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A conserved role of a DEAD box helicase in mRNA masking

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ABSTRACT

Clam p82 is a member of the cytoplasmic polyadenylation element-binding protein (CPEB) family of RNA-binding proteins and serves dual functions in regulating gene expression in early development. In the oocyte, p82/CPEB is a translational repressor, whereas in the activated egg, it acts as a polyadenylation factor. Coimmunoprecipitations were performed with p82 antibodies in clam oocyte and egg lysates to identify stage-regulated accessory factors. p47 coprecipitates with p82 from oocyte lysates in an RNA-dependent manner and is absent from egg lysate p92-bound material. Clam p47 is a member of the RCK/p54 family of DEAD box RNA helicases. Xp54, the *Xenopus* homolog, with bona fide helicase activity, is an abundant and integral component of stored mRNP in oocytes (Ladomery et al., 1997). In oocytes, clam p47 and p82/CPEB are found in large cytoplasmic mRNP complexes. Whereas the helicase level is constant during embryogenesis, in contrast to CPEB, clam p47 translocates to nuclei at the two-cell stage. To address the role of this class of helicase in masking, Xp54 was tethered via 3′ UTR MS2-binding sites to firefly luciferase, following microinjection of fusion protein and nonadenylated reporter mRNAs into *Xenopus* oocytes. Tethered helicase repressed luciferase translation three- to fivefold and, strikingly, mutations in two helicase motifs (DEAD → DQAD and HRIGR → HRIGQ), activated translation three- to fourfold, relative to MS2. These data suggest that this helicase family represses translation of maternal mRNA in early development, and that its activity may be attenuated during meiotic maturation, prior to cytoplasmic polyadenylation.

Keywords: CPEB; oocyte maturation; RCK/p54 helicase; translational control; *Spisula solidissima*

INTRODUCTION

Control of translation of maternal mRNAs regulates completion of meiosis in oocytes, and entry into and progression of the cleavage stages during early development in organisms ranging from flies, clam, and *Xenopus* to mouse; essentially in the absence of transcription. mRNAs encoding cyclins, c-mos, and the small subunit of ribonucleotide reductase are stored in a masked form in oocytes, and are translationally activated as oocytes are induced to complete meiosis by hormones or sperm. Translationally regulated maternal mRNAs also dictate sexual fates in the *Caenorhabditis elegans* hermaphrodite germline and the specification of pattern along the anteroposterior body axis in *Drosophila* by generation of protein gradients from localized mRNAs (Wickens et al., 2000).

mRNAs that are recruited to polysomes during meiotic maturation undergo polyadenylation, whereas housekeeping mRNAs such as actin and ribosomal protein mRNAs that are released from polysomes are deadenylated. Subsequent studies showed that 3′ UTR U-rich elements (cytoplasmic polyadenylation elements [CPE]), consensus $U_{4–8}A_{1–2}U$, in relatively close proximity to the ubiquitous nuclear polyadenylation hexanucleotide AAUAAA, were required to promote cytoplasmic polyadenylation and translational activation (Richter, 1996).

CPEs are specifically recognized by CPEB, one of best characterized regulators of translation in early development. *Xenopus* CPEB (Hake & Richter, 1994) is the founder member of a growing family of proteins in both vertebrates and invertebrates including *Drosophila orb* (Lantz et al., 1994), clam (*Spisula*) p82 (Walker et al., 1999), *C. elegans* CPB-1–4 (Luitjens et al., 2000), and the more closely related mouse, zebrafish, and human homologs (Gebauer & Richter, 1996; Bally-Cuif et al., 1998; Welk et al., 2001). All CPEBs share the two C-terminal RNA recognition motifs (RRMs) upstream of an unusual zinc finger motif that altogether promote efficient RNA binding (Hake et al., 1998), whereas the N-termini are far more varied in sequence. CPEB promotes polyadenylation (Hake & Richter, 1994; Stebbins-Boaz et al., 1996) by its increased affinity for CPSF in

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maturing eggs following phosphorylation with Eg2 kinase (Mendez et al., 2000a, 2000b). Interestingly, CPEB has an additional and opposing role in early development. In clam and *Xenopus* oocytes, CPE/CPEB repress translation (de Moor & Richter, 1999; Minshall et al., 1999; Barkoff et al., 2000) as well as promoting polyadenylation in maturing eggs. Multiple CPEs appear to be necessary and sufficient to mask cyclin B1 mRNA (de Moor & Richter, 1999; Barkoff et al., 2000; Nakahata et al., 2001), though additional elements and *trans*-acting factors may operate in other mRNAs such as wee1 (Charlesworth et al., 2000). Intriguingly, it seems that CPEB's function is not confined to oogenesis, but is in fact restricted to spermatogenesis in developing nematodes (Luitjens et al., 2000) and extends to synapses in rodents (Wu et al., 1998).

