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Dual roles of p82, the clam CPEB homolog, in cytoplasmic polyadenylation and translational masking

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ABSTRACT

In the transcriptionally inert maturing oocyte and early embryo, control of gene expression is largely mediated by regulated changes in translational activity of maternal mRNAs. Some mRNAs are activated in response to poly(A) tail lengthening; in other cases activation results from de-repression of the inactive or masked mRNA. The 3′ UTR *cis*-acting elements that direct these changes are defined, principally in *Xenopus* and mouse, and the study of their *trans*-acting binding factors is just beginning to shed light on the mechanism and regulation of cytoplasmic polyadenylation and translational masking. In the marine invertebrate, *Spisula solidissima*, the timing of activation of three abundant mRNAs (encoding cyclin A and B and the small subunit of ribonucleotide reductase, RR) in fertilized oocytes correlates with their cytoplasmic polyadenylation. However, in vitro, mRNA-specific unmasking occurs in the absence of polyadenylation. In Walker et al. (in this issue) we showed that p82, a protein defined as selectively binding the 3′ UTR masking elements, is a homolog of *Xenopus* CPEB (cytoplasmic polyadenylation element binding protein). In functional studies reported here, the elements that support polyadenylation in clam egg lysates include multiple U-rich CPE-like motifs as well as the nuclear polyadenylation signal AAUAAA. This represents the first detailed analysis of invertebrate *cis*-acting cytoplasmic polyadenylation signals. Polyadenylation activity correlates with p82 binding in wild-type and CPE-mutant RR 3′ UTR RNAs. Moreover, since anti-p82 antibodies specifically neutralize polyadenylation in egg lysates, we conclude that clam p82 is a functional homolog of *Xenopus* CPEB, and plays a positive role in polyadenylation. Anti-p82 antibodies also result in specific translational activation of masked mRNAs in oocyte lysates, lending support to our original model of clam p82 as a translational repressor. We propose therefore that clam p82/CPEB has dual functions in masking and cytoplasmic polyadenylation.

Keywords: 3′UTR; masked mRNA; RNA-binding proteins; translational control

INTRODUCTION

In maturing oocytes and early embryos, regulated recruitment of certain mRNAs onto polysomes, in the midst of other mRNAs undergoing polysomal dissociation, is one of the principal modes of control of gene expression (Curtis et al., 1995; Wickens et al., 1996). The identities of some members of these two classes of mRNA are known; for example *c-mos*, cyclins, histones, and the small subunit of ribonucleotide reductase (RR) mRNAs fall into the former class whereas housekeeping protein mRNAs encoding actin, tubulin, and ribosomal proteins fall into the latter. In view of the identities of the translationally activated mRNAs, it is not difficult to appreciate why their regulated expression is of paramount importance, at a stage of development when transcription is silenced. Despite considerable efforts in several laboratories, we do not yet understand in detail the mechanism of translational activation during early development. What is becoming clearer is that there are two major routes, namely cytoplasmic polyadenylation and de-repression; depending on the organism and more importantly probably the mRNA, one or the other and sometimes a mix of both mechanisms is employed.

In *Xenopus*, poly(A) lengthening of cytoplasmic mRNAs requires a U-rich tract, consensus $U_{4-6}A_{1-2}U$, known as the cytoplasmic polyadenylation element (CPE) in conjunction with a nearby nuclear signal (NPE) AAUAAA. While the presence of these two *cis*-elements allows the mRNA to undergo polyadenylation following

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progesterone-induced maturation, the sequence, context, and position of the CPE can influence the timing and extent of polyadenylation, which varies for different mRNAs (Sheets et al., 1994; Richter, 1996). These 3′ UTR elements function through specific *trans*-acting factors: CPEB (Hake & Richter, 1994; Gebauer & Richter, 1996) and CPSF (cleavage and polyadenylation specificity factor; Bilger et al., 1994) in conjunction with cytoplasmic poly(A) polymerase (Ballantyne et al., 1995; Gebauer & Richter, 1995). In the case of several mRNAs, the added A residues or the process of addition of A residues directly promotes translational recruitment, through a mechanism that is not yet understood (Richter, 1996; Wickens et al., 1996). A possible mechanistic explanation is suggested by the observation that cap ribose methylation of *Xenopus* B4 mRNA requires ongoing polyadenylation (Kuge & Richter, 1995). However, cytoplasmic polyadenylation is not always sufficient for the activation of protein synthesis, as shown recently for *Xenopus* FGFR mRNA (Culp & Musci, 1998). Cytoplasmic polyadenylation is an evolutionarily conserved mechanism in the translational activation of mRNAs in the early development of *Xenopus*, mouse, and *Drosophila* (Verrotti et al., 1996). The motifs and their binding factors that support this poly(A) extension in organisms other than *Xenopus* have not been characterized in detail.

The now classic studies of Rosenthal and Ruderman established the surf clam mollusc *Spisula solidissima* as a model system for the study of translational regulation in early development (Rosenthal et al., 1980, 1983; Rosenthal & Ruderman, 1987). Three abundant mRNAs, encoding cyclins A, B, and RR, are held in an inactive or masked state in the oocyte. Fertilization, which in this organism triggers meiotic maturation as well as subsequent development of the embryo, releases clam oocytes from prophase I arrest and the oocytes enter first meiotic M phase about 10 min later (Hunt et al., 1992 and references therein). The masked mRNAs are loaded onto polysomes within 20–25 min following fertilization, at a time when translationally regulated mRNAs undergo poly(A) extension (Rosenthal & Ruderman, 1987; Turner et al., 1995). The housekeeping class of mRNAs dissociates from polysomes in maturing oocytes concomitantly with their deadenylation, also observed about 25 min postactivation (Rosenthal & Ruderman, 1987). Sequence inspection of the 3′ UTRs of five translationally regulated *Spisula* mRNAs showed that they all contained the NPE AAUAAA (or AUUAAA), 8–12 nt from the A tail (Standart & Dale, 1993). The four translationally up-regulated mRNAs contained several copies of CPE-like U_{4-6}AA/U motifs in their 3′ noncoding regions, in contrast to actin mRNA which does not have tracts longer than 3Us throughout its 1.1-kb-long 3′ UTR. Initial studies showed that polyadenylation in clam lysates is stage- and RNA-specific (Standart & Dale, 1993).

Stage-specific differential translational activity is also preserved in cell-free lysates prepared from clam oocytes and activated eggs or early embryos, assayed in the rabbit reticulocyte lysate. Relief of repression of masked mRNAs in oocyte lysates can be achieved by several means including gel filtration in 0.5 M KCl-containing buffers, scission of RR mRNA 3′ UTR using complementary oligos and RNAse H that specifically unmasks RR mRNA, and using antisense RNA directed to the 3′ UTRs of RR and cyclin A mRNA (Standart et al., 1990). We reasoned that translational activation was due to the removal of a salt-labile repressor from the 3′ UTR masking element, delineated in the latter approaches to approximately the central portions of RR and cyclin A 3′ UTRs (Standart et al., 1990). An oocyte protein of 82 kDa (p82) selectively binds the RR masking element in UV-crosslinking assays and undergoes phosphorylation to 92 kDa upon fertilization or parthenogenetic activation. p82 associates with masked mRNAs in low salt but is removed from the RNP peak by 0.5 M KCl, which, coupled with gel-filtration, activates these mRNAs (Standart et al., 1990; Walker et al., 1996).

