end of the mRNA. To decipher which steps of translation initiation are affected by masking, we used an internal ribosome entry sequence, which circumvents the need for the cap and its binding factor eIF-4E. A similar strategy has been used to demonstrate that in *Drosophila*, the masking of *caudal* mRNA by the binding of Bicoid to its 3' UTR is abolished by internal initiation, while the repression of *hunchback* mRNA by Pumilio is unaffected (Dubnau and Struhl, 1996; Wharton *et al.*, 1998). Similarly, translational repression of 15-lipoxygenase mRNA by hnRNP K and hnRNP E1 is not abolished by internal initiation (Ostareck *et al.*, 1997). As described in Figure 5B, CPEs repress internal initiation of translation much

less than normal cap-dependent translation (2.5-fold versus 20-fold). This implies that the CPE-bound masking complex interferes with the translation initiation factor interactions that are not necessary for cap-independent initiation. A schematic representation of our current model is shown in Figure 7. We envision that multiple CPEB protein molecules bind to cyclin B1 mRNA before maturation. They interact specifically with other, as yet unknown, proteins to render the cap inactive in the initiation of translation, either by competing for cap binding with eIF-4E or by blocking the association of eIF-4E with eIF-4G. This last proposition is reminiscent of the general translation repression mediated by the 4E-BP family. These proteins bind eIF-4E in the same region that associates with eIF-4G, thus inhibiting cap-dependent initiation (Proud and Denton, 1997; Kleijn *et al.*, 1998). Possibly, the CPE-bound masking complex includes such a eIF-4E-binding protein, which would make the translational inhibition mediated by this protein mRNA-specific. The results outlined in Figure 4 demonstrate that the mechanism of unmasking is dependent on cytoplasmic polyadenylation. It is possible that polyadenylation dis-

rupts an interaction between the cap or eIF-4E and the putative CPEB-binding protein. Alternatively, or perhaps in addition, the poly(A) tail elongation by poly(A) polymerase and modification of the cap by the associated 2'-O-methyltransferase could enhance the affinity of the mRNA for the translation initiation machinery and shift a competitive equilibrium, leading to unmasking and efficient translation (Kuge and Richter, 1995; Hentze, 1997; Le *et al.*, 1997; Craig *et al.*, 1998; Kuge *et al.*, 1998; Preiss and Hentze, 1998). The phosphorylation and partial degradation of CPEB in the maturing oocyte may also contribute to unmasking, but these events are insufficient to induce unmasking in the absence of polyadenylation. In the injected oocyte, the large excess of CPEs most probably titrates CPEB and its associated proteins from the cyclin B1 mRNA, releasing the cap for translation initiation without the requirement of cytoplasmic polyadenylation.

While we were preparing this manuscript, Stutz *et al.* (1998) reported that the CPE-containing region of tPA mRNA (referred to as an ACE, 41 nucleotides long) is a masking element in mouse oocytes. This indicates that both masking and cytoplasmic polyadenylation are conserved functions of the CPE in vertebrates (Stutz *et al.*, 1998). However, in contrast to our findings with cyclin B1 mRNA, the unmasking of tPA does not require the hexanucleotide. As depicted in Figure 4B, even the addition of a short poly(A) tail does not relieve the masking mediated by CPEs in the mature *Xenopus* oocyte. This could reflect a difference between these two species or between the CPE-containing mRNAs.

In *Drosophila*, the mRNA for cyclin B is translationally repressed and localized to the prospective germ plasm. Strikingly, a small deletion that abolishes the repression but not the localization of this mRNA contains a sequence that strongly resembles a CPE (Dalby and Glover, 1993). Similarly, translation repression of cyclin A and ribonucleotide reductase mRNAs in clam oocytes is mediated by 3' UTR elements containing CPE sequences, which might suggest that the translational repression of cyclin mRNA by CPEs is widely conserved in evolution (Standart *et al.*, 1990).

The data presented in Figures 2 and 3 suggest that an additional, weaker, translational repressor seems to be located in the cyclin B1 3' UTR. This repressor, which is present in the sB1 mutant lacking all CPEs (sB1-CPE0), can both unmask *in trans* and mask *in cis*, albeit much less efficiently and reliably than sB1 or the CPEs alone. In UV crosslinking assays, sB1 and sB1-CPE0 both specifically label a protein of ~50 kDa, which is not detected when CPE1 RNA is used as a probe (results not shown). Possibly, this represents a second masking protein that contributes to the translational silencing of cyclin B1 mRNA.

In conclusion, we propose that CPEB mediates translation repression in the immature oocyte and translational activation by cytoplasmic polyadenylation in the maturing oocyte. The switch between these two functions is likely to be controlled by CPEB-associated proteins. Thus, our future efforts will be directed towards the isolation and identification of these proteins. One eventual goal will be to assemble appropriate translational control of cyclin B1 mRNA *in vitro* with purified components.