In *Xenopus*, masking by CPEs requires cap-dependent translation (de Moor & Richter, 1999). Before maturation, CPEB sequesters the cap-binding factor eIF4E, indirectly, through a 150-kDa protein named maskin, thus preventing productive eIF4F complex formation and hence ribosome recruitment. Upon progesterone-treatment, maskin releases eIF4E, and active translation can ensue (Stebbins-Boaz et al., 1999). Disruption of maskin-eIF4E contacts occurs at about the same time as polyadenylation but details of the release of eIF4E or the link with poly(A) extension are not known. Polyadenylation may activate translation by providing additional PABP-binding sites and thus strengthen PABP-eIF4G contacts, shown in yeast and mammalian cells to synergistically promote translation (Sachs, 2000). Recent reports attest to the stimulatory role that PABP, and, in particular, the binding of eIF4G with PABP, plays in translation and in oocyte maturation in *Xenopus* (Gray et al., 2000; Wakiyama et al., 2000).

During meiotic maturation and early embryogenesis, CPEB is modified by phosphorylation and subsequent proteolysis in vertebrates (Hake & Richter, 1994; Bally-Cuif et al., 1998; Tay et al., 2000), and clam (Walker et al., 1999). At germinal vesicle breakdown (GVBD), CPEB is phosphorylated by cdc2 kinase, resulting in an apparent size shift on SDS-polyacrylamide gels (Hake & Richter, 1994; Walker et al., 1999). In clams, unmodified CPEB migrates at 82 kDa (p82) whereas the phosphorylated form migrates at 92 kDa (p92). *Xenopus* CPEB is also phosphorylated earlier in maturation by Eg2 kinase, which enhances CPSF binding (Mendez et al., 2000a, 2000b). However, the Eg2 kinase sites are not conserved in clam, fly, or nematode CPEBs, and at least in clam, early phosphorylation is performed by MAP kinase (Katsu et al., 1999).

To extend our understanding of CPEB in translational control, we used immunoprecipitation to identify interacting proteins of clam p82/CPEB. We report here on a 47-kDa protein that associates with clam p82 in oocytes, in an RNA-dependent manner, and encodes a member of the RCK/p54 subfamily of DEAD box RNA helicases. The *Xenopus* homolog of clam p47, Xp54, is an integral component of oocyte mRNP, and possesses helicase activity (Ladomery et al., 1997). We provide evidence, using the tethered approach, that *Xenopus* Xp54 represses translation and propose that the RCK/p54 helicase family has a conserved function in masking maternal mRNA.

RESULTS

Identification of clam p82/CPEB interacting partners in oocyte and egg lysates

Three rabbit sera were raised against full-length bacterially expressed clam p82/CPEB. All three detected clam p82 with great specificity and affinity in western blots of oocyte proteins (see Minshall et al., 1999, for one example), and all three recognized the highly conserved C-terminal portion of the protein, corresponding to the RNA-binding domain of this protein family (Hake et al., 1998; Walker et al., 1999), rather than the variable N-terminal portion (data not shown).

We performed an extensive analysis of proteins that specifically coimmunoprecipitate with CPEB in oocyte and in egg lysates to identify potential stage-regulated partner polypeptides. In these experiments, preimmune and anti-p82/CPEB rabbit antibodies were covalently coupled to protein-A Sepharose, so that bound proteins eluted with SDS buffer could be analyzed by protein staining to reveal the specificity and range of coimmunoprecipitating proteins. Lysates were used untreated or after RNAse A treatment to distinguish between protein- and RNA-dependent interactions.