In Walker et al. (in this issue), we identified and cloned clam p82 as a homolog of *Xenopus* CPEB that mediates cytoplasmic polyadenylation of several *Xenopus* mRNAs, including cyclin, c-mos, and B4 mRNAs by virtue of its affinity for their CPE motifs (Hake & Richter, 1994; Stebbins-Boaz et al., 1996). In this article, to explore the functional significance of this homology, we first define the CPE motifs in clam RR 3′ UTR that support polyadenylation in vitro, and show that both a 3′ proximal CPE and an NPE motif are required for efficient polyadenylation in egg lysates. Deletion of this CPE abrogates p82 binding. Purified anti-p82 antibodies, but not preimmune antibodies, effectively neutralize polyadenylation, lending strong support to the identification of clam p82/CPEB as a functional homolog of *Xenopus* CPEB. Furthermore, these same specific antibodies translationally activate masked mRNAs in clam oocyte lysates, in a high-salt-dependent manner, implying that p82 mediates the translationally repressed state of masked mRNAs.

RESULTS

p82 has multiple binding sites in RR 3′ UTR, with varying affinities

We first determined the number and hierarchy of p82 binding sites in p82's most abundant RNA substrate, the RR 3′ UTR. Previously we showed that p82 has a high affinity for the RR 3′ UTR masking element in UV-crosslinking experiments, relative to RNA corresponding to the RR 5′ UTR, RR ORF, and part of 3′ UTR not containing the masking element (Walker et al., 1996). Here we extend those observations by perform-

ing competition experiments using four RNA competitors that span the whole 3′ UTR of RR mRNA, to more precisely assign p82 binding site preference. The boundaries of these four domains of the 3′ UTR, one of which represents the masking element, were chosen so as to yield roughly equal-sized transcripts (Fig. 1A). Because the whole UTR is approximately 70% AU, there was no reliable way in which to predict any secondary structure (if any were present) of such domains. They were subcloned as PCR products into transcription vectors that were used to produce ^{32}P-labeled competitors of low specific activity. In the UV-crosslinking assay, in which the masking element (ME or II) RNA was used as probe, the oocyte extracts were preincubated with increasing doses of competitor RNA before addition of the probe (30, 60, 125, and 250× molar excess; Fig. 1B). The most effective competitor for p82 binding was the masking element RNA, as noted previously (Walker et al., 1996). In contrast, the 5′-most competitor (I) did not diminish p82 binding to any significant extent, even at the highest concentration used. The two remaining competitors (III and IV), 3′ to the masking element, although less efficient at preventing p82 crosslinking than ME RNA, were able to act as competitors to differing extents at the highest doses tested. We conclude that p82 binds at several sites in the RR 3′ UTR, the relative order of binding affinity being ME/II > IV > III. Interestingly, all these three RNAs contain CPE-like U-rich motifs (see below), whereas RNA I is devoid of such tracts.

The proposal that p82 acts as a repressor of translation was based partly on the use of masking element antisense RNA to prevent binding of and displace translational inhibitors from endogenous masked mRNP in vitro (Standart et al., 1990). In Figure 1C we show that RNA corresponding to the antisense of the masking element RNA, when duplexed with full-length RR 3′ UTR probe RNA, acted as a very efficient competitor of p82 binding to clam oocyte proteins, confirming the high affinity binding of this protein to

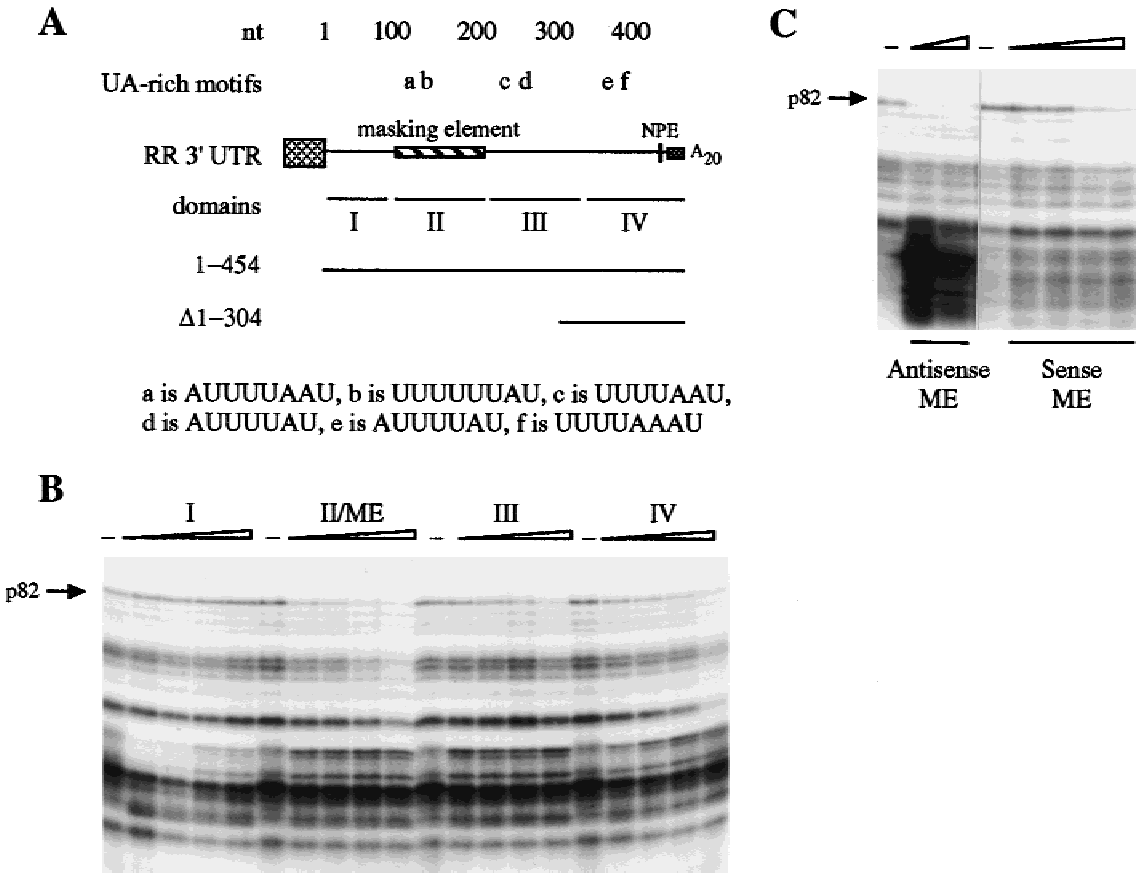

FIGURE 1. p82 binding sites in ribonucleotide reductase 3′ UTR. **A**: Top: schematic map of RR 3′ UTR, indicating the location of the 6 (a–f) CPE-like U-rich motifs, the nuclear polyadenylation signal (NPE) AAUAAA, and the 3′ terminal adenylate (A_{20}) tract. Below are indicated the length and positions of in vitro transcribed RNAs (domains I–IV, full-length UTR (1–454 nt), and a truncated RNA (Δ1–304) used as probes and competitors in UV crosslinking (Figs. 1 and 3) and/or polyadenylation substrates (Figs. 2 and 3). **B**: UV-crosslinking assay of clam oocyte proteins preincubated with domain I–IV RNAs (30, 60, 125, and 250× molar excess) and irradiated in the presence of ^{32}P labeled masking element (domain II) RNA. −: absence of competitor RNA. Arrow points to p82. **C**: UV-crosslinking assay of oocyte proteins with ^{32}P labeled full-length RR 3′ UTR (−). Left: pre-annealed with 10 or 20× molar excess of antisense domain II RNA. Right: following preincubation with 30, 60, 125, and 150× excess sense domain II RNA. Arrow points to p82.