Materials and methods

Oocyte preparation and injection

Ovarian lobes were removed from full-grown *Xenopus laevis* females and treated with collagenase and dispase (Kuge and Richter, 1995). Stage VI oocytes were cultured in Barth's medium and, occasionally, with progesterone (10 μ M) to induce oocyte maturation. Four to five oocytes per sample were injected with competitor RNAs (70–80 nl per oocyte at a concentration of 20 μ M; 1.4–1.6 pmol or ~65 ng for these RNAs), followed by incubations for 12–15 h, unless indicated otherwise. Oocytes were also injected with CAT mRNAs (30–50 nl per oocyte at a concentration of 10 nM; 0.3–0.5 fmol or ~100 pg), which were trace-labelled with [³²P]UTP to estimate the injected volume. When indicated, the CAT mRNA solution also contained cap analog (⁷mGpppG) at a concentration of 4 mM. The CAT mRNA-injected oocytes were incubated for 7–10 h, or ~1–2 h after 90% of the oocytes had matured. For each sample 4 or 5 oocytes were then collected and stored frozen until processed further.

Plasmid construction and RNA synthesis

Plasmids pscyclin B1 (sB1), pscyclin B1-CPE0 (sB1-CPE0), pB4.3F (B4 3' UTR) and Cl2 Δ 1-454 (Cl2) have been described previously (Simon and Richter, 1994; Stebbins-Boaz *et al.*, 1996; De Moor and Richter, 1997). A hexanucleotide mutant (AAUAAA to AAUAGA) of pscyclin B1 was created by PCR using pscyclin B1 with primers 5' sB1 (CGGGATCCTGTTGGCACCATTGTGCTC) and 3' sB1-dHex (GCGAATTCATGTTAAATGAGCTCTATTAAAAACC). The PCR product was digested with *Eco*RI and *Bam*HI and cloned into the corresponding sites in pBluescriptII KS- (Stratagene). pT3-CPE1 (CPE1) was created by inserting oligonucleotides CPE-a (AATTCATTAAAAAC) and CPE-s (AATTGTTTAAATG) into the *Eco*RI site of pBluescriptII SK- in the T3 sense direction. Mutants of this CPE were cloned in an identical manner (for sequences, see Figure 2B). Multiple CPEs were constructed by sequential insertions of oligonucleotides into the recreated *Eco*RI site. A plasmid containing a single hexanucleotide, pT3-Hex, was created by inserting oligonucleotides Hex-a (AGCTGAGCTCTTTTATTA) and Hex-s (AGCTTAATAAGAGCTC) into the *Hind*III site of pBluescriptII SK- in the T3 sense direction. Plasmids containing the GM-CSF, TNF α and SYN AREs were derived from pBBB plasmids donated by A.-B. Shyu (Shaw and Kamen, 1986; Chen *et al.*, 1992; Xu *et al.*, 1997). Sau3A fragments containing the AREs were cloned into the *Bam*HI site of pBluescriptII SK-. Plasmids containing T7 sense-orientated inserts (pT7-GM-CSF, pT7-TNF, pT7-Syn) were used for the oocyte injections, while pT3-GM-CSF was used in the gel-shift assay.

The CAT mRNAs were made using the CAT coding region from pLeaderscript (De Moor *et al.*, 1995) fused to various 3' UTRs. For the cyclin B1 3' UTR and its mutants, the *Bam*HI CAT fragment was cloned into the *Bam*HI site (pT3CAT). For the synthetic CPEs, a *Hinc*II-*Bam*HI CAT fragment was blunt-ended and inserted in the *Sma*I site and the T3 sense orientation was selected (pCT3). The CAT constructs with the EMCV IRES were constructed by first ligating the *Eco*RI fragment from pBS-ECAT into the *Spe*I and *Eco*RI sites of pBluescript II SK- using an oligonucleotide linker (CTAGTGCCCGGGAC and AATTGCCCGGGCA). In the T3 sense orientation, this destroys the *Eco*RI site at the beginning of the IRES, but not the *Eco*RI site in the CAT coding region. Subsequently, the rest of the CAT coding region and the 3' UTR elements were added by inserting the *Eco*RI fragments of T3CAT (pT3ECAT) and CT3-3CPE1 (pT3ECAT-3CPE1) into the *Eco*RI site, and selecting for the T3 sense orientation.

To generate transcription templates for CAT mRNAs with short poly(A) tails, the appropriate plasmid was amplified by PCR using a sense oligonucleotide annealing upstream of the T3 promoter and an antisense oligonucleotide with an appendage of 30 T residues that annealed in the polylinker downstream of the 3' UTR.