We found the following to be consistent observations in at least four batches each of oocyte and egg lysates; we show the results with one oocyte lysate and one egg lysate immunoprecipitated with antibodies from preimmune and three immune sera (Fig. 1). As expected, in oocyte immunoprecipitates, p82/CPEB is a major silver-stainable band. p92/CPEB is present in egg immunoprecipitated material (see the western in Fig. 3), but the phosphorylated clam CPEB apparently silver stains with a lighter hue than its unmodified form. In addition, several abundant or moderately abundant polypeptides specifically coprecipitated with CPEB, but not with preimmune IgG-coated beads. In particular, a 47-kDa protein (p47) is coimmunoprecipitated in oocytes, but is absent from egg precipitates and from oocyte ones pretreated with RNase A. Secondly, unlike p47, p60 is found in both oocyte and egg lysates, but its coprecipitation also requires RNA. We also noted the presence of higher molecular weight polypeptides that specifically pelleted with immune IgG-bound beads, including one migrating at about 105 kDa. We have thus discerned a variety of patterns of possible clam CPEB partners, whose changes in association and

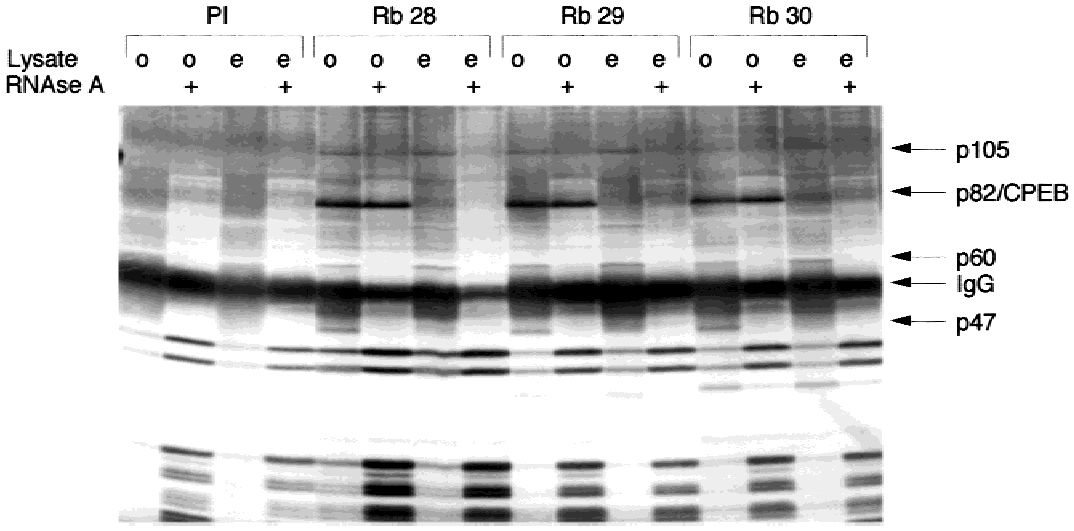

FIGURE 1. Proteins that coimmunoprecipitate with clam p82/p92 in oocyte and egg lysates. Proteins that coimmunoprecipitate with preimmune (PI) and three rabbit anti-p82 antisera (Rb 28, Rb 29, and Rb 30) covalently bound to protein A-Sepharose, from clam oocyte (o) and egg (e) lysates. In indicated lanes, extracts were preincubated with RNAse A prior to immunoprecipitation (+). Proteins were separated on a 15% polyacrylamide gel and detected by silver nitrate staining. The positions of p82/CPEB, p47, p60, p105, and IgG are shown.

RNA dependence presumably reflect global structural changes in mRNP upon oocyte activation. Following large-scale purification, peptide sequences obtained by mass spectroscopy from the 39-, 47-, 60-, and 105-kDa proteins were used to search databases. (The 39-kDa protein, subsequently shown by sequencing to be aldolase, only coprecipitated with rabbit 30 antibody, and has not been pursued further.) Here we report the identification of p47.