ME RNA. Indeed, only p82 binding to the probe RNA is prevented by the partial duplex in the masking element region. In an analogous experiment in which the ME RNA was included as a sense strand competitor, more than ten times molar levels relative to antisense RNA were needed to observe efficient competition (Fig. 1C).

Role of multiple redundant CPE-like sequences and the hexanucleotide in RR 3′ UTR polyadenylation

As reported previously, approximately 125–150 A residues are added to RR 3′ UTR transcripts in a stage-regulated manner in vitro; polyadenylation is observed in lysates prepared from activated eggs, but not in those made from oocytes (Standart & Dale, 1993). On visual inspection, 6 motifs (a–f) resembling CPEs are scattered throughout the 454-nt-long RR 3′ UTR. For this assignment, the minimal CPE motif was taken to be $U_4A_{1-3}U$ (Richter, 1996; Stebbins-Boaz et al., 1996). Two such sequences reside within the masking element portion of the UTR itself, the second of which has the longest U-tract CPE, U_6AU (see Fig. 1A). Our analysis concentrated on the CPEs in the masking element (a and b), and the two near the 3′ terminus (e and f). We tested their role in polyadenylation by deleting individual, or a pair of, CPEs by PCR-mediated mutagenesis of the RR 3′ UTR plasmid, and transcribing the mutated DNAs in vitro. These labeled RNAs were then incubated in clam extracts prepared from both oocytes and activated eggs, phenol-extracted and analyzed on denaturing gels. None of the RR 3′ UTR RNAs were extended in oocyte extracts (Fig. 2A, top panel), as predicted from the behavior of RR mRNA in vivo, which undergoes polyadenylation only after fertilization (Rosenthal & Ruderman, 1987). We note that these RNAs in fact appear to undergo deadenylation in oocyte lysates, as judged by the loss of the cloning tracts of A_{20} residues present at their 3′ termini. In contrast, 85% of wild-type RR 3′ UTR RNA was modified in the egg lysate (Fig. 2A, bottom panel). When the effect of individual CPE deletions was tested, it was clear that the loss of any one motif did not significantly impair the efficiency of poly(A) extension; for example Δa, Δb and Δe RNAs behaved very much like wild type. Loss of both Δa and Δb motifs did not affect polyadenylation either. The single CPE mutation that lowered the efficiency of polyadenylation by any significant extent was the loss of the 3′-most terminal motif f (Fig. 2A), though even in this case, almost 70% of input RNA underwent poly(A) extension. We concluded that the RR 3′ UTR may contain multiple, functionally redundant U-rich CPE-like motifs.

In *Xenopus* and mouse (the only organisms examined to date in detail), cytoplasmic polyadenylation requires the presence of the nuclear polyadenylation signal AAUAAA proximal to a CPE (Richter, 1996). The function of the hexanucleotide is typically abolished by a U to G mutation (Fox et al., 1989; Paris & Richter, 1990). In *Drosophila*, the exact signals for cytoplasmic polyadenylation remain to be delineated (see Discussion). The role of the hexanucleotide in clam RNA polyadenylation was examined by mutagenizing the RR 3′ UTR AAUAAA 11 nt upstream from the A-tail to AAGAAA, and examining the ability of the resulting RNA to support polyadenylation. In contrast to *Xenopus*, clam lysates modify this RNA to ~70% of wild-type levels (Fig. 2B; Standart & Dale, 1993).

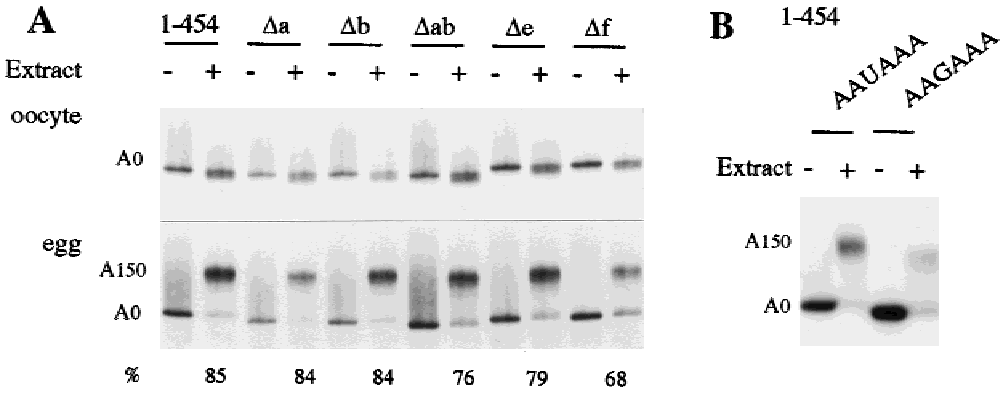

FIGURE 2. Multiple CPEs in the RR 3′ UTR. **A**: Oocyte (top panel) and egg lysate polyadenylation assays (bottom panel) with full-length RR 3′ UTR (1–454), or 1–454 RNA in which CPE a, b, a and b, e, and f were deleted (Δa, Δb, Δab, Δe, and Δf; see Fig. 1A). Capped ^{32}P-labeled RNAs were analyzed by denaturing polyacrylamide gel electrophoresis before (−) and after (+) addition of lysate and subsequent incubation for 2 h at 18 °C, followed by phenol extraction. Extension of the RNAs by 150 residues was estimated from labeled DNA markers (not shown). **B**: Polyadenylation in egg lysate of wild type 1–454 RNA (AAUAAA) and 1–454 RNA with mutated NPE (AAGAAA). −, +: as in **A**. Note that the differing sizes of the RNA substrates are because of the different cloning cassettes in the wild-type and mutated constructs.

Polyadenylation activity correlates with p82 binding

Because of the redundancy of the potential CPEs in the full-length RR 3′ UTR we could not easily examine their role in polyadenylation in *Spisula* lysates or ask whether polyadenylation correlates with p82 binding. To do so, a set of truncated RR 3′ UTR RNAs, lacking the first 304 nt, and being either wild type in sequence or lacking CPEs e and f (Fig. 1A) were assayed as polyadenylation substrates. The truncated RNA was efficiently modified (Fig. 3A). While a mutation resulting in the loss of CPE motif e diminished polyadenylation efficiency, a truncated RNA lacking CPE motif f was completely impaired in polyadenylation (Fig. 3A). Further manipulation of the plasmid sequences that resulted in mutating the hexanucleotide AAUAAA to AAGAAA (U-G) motifs showed that both a 3′-proximal CPE and the hexanucleotide participate in polyadenylation, seen in the case of Δ1-304, Δe RNA in particular. Inactivating the hexanucleotide in this RNA reduces the partial activity of the less severe CPE deletion (Fig. 3A). As shown above, in the context of the full-length 3′ UTR RNA, a U to G mutation of the hexanucleotide only partially inhibits polyadenylation (Fig. 2B).