For transcription, pscyclinB1 and its mutants were linearized with *Eco*RI, pT3-CPE1, its derivatives and pT3-Hex with *Hinc*II, pT7-GM-CSF, TNF and -Syn with *Xba*I, pT3-GM-CSF with *Eco*RI, pT3CAT and pT3ECAT and their derivatives with *Eco*RV, and pCT3 and its derivatives with *Hinc*II. All transcriptions were performed with T3 RNA polymerase, except for the pT7-ARE plasmids, which were transcribed with T7 RNA polymerase. Transcriptions for competitor and CAT RNAs were executed with the MessageMachine kit (Ambion), using trace [³²P]UTP label (<1 μ Ci) to quantify the RNA synthesized. RNAs were diluted to equal concentrations, denatured in 80% formamide, and analyzed on agarose gels in 1 $\times$ TBE (10.8 g/l Tris base, 5.5 g/l boric acid, 0.93 g/l EDTA)

stained with ethidium bromide. Probe RNA of high specific activity was made at 37°C as described previously (De Moor and Richter, 1997).

H1 kinase assays, Western and Northern blots

H1 kinase assays, Western blots and Northern blots were processed as described (De Moor and Richter, 1997).

UV crosslinking and RNA gel retardations

Crushed oocyte extract was used as a source of CPEB, prepared as described (Patrick *et al.*, 1989). As an alternative, CPEB was synthesized with the TNT-coupled transcription/translation systems (Promega) using the plasmids pHisCPEB and pHisCPEB-C⁵²⁹A/H⁵⁴⁷A (A₃A₇), an RNA-binding deficient double point mutant (Stebbins-Boaz *et al.*, 1996; Hake *et al.*, 1998). RNA probe (1 µl, 1 nM), with or without competitor RNA, was incubated with 0.2 µl extract or 1 µl TNT lysate for 20 min at room temperature in 200 mM KCl, 2 mM MgCl₂, 5 mM dithiothreitol, 20 mM HEPES pH 7, 5% glycerol, 0.2 mg/ml bovine serum albumin (BSA) (RNase free). Two µl of non-specific competitors was added (25 mg/ml heparin, 0.5 mg/ml *Escherichia coli* tRNA) and the mixture was incubated for a further 10 min. If indicated, anti-CPEB serum or normal rabbit serum was added after the non-specific competitors. Immediately after the end of this incubation, the mixture was subjected to electrophoresis on a 4% acrylamide gel (acrylamide:bisacrylamide: 40:0.5, 0.5 × TBE, 1.5 mm thick). The gels were dried and exposed to phosphorimaging screens.

UV crosslinking was executed on ice in 5 µl reactions containing 100 mM KCl, 2 mM MgCl₂, 5 mM dithiothreitol, 20 mM HEPES pH 7, 0.5 mg/ml heparin, 0.5 µl oocyte extract, 50 000 c.p.m. RNA probe. Subsequently, the mixture was digested with RNase A and applied to an SDS-polyacrylamide gel.

CAT assays

For CAT assays, 4 or 5 oocytes were homogenized in 200 mM Tris-HCl pH 8 (50 µl/oocyte), centrifuged for 1 min at 4°C and the supernatant collected and kept on ice. Fifty µl of this supernatant was used to determine the injected volume by Cerenkov counting. Twenty to one hundred µl extract was incubated with 200 mM Tris-HCl pH 8 or water to 100 µl, 5–10 µl acetyl coenzyme A (0.5 mg/ml), and 0.1 µCi ¹⁴C-chloramphenicol 37°C for 60 min. For the reliable detection of low amounts of CAT, the extract was preincubated for 10 min at 65°C and the reaction was incubated for 15 h at 37°C. The mixture was extracted with ethyl acetate and applied to silica gel TLC plates as described (Jones *et al.*, 1983). The percentage chloramphenicol conversion was determined using phosphorimaging screens after several days exposure. The percent conversion per fmol of mRNA was calculated using the injected mRNA concentration (10 nM) and the injected volume (30–50 nl), as indicated above.

Acknowledgements

We thank J.L.Maller for providing the cyclin B1 antibody, A.-B.Shyu for donating the pBBB plasmids containing the cytokine AU-rich elements, and E.Wimmer for the EMCV IRES-containing CAT clone. We also thank R.Mendez, B.Stebbins-Boaz, Q.P.Cao, J.Tay, N.Vlachakis, L.Wu and I.Groisman for helpful suggestions and stimulating discussions. This work was supported by NIH grant GM46779.

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Received December 8, 1998; revised and accepted February 19, 1999