Clam p47 is a member of the RCK/p54 family of DEAD box helicases

p47 contained four peptides found in a maternally expressed *Drosophila* protein called ME31B (de Valoir et al., 1991), and other members of what is known as the RCK/p54 family of DEAD box RNA helicases, after the human protein (Akao et al., 1992; Lu & Yunis, 1992; see Fig. 2). Members of the DEAD box superfamily of proteins are widely distributed in nature and are involved in a variety of cellular processes, including splicing, ribosome biogenesis, RNA transport, degradation, and translation, though their precise contribution to most of these processes is not known. They are characterized by eight conserved domains, including the eponymous tetrapeptide DEAD (or the variant DexH/DEAH) motif, with roles in catalysis and substrate binding. These enzymes use NTP (usually ATP) hydrolysis to unwind short RNA duplexes in a nonprocessive manner and may also influence rearrangements of large RNA structures or protein–RNA interactions. Their ATPase activity is stimulated or dependent on RNA binding but it is unclear to what extent DEAD box proteins recognize specific RNA sequences (reviewed in Fuller-Pace, 1994; Lüking et al., 1998; de la Cruz et al., 1999). The prototype of the DEAD box superfamily is the translation initiation factor eIF4A, with a role (along with eIF4B) in unwinding structured leader sequences (Pause et al., 1994). In view of the role of eIF4A in translation, it was important to test whether clam p47 was indeed a member of the RCK/p54 or the eIF4A helicase subfamily of RNA helicases. Clam eIF4A, a polypeptide of 49 kDa detected with an antibody raised against bovine eIF4A, did not coimmunoprecipitate with CPEB in either oocyte or egg lysates (data not shown). The distinction between p47/RCK and eIF4A was corroborated during cloning.

Clam p47/RCK helicase

Clam p47 helicase was cloned as described in Materials and Methods, based on sequences shared by the RCK/p54 family of DEAD box helicases. The 1917-nt-long cDNA appears to be nearly complete, as the mRNA size, according to northerns, was around 2.2 kb (not shown). The ORF contained 449 amino acids with a predicted molecular weight of 50.7 kDa, close to its observed migration on SDS-PAGE. The conceptual protein sequence contained all four peptide sequences obtained by sequencing of clam p47 (Fig. 2).

Clam p47 is aligned in Figure 2 with the RCK/p54 family members including human RCK (Lu & Yunis, 1992), mouse p54 (Seto et al., 1995), *Xenopus* Xp54 (Ladomery et al., 1997), *Drosophila* ME31B (de Valoir et al., 1991), *C. elegans* Cgh-1, *Schizosaccharomyces pombe* Ste13 (Maekawa et al., 1994), and *Saccharomyces cerevisiae* Dhh1 (Strahl-Bolsinger & Tanner, 1993). The degree of homology between members