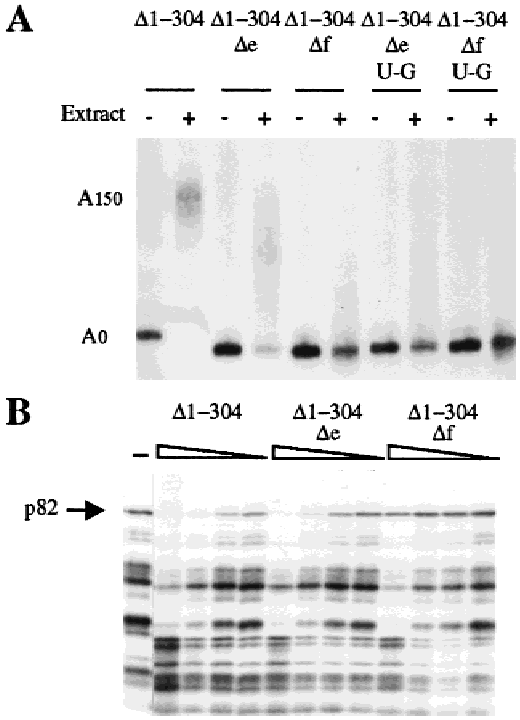

FIGURE 3. Role of the 3′ terminal CPE motifs and the hexanucleotide AAUAAA in polyadenylation of RR 3′ UTR. **A**: Polyadenylation assay in egg lysate of truncated 3′ terminal RR 3′ UTR, Δ1–304 RNA (see Fig. 1A), and Δ1–304 RNA with deleted CPE motif e and f (Δ1–304 RNA Δe and Δf), and CPE deleted RNAs with additional AAGAAA mutations (U-G). **B**: UV-crosslinking assays of oocyte proteins with ^{32}P labeled masking element probe (−) and following preincubation with 250, 125, 60, and 30× molar excess of competitor RNAs (Δ1–304, Δ1–304 Δe, and Δ1–304 Δf). Arrow indicates p82.

These experiments indicate that the terminal U-rich motif f, although necessary for polyadenylation, is not sufficient, as the effect of loss of motif e shows. Furthermore, the AAUAAA hexanucleotide function can be detected in a partially compromised polyadenylation substrate. Thus both the U-rich CPE motifs and the hexanucleotide play a role in polyadenylation in clams; however their precise contribution to the overall activity is difficult to dissect in the context of multiple CPEs.

We next examined the ability of these compromised polyadenylation substrates to bind p82 in UV-crosslinking assays. The labeled probe RNA (the masking element RNA) was incubated with oocyte extract in the presence of increasing amounts of cold competitor RNA, ranging from 30 to 250 molar excess. The competitor RNAs included the wild-type truncated RNA, and the truncated RNAs with deletions of CPEs e and f (Fig. 3B). While loss of CPE e motif reduces only slightly the ability of the competitor RNA to bind p82, loss of CPE f motif completely inactivates its competitive binding. Thus the relative requirement of CPE e and f motifs in polyadenylation (Fig. 3A) is mirrored by their relative affinity for p82 (Fig. 3B). Similar results were obtained in UV-crosslinking assays using egg lysates, but are not as simple to interpret because of the typical smeariness of the multiply phosphorylated p92 (data not shown).

p82 neutralization reduces polyadenylation

To confirm the role of clam p82 in polyadenylation, we tested the effect of neutralizing polyadenylation activity in clam egg lysates by incubation with purified, specific p82 antibodies. Anti-p82 serum, obtained from rabbits immunized with gel-purified His_6-tagged p82 (as detailed in Materials and Methods), was highly specific for the 82 kDa and 92 kDa polypeptides on Western blots of clam oocyte and egg S10 lysate proteins (Fig. 4A). The preimmune serum did not cross react with any clam proteins under the same conditions (not shown). For use in polyadenylation assays, the immune and preimmune antibodies were purified on protein A-Sepharose and added to clam lysates to a final approximate concentration of 2 mg/mL (see Materials and Methods).

In these polyadenylation assays, as seen previously, the labeled RNA substrate (the RR 3′ UTR; see Fig. 2), was not modified by incubation in the oocyte extract and was extended by about 150 A residues in the egg lysate (Fig. 4B, lanes 1–4). The overall extent of polyadenylation in the control reactions is somewhat lower than seen previously, presumably because of lysate dilution by the antibody or buffer. Neither of the purified IgGs had any significant effect on the RNA incubated in the oocyte extract (Fig. 4B, lanes 5 and 6). Strikingly, however, the immune antibodies almost completely abolished the polyadenylation activity of the egg lysate

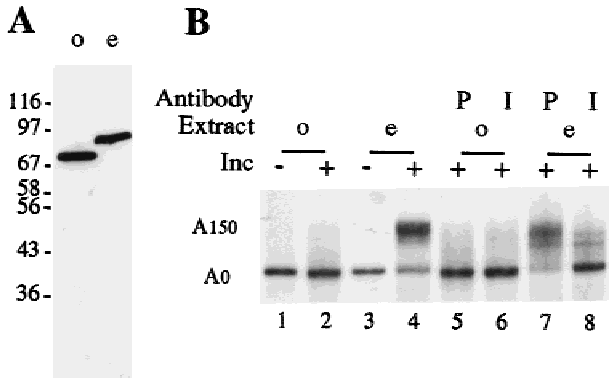

FIGURE 4. p82 neutralization prevents polyadenylation A. Western blot of clam oocyte and egg S10 proteins, revealed with rabbit anti-p82 antibodies and ECL. **B**: Polyadenylation assays with RR 3′ UTR RNA in oocyte (o) and egg (e) lysates, in the presence of protein A–Sepharose-purified preimmune (P) or anti-p82 (I) antibodies.

(Fig. 4B, lane 7). In contrast, the preimmune antibodies purified by the same means decreased RNA polyadenylation only partially (Fig. 4B, lane 8). Very similar data were obtained when egg lysates were depleted of p82 with protein A-Sepharose-bound antibodies (not shown). However, because of the abundance of p82/p92 in clam lysates (estimated to be ~50 μg/mL; data not shown), several consecutive depletions had to be carried out to ensure complete loss of p82/p92, with concomitant loss of control polyadenylation activity in mock depletions using preimmune antibodies, presumably because of the multiple manipulations.

These results are consistent with those shown in Figure 3 and indicate that clam p82 plays a positive role in polyadenylation. Removal of p82 binding sites from an RNA substrate impairs or abolishes polyadenylation activity, and neutralization/depletion of p82 from active extracts inhibits polyadenylation of wild-type RNA. We have not been able to demonstrate directly this proposed function of p82 because of our inability so far to express sufficient quantities of soluble recombinant protein (see Discussion).

High salt dependence of translational unmasking by anti-p82 antibodies

To examine p82 masking function, we exploited the observation that in vitro-translated clam lysates faithfully mimic the stage-specific pattern of protein synthesis observed in vivo upon fertilization (Standart et al., 1990). We tested anti-p82 antibody addition to clam oocyte and activated egg lysates, subsequently assayed for protein synthesis, to see whether neutralizing p82 resulted in mRNA-specific and stage-dependent translational effects. In particular, in view of our proposed role of p82 as a translational repressor, we were asking whether loss of p82 function led to translational activation of masked oocyte mRNAs. Previously we showed that global translational unmasking of masked mRNP in oocyte lysates can be achieved by a combination of adding KCl to 0.5 M followed by gel filtration, though either step alone was ineffective (Standart et al., 1990). Recent experiments indicated that under these high salt conditions p82 was removed from mRNP (Walker et al., 1996).

The protein A–Sepharose-purified antibodies, both preimmune and immune, were incubated with clam lysates for 15 min at 4 °C and the mixtures subsequently translated in the nuclease-treated rabbit reticulocyte lysate. All complete translation reactions were assayed at a final concentration of 100 mM KCl. In some assays, KCl was added to the clam lysate/IgG mix to 0.5 M KCl, before the addition of rabbit lysate, to dissociate masked mRNP. In this case, KCl was omitted from the added rabbit lysate. Results obtained in a representative experiment are shown in Figure 5. The pattern of protein synthesis in clam oocyte and egg lysates is markedly different, in the absence of added antibody, as seen particularly clearly in the case of masked cyclin A mRNA (A) and that encoding the small subunit of ribonucleotide reductase. (Cyclin B protein, made in the egg, runs at a similar position to an oocyte-translated protein and so is less easy to use to score unmasking). These mRNAs are only efficiently translated in activated egg lysates (Fig. 5A). Antibody addition in normal salt conditions does not alter translation to any great extent. Strikingly, however, purified anti-p82 antibodies in the presence of 0.5 M KCl result in translational activation of cyclin A and ribonucleotide reductase mRNA. This unmasking is specific to oocyte lysates, and to anti-p82 antibodies. In lysates prepared from activated eggs, unmasking has already occurred (Fig. 5A). To test whether the salt and/or antibody additions in some artifactual manner could alter the translational pattern in the cell-free system, we performed essentially the same experiment but used purified oocyte and egg RNA, rather than oocyte and egg lysate. As shown in Figure 5B, the pattern of protein synthesis programmed by oocyte RNA was unaffected by either salt treatment or presence of antibody (preimmune and p82-specific). The same result was obtained with egg RNA (not shown).

Upon close examination of the synthetic capacity of antibody-activated lysates, we noted that of the two well-characterized mRNAs, cyclin A mRNA is more robustly activated than RR mRNA. Part of the explanation for this difference may lie in the varying degree of translational silencing in the first place; cyclin A mRNA in oocyte lysates is less efficiently repressed than RR mRNA (Standart et al., 1990 and Fig. 5A). Moreover, the translation of many mRNAs is affected by anti-p82 antibody in a high-salt-dependent manner, in addition to those encoding A and RR, implying that p82 may interact and translationally regulate many maternal

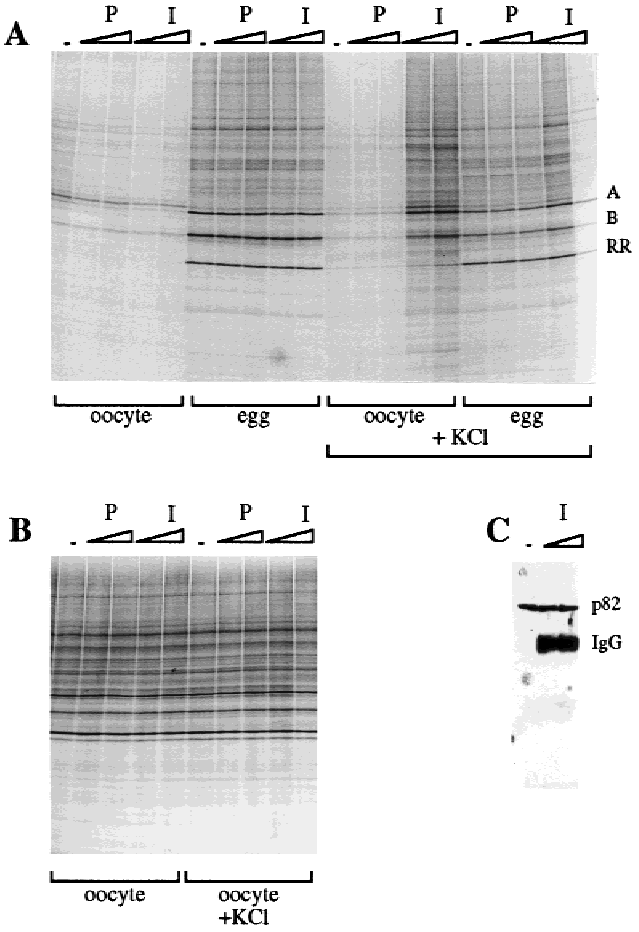

FIGURE 5. Anti-p82 antibodies translationally activate masked mRNA in oocyte lysates treated with 0.5M KCl. **A:** Control buffer (−), protein A–Sepharose-purified preimmune (P) and anti-p82 antibodies (I) (see Fig. 4) were preincubated with oocyte or egg lysates, without or with (+ KCl) 0.5 M KCl prior to translation in the nuclease-treated rabbit reticulocyte lysate in the presence of ^{35}S Met. Positions of cyclins A and B, and ribonucleotide reductase are indicated (A, B, and RR, respectively). **B**: Control buffer (−), protein A–Sepharose-purified preimmune (P) and anti-p82 antibodies (I) were preincubated with phenol-extracted oocyte RNA, without or with (+ KCl) 0.5 M KCl prior to translation in the nuclease-treated rabbit reticulocyte lysate. **C**: Western blot of translation reactions (oocyte, I, plus salt) revealed with anti-p82 antibodies and ECL.

mRNAs. From the previous experiment we know that exogenous RR 3′ UTR transcripts at any rate are not polyadenylated in the presence of purified IgG in either oocyte or egg lysates, so antibody-mediated unmasking is not a result of poly(A) extension. Furthermore, affinity of antibodies to p82 in lysates does not significantly differ between low-salt and high-salt conditions (data not shown). Western blotting of the translation reaction mixtures revealed that the purified IgGs did not promote p82 phosphorylation (Fig. 5C), eliminating another possible explanation for unmasking. We infer that anti-p82 antibodies resulted in translational activation of masked mRNP by removal of a repressor protein from the mRNA. This removal, or antibody binding as such, was increased in high-salt-treated lysates. In other words, these antibody experiments mimic our original antisense RNA work (Standart et al., 1990) and together, strongly suggest that p82 represses translation in the oocyte.

p92 does not associate with polysomal mRNA after fertilization

An important question regarding p82 and its phosphorylated form, p92, is whether the protein remains associated with the maternal cyclin A and ribonucleotide reductase mRNAs after translational activation. UV-crosslinking studies using oocyte and egg extracts suggest that both p82 and p92 bind the masking element RNA, with comparable affinities as judged from competition studies (Walker et al., 1996 and unpubl.). Indeed, Hake and colleagues also recently concluded that phosphorylation of *Xenopus* CPEB did not markedly affect its RNA binding in UV-crosslinkng assays (Hake et al., 1998). On the other hand, gel retardation assays suggest that RNP conformation alters on activation, with clam oocyte and egg proteins forming complexes of radically different sizes with the masking element RNA, in part because of p82 phosphorylation (Walker et al., 1996). Changes in native complexes could, of course, result from alterations in protein–protein interactions, protein–RNA interactions, or a mixture of both. We sought another method by which to examine p82-mRNA association in oocytes and activated egg lysates that could elucidate the role of p82 in cytoplasmic polyadenylation and unmasking.