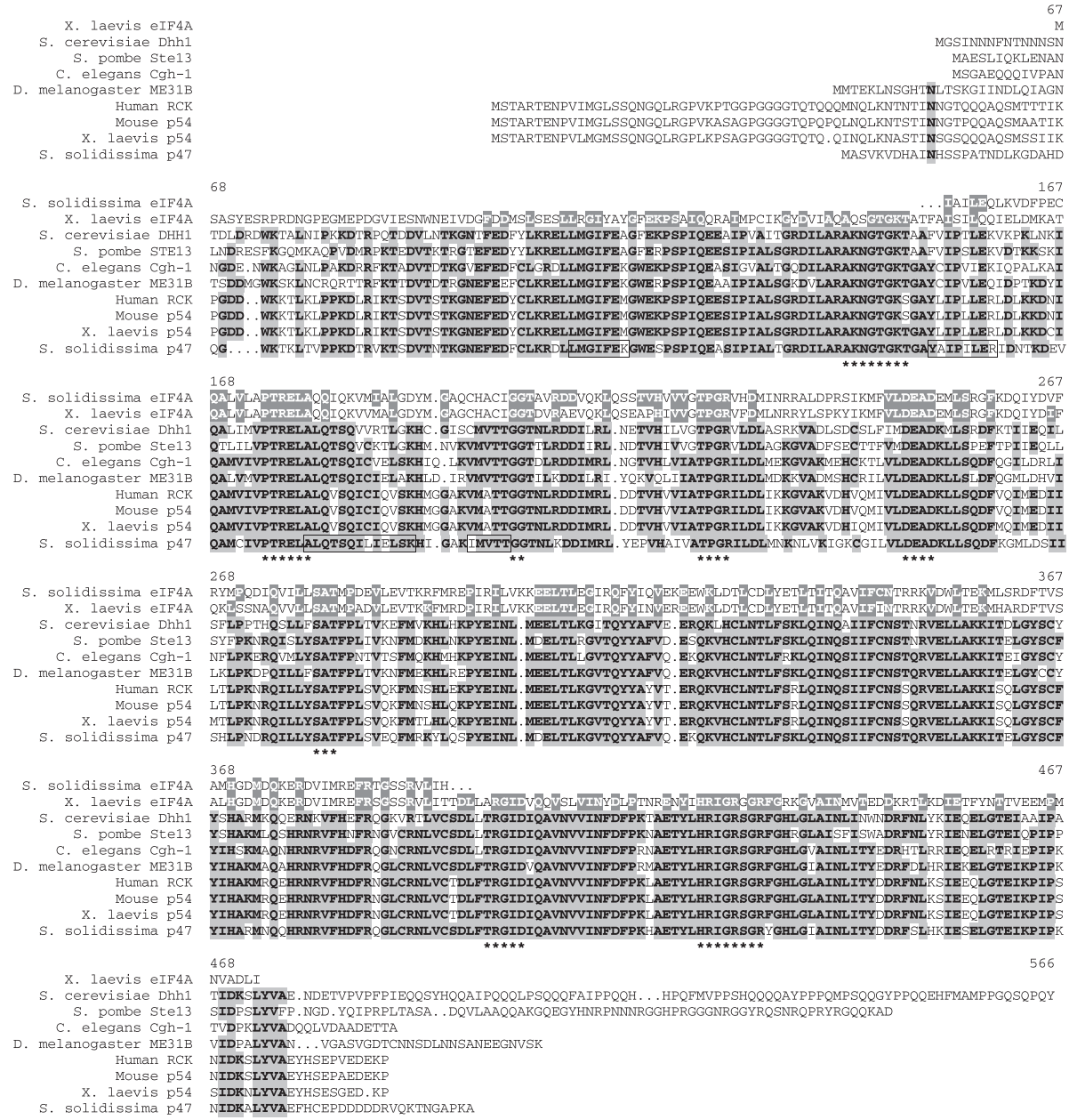

FIGURE 2. Clam p47 is a member of the RCK family of DEAD box helicases. Alignment of amino acid sequences of eight members of the RCK/p54 subfamily of DEAD box helicases. Alignments were made using the PILEUP algorithm (GCG version 15). Comparison is made between five invertebrates, clam p47 (AF399934), *S. cerevisiae* Dhh1 (X66057), *S. pombe* ste13 (D29795), *C. elegans* Cgh-1 (AC006605), and *D. melanogaster* ME31B (M59926), and three vertebrates, human RCK (D17532), mouse p54 (D50494), and *X. laevis* Xp54 (X92421). Included in the alignment are also sequences of *Xenopus* eIF4A (Y12590) and a partial sequence of clam eIF4A (AF399935). The eight motifs typical of DEAD box helicases are indicated by asterisks. Sequences conserved between RCK helicases are highlighted in pale gray, and those common to RCK and eIF4A helicases are highlighted in dark gray (white letters). The four peptide sequences obtained from clam p47 are boxed.

of the RCK helicase family is strikingly high throughout the helicase core, with differences being confined to the N and C termini. Such variable extensions have been suggested in other helicases to mediate substrate specificity or subcellular localization; no obvious conserved RNA-binding or localization domains reside in these extensions. The clam and *Xenopus* proteins share 77% of identical residues (85% homologous) whereas clam and the yeast proteins contain 68% identical (77% homologous) residues. All of the sequences share the motifs typical of DEAD box helicases (Fig. 2).

During the course of cloning, a partial clone encoding clam eIF4A was also obtained and is included in the

alignment along with *Xenopus* eIF4A. In Figure 2, amino acid residues conserved in RCK helicases are highlighted in pale gray, whereas those common to RCK and eIF4A helicases are shown in dark gray. Though both belong to the DEAD box helicase superfamily, they are distinguishable by family-specific motifs. In particular, the clam p47 peptide sequences clearly place this protein among the RCK helicase family, in contrast to the eIF4A family.

Relatively little, until very recently, was known about RCK helicases (see Discussion). Their functions are by no means confined to oogenesis—whereas *Drosophila* ME31B (de Valoir et al., 1991), *Xenopus* Xp54 (Ladomery et al., 1997), mouse RCK (Paynton, 1998), and clam p47 are maternally expressed, close homologs have been identified in *Arabidopsis*, yeast, and protozoa (malaria and trypanosomes). Human RCK/p54, a lymphoma-linked chromosomal translocation junction gene on 11q23 (Lu & Yunis, 1992) is overexpressed in colorectal tumors (Nakagawa et al., 1999). In *S. pombe* and *S. cerevisiae*, the orthologs ste13