Clam oocyte and activated egg lysates were fractionated on 15–50% polysomal sucrose gradients and the A_{260}-nm absorbance profile of each gradient was measured (Fig. 6, top panels). To determine the distribution of different mRNAs between mRNP and polysomes in both stages, RNA phenol extracted from the gradient fractions was translated in vitro (Fig. 6, middle panels) while the proteins from the fractions were analyzed on Western blots to locate p82/p92 (Fig. 6, bottom panels).

In oocytes, we noted a complex and rather broad pattern of mRNA distribution around the 80S peak, with some unidentified mRNAs migrating in lighter fractions than those encoding cyclins A and B and RR mRNAs that were present in the heaviest mRNP fractions (Rosenthal et al., 1983). Oocyte lysates do not support significant synthesis of cyclin A and B and RR proteins (Fig. 5) and their mRNAs were excluded from polysomal fractions. Activation of oocytes, though resulting in an almost undetectable effect on the overall absorbance profile, moves a large fraction of the maternal cyclin A and ribonucleotide reductase mRNAs onto polysomes (compare Fig. 6A and 6B). A control gradient, run with activated egg extract incubated with 30 mM EDTA, showed that this treatment removed all mRNA from this region of the gradient, confirming their polysomal association (data not shown).

The oocyte p82 protein copurifies precisely with the broadly distributed untranslated mRNP (Fig. 6A). Copurification of many different mRNAs with p82 supports the data shown in Figure 5B, indicating that p82 interacts with a wide selection of mRNAs. In activated egg extracts, although a significant proportion of cyclin and RR mRNAs are in polysomes, only small amounts of p92 were detected in this region, with much of the p92 found in fractions lighter than 80S (Fig. 6B). We note that some p92 appeared to be dephosphorylated in the egg gradient, possibly resulting from coenrichment of a phosphatase during the 2-h centrifugation step. This data suggests that p92 has a weaker association with the translationally activated maternal cyclin A and RR mRNAs in eggs.

DISCUSSION

This study provides evidence that clam p82/CPEB is a functional homolog of *Xenopus* CPEB and plays a positive role in CPE-dependent polyadenylation. In Walker et al. (this issue) we indicated that cloned p82 shares considerable sequence homology with CPEB from frog, mouse, and goldfish, particularly in the RNA-binding domains and was therefore likely to function in polyadenylation. Here we show in binding and polyadenylation assays that p82 binding sites resemble the CPE motifs defined largely in *Xenopus* studies (Richter, 1996) and shown to be evolutionarily conserved (Verrotti et al., 1996) and that these U-rich motifs are required for clam RNAs to undergo poly(A) extension in vitro (Figs. 1–3). Furthermore, specific anti-p82 antibodies abrogate polyadenylation in clam egg lysates (Fig. 4), just as anti-CPEB antibodies inhibit cytoplasmic polyadenylation in *Xenopus* egg lysates and in progesterone-matured oocytes (Hake & Richter, 1994; Stebbins-Boaz et al., 1996).

The role of CPEB in cytoplasmic polyadenylation has thus been confirmed in the marine invertebrate, *Spisula solidissima*. Additionally, Hake & Richter (1994) were able to demonstrate partial recovery of polyadenylation activity by adding to depleted lysates reticulocyte lysate-made CPEB; we have so far been unable to satisfactorily perform such an add-back experiment. While on the one hand the amounts of p82 synthesized in vitro may be insufficient to compensate for depleted endogenous p82, on the other hand, our attempts to make soluble recombinant protein in *Escherichia coli* have not been successful either (data not shown). We are currently attempting to obtain active p82 in baculovirus-infected Sf9 cells. While the weight of evidence in both *Xenopus* and clam supports the positive

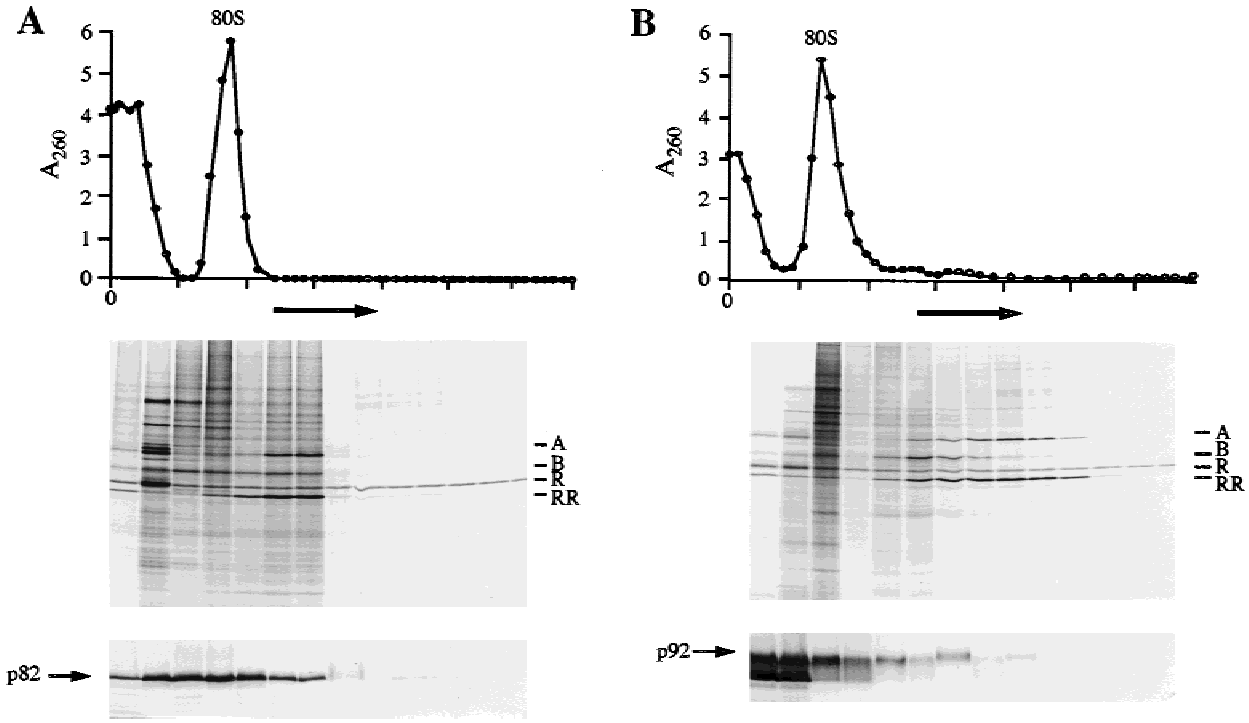

FIGURE 6. p92 does not associate with translationally activated mRNA. Oocyte (**A**), and egg (**B**) lysate prepared 60 min after activation were analyzed on polysome sucrose gradients, with arrows indicating direction of sedimentation. Top panel: A_{260} absorbance profile. Middle panel: reticulocyte lysate translation products, labeled with ^{35}S Met, directed by RNA phenol extracted from gradient fractions. Bottom panel: Western blot of fractions revealed with anti-*Xenopus* CPEB antibodies and alkaline phosphatase-linked secondary antibodies. The positions of cyclin A, cyclin B, and ribonucleotide reductase (A, B, and RR) are indicated on right. R: endogenous rabbit reticulocyte lysate labeled protein. p82/92 is indicated on left.

role of p82/CPEB in cytoplasmic polyadenylation, it is not yet clear whether the protein has an intrinsic activity, or whether it acts as a bridge to components of CPSF and/or poly(A) polymerase. The latter scenario is supported by the work of Bilger et al. (1994), who demonstrated the participation of the nuclear polyadenylation factors in CPE-dependent cytoplasmic polyadenylation. As both CPEB and p82 interact with their maternal mRNA substrates in immature oocytes as judged by UV-crosslinking assays, differential RNA binding as such cannot be the explanation for stage-specific polyadenylation. Possibly p82 and CPEB phosphorylation modifies their interaction with polyadenylation factors, though this possibility has been ruled out, at least in the case of *Xenopus* class I polyadenylation RNAs including c-mos, cyclin A1, and B4 mRNAs (Ballantyne et al., 1997; de Moor & Richter, 1997). We are currently identifying polypeptides that specifically coimmunoprecipitate with p82/p92 in clam oocytes and egg lysates (N. Minshall and N. Standart, unpubl.). Indeed, among several specific p82-interacting polypeptides, we have identified some that exhibit stage-specific p82-binding; their detailed examination may shed light on the regulation of polyadenylation in early embryos.

Originally p82 was identified as the major protein selectively binding the RR and cyclin A 3′ UTR masking elements, defined in unmasking assays in vitro (Standart et al., 1990; Walker et al., 1996). On the basis of these assays, we postulated that the binding of p82 to the 3′ UTR results in translational repression. It was therefore with considerable surprise that we identified p82 as a CPEB family member (Walker et al., 1998). However, functional studies, discussed above, confirmed this classification. We also tested whether p82 acts as a repressor in the oocyte by a modified unmasking assay, using specific purified anti-p82 antibodies in place of antisense RNA. In line with our previous observations, translational activation of masked mRNP was observed when the specific antibodies were incubated with oocyte lysate in high-salt conditions (Fig. 5). In these conditions, mRNP dissociation results in p82 release (Walker et al., 1996). We reasoned that unmasking was not due to polyadenylation, because no activity was detectable in oocyte lysates, and anti-p82 antibodies prevented rather than promoted polyadenylation in egg lysates (Fig. 4). We also ruled out the possibility that the specific antibodies in some unknown manner resulted in p82 phosphorylation, which could possibly have indirectly led to unmasking (Fig. 5; Walker et al., 1996). These data strongly suggested that the underlying unmasking mechanism in our experiments relied on the sequestration of a repressor protein by anti-p82 antibody. One possible model of how anti-p82 antibody relieved repression supposes that it prevented rebinding of the repressor protein to mRNA, as salt concentration is lowered to allow translation. Alternatively, it may be envisaged that antibody binding alters protein conformation in such a manner so as to mimic phosphorylation. Consistent with its proposed repressor function, p92 is not associated with the actively translating cyclin A and RR mRNAs in activated egg lysates (Fig. 6) and clam p82 and *Xenopus* CPEB are both degraded in maturing oocytes and early embryos (Hake & Richter, 1994; Walker et al., 1998). Lastly, evidence that *Xenopus* CPEB may be functioning as a translational repressor in immature oocytes was obtained in microinjection studies using chimeric luciferase reporters fused to frog ubp3 and clam RR 3′ UTRs (A. Searfoss & M. Wormington, in prep.). Clearly, it will be very important to test directly for this second p82/CPEB function once soluble protein is obtained (see above).

We note that antibody-mediated activation of translation, although surprising, is not entirely without precedent. In *Xenopus* oocytes, two major proteins, mRNP3 and mRNP4, are associated with maternal mRNA (Murray et al., 1991). mRNP3 is very similar in sequence to, and mRNP4 is identical to, FRGY2, an independently characterized *Xenopus* oocyte-specific transcription factor, subsequently classified as a prototype of the Y-box family of proteins (Wolffe, 1994). Evidence supporting the role of FRGY2 in sequence nonspecific translational repression includes reconstitution of mRNA with FRGY2 (Richter & Smith, 1984; Kick et al., 1987), expression of FRGY2 in somatic cells (Ranjan et al., 1993), and overexpression in *Xenopus* oocytes (Bouvet & Wolffe, 1994). Furthermore, antibodies to FRGY2 relieve the inhibition of translation when injected into *Xenopus* oocytes (Braddock et al., 1994; Gunkel et al., 1995).

The rabbit anti-p82 antibodies do not cross-react with *Xenopus* CPEB on Western blots (N. Minshall & N. Standart, unpubl.), so we cannot test their effect on *Xenopus* oocyte maturation (clam oocytes cannot be microinjected). Stebbins-Boaz et al. (1996) reported that anti-CPEB antibodies prevent progesterone-induced maturation by inhibiting steps leading to the normal increase in H1 kinase seen in maturing oocytes, consistent with CPEB being an activator of (mos) mRNA expression. However the phosphorylation and stability of CPEB were not examined; preliminary data in our laboratory suggests that preventing p82 phosphorylation stabilizes the protein (G. Thom & N. Standart, unpubl.). In a possible scenario, stable and RNA-bound CPEB would mask the expression of mRNAs lying between mos and cyclin B1, a class II mRNA (Ballantyne et al., 1997).

How do 3′ UTR translational repressor proteins work? The growing body of evidence, gathered from studies of RNA stability and translation showing that the 5′ and 3′ ends of the mRNA interact (Caponigro & Parker, 1995; Tarun & Sachs, 1996; Gunkel et al., 1998; Preiss & Hentze, 1998), has led to models in which 3′ UTR binding repressors interfere with this interaction and

prevent contacts necessary for initiation. Recent studies suggest that such contacts do not necessarily involve the 5′ cap structure or cap-binding factors. hnRNP K and E, which bind a pyrimidine-rich repeat motif in lipoxygenase 3′ UTR, inhibit translation initiation at the level of 80S ribosome formation. Furthermore, since they control both cap-dependent and internal ribosome entry site-mediated translation in vitro, these proteins exert their repression at a step downstream of the convergence of the two translation initiation systems, that is ribosome assembly at the AUG codon (Ostareck et al., 1997). Interestingly, Nanos and Pumilio, which collaborate to regulate the translation of maternal *hunchback* mRNA, can also repress cap-independent translation from an IRES in vivo (Wharton et al., 1998). Whether this model of inhibition of translation will be applicable to other 3′ UTR repressors (e.g., this work; Webster et al., 1997; Culp & Musci, 1998) remains to be seen. In any case, since no specific developmental translational repressor has yet been cloned that is capable of reconstituting repression in vitro, it may be necessary to invoke the possibility that such repressors exert their effects through or in collaboration with other factors.

How can both functions of clam p82/CPEB be reconciled? In other words, how does the same protein act both as a repressor and as an activator of mRNA expression? Our data strongly suggest that the repressor functions at the level of translation in the quiescent oocyte whereas the activator functions at the level of polyadenylation in the activated egg and early embryo. Robust translational activation of the masked mRNAs, triggered by fertilization, may result from the combination of de-repression coupled with poly (A) lengthening. It is doubtless noteworthy that the two functions are temporally and cell-cycle stage distinct, and that the protein is modified between the two stages (Walker et al., 1998). Clearly, further investigation will be warranted to characterize the mode of action of p82/CPEB, which would be particularly facilitated by reconstitution of repression and polyadenylation in vitro.

MATERIALS AND METHODS

RR 3′ UTR plasmids

DNAs were subcloned into *Eco*RI and *Bam*HI (underlined below) cut pGEM1 following standard PCR reactions with pfu DNA polymerase (Stratagene) and appropriate oligonucleotides (all 5′–3′, upstream/downstream): the complete RR 3′ UTR (nt 1–454; CCG GAA TTC ACT TCT GAA AAA CCA/GGG GGA TCC T20 C); domain I (nt 1–78; CCG GAA TTC ACT TCT GAA AAA CCA/CGC GGA TCC GTC GAT GAA CCA CAA); domain II, the masking element (nt 83–216; CCG GAA TTC TGG AAG CGT TGT TGG/CGC GGA TCC AAA CCA AAT TTA CAA AAA T); domain III (nt 228–354; CCG GAA TTC CAC ATT TAC ATT TAG/CGC GGA TCC ATA CAC ATA CTC TCG C); domain IV (nt 368–454; CCG GAA TTC TTA CTC TTA ATG TGC/GGG GGA TCC T20 C) and Δ1–303 (nt 305–454; CCG GAA TTC ATT GTT GGA AGA G/GGG GGA TCC T20 C) using as a template DNA plasmid DRS3 (Walker et al., 1996). Deletion mutants were obtained by a modified Megaprimer method used basically as described (Picard et al., 1994), except that the rate of cooling between the melting and annealing temperatures for step 2 was reduced to 0.05 °C/s. Antisense oligos spanning the region to be deleted included Δa (resulting in loss of ATTTTAAT, TTA TAA TTT GCA CTA TTG TTT TAA AAC CAA TGA AAC), Δb (resulting in loss of TTTTTTATTA, CAT GCA TTA TTG CAC ACG TAA AAT TAT AAT TTG C), Δe (resulting in loss of TTTTAT, GAT GAG ATT TAA AAC CTG GCA CAT TAA GAG TAA G), and Δf (resulting in loss of TTTTAAAT, CAT TAG ATA CAT GAT GAG CCT GAT AAA ATG CAC AT). The point mutation AAUAAA to AAGAAA (using CAT TAA ATT TCT TAC ATA TTA C) was initially achieved by a standard M13-mediated mutagenesis protocol in plasmid DRS3, and subsequently recreated by PCR in Δ1–304Δe and Δ1–304 Δf. All DNAs were checked by sequencing and were linearized with *Bam* H I prior to transcription.

In vitro transcription and UV crosslinking

Preparation of RNA probes and UV crosslinking are described in Walker et al. (1998). RNA polyadenylation substrates were transcribed in the presence of GpppG to prevent degradation (Paris & Richter, 1990). Conditions for the large-scale production of competitor RNA using T7 RNA polymerase were optimized as follows: for a 100 μL reaction, 40 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$, 5 mM DTT, 5 mM of each NTP, 8 μg linearized DNA template, ~240 U T7 RNA polymerase, ~100 U RNA guard, 0.5 U inorganic pyrophosphatase (Sigma) and a trace of [α^{32}P] UTP to allow quantitation. The reaction mixture was incubated at 37 °C for 2 h. At the end of incubation, prior to phenol extraction, an equal volume of 10 mM Tris (pH 8.0), 10 mM EDTA buffer was added to mop up the excess Mg^{2+} ions.

Preparation of anti-p82 antibody

Anti-p82 antibody was obtained from rabbits immunized with gel-purified, recombinant histidine tagged p82. The p82 expression plasmid was constructed by inserting the complete open reading frame of p82 as an *Nde*I–*Xho*I fragment into pET21b (Novagen) as detailed (Walker et al., 1998). Transformed BL21(DE3) was induced with 0.4 mM IPTG, cells resuspended in 0.01–0.02 vol. 300 mM NaCl, 50 mM sodium phosphate buffer (pH 7.8), and lysed using a french pressure cell and subsequent addition of Triton X-100 to 1% (Walker et al., 1998). The cell debris, including the insoluble His-tagged p82, was pelleted at 12,000 *g* for 20 min and was resuspended in 300 mM NaCl, 50 mM sodium phosphate buffer (pH 7.8), 1% Triton X-100. Aliqouts were run on SDS-polyacrylamide preparative gels, and stained in 0.25 M KCl at 4 °C for 10 min or until white bands were seen. p82 was cut from the gel and electroeluted overnight. Rabbit serum was obtained after four injections at three weekly intervals, each containing ~15–30 μg protein. When required, immune and preimmune sera was purified by incubation of rabbit serum

with protein A-Sepharose (Pharmacia) as described in Harlow & Lane (1988). Western blots were detected by ECL.

Polyadenylation assays

Neutralized extracts were obtained by incubation of 10 μL clarified S10 extract (Walker et al., 1998) with 5 μL protein A-antibody (20–40 μg), 1 h on ice. Ten microliters of neutralized extract were incubated with ~1 ng (~40,000 cpm) of ^{32}P-labeled RNA, 5 mM creatine phosphate and 250 ng creatine kinase at 18 °C, with 5 μL samples taken at 0 and 2 h. The samples were then treated with 50 μg/mL of proteinase K in 0.5% SDS, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA at 37 °C for 20 min. Following addition of an equal volume of 2 × RNA extraction buffer (0.2 M LiCl, 2% SDS, 0.1 M Tris-HCl (pH 7.6), 2 mM EDTA) and phenol/chloroform extraction, RNA was ethanol precipitated and washed in 80% ethanol. Pellets were resuspended in H_2O prior to electrophoresis of ~1,000–2,000 cpm aliquots through 4.5% polyacrylamide-7 M urea-TBE gels for 1 h, 45 min at 8 W. Following fixation, the gels were dried and autoradiographed.

In vitro translation

Phenol-extracted RNA was translated in the nuclease-treated rabbit reticulocyte lysate in the presence of [^{35}S]-methionine at 0.5 μCi/μL (Kaminski & Standart, 1996). In the experiment shown in Figure 5, protein A–Sepharose-purified antibodies were diluted (from a stock of 4–8 mg/mL) 8- or 16-fold into water, and 1 μL was added to 2 μL of clam oocyte or extract with or without an additional 500 mM KCl. Antibodies were incubated with the extract for 15 min on ice prior to addition of 8 μL rabbit reticulocyte translation mix and incubation at 30 °C for 1 h.

Polysome gradients

Linear 15–50% 33-mL sucrose gradients were prepared in 0.125 M KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 10 mM HEPES (pH 7.0), 0.5 mM DTT. One-half milliliter of 12k supernatant oocyte or activated egg extracts was thawed on ice, diluted in 2.5 mL of this buffer, and layered onto the gradients. To disassemble polysomes, diluted egg extract was incubated with EDTA (to 30 mM) on ice for 15 min. Gradients were centrifuged in a Beckman SW28 rotor at 90,000 *g* for 2 h at 4 °C. RNA was precipitated from half of each fraction with ethanol and 25 μg tRNA at −80 °C. The pellet was taken up in 0.5 mL SDS buffer (0.2 M LiCl, 2 mM EDTA, 2% SDS, 0.1 M Tris-HCl, pH 7.6), extracted with phenol/chloroform, and precipitated with 3 volumes of 96% ethanol. The RNA pellet was taken up in 0.2 mL RNA buffer (SDS buffer without SDS) and reprecipitated with ethanol. Finally the pellet was taken up in 15 μL water and 2 μL were used for in vitro translation in the reticulocyte lysate. Proteins were precipitated from the gradient fractions with 10% TCA on ice and the pellet washed in acetone and resuspended in SDS sample buffer prior to Western blotting.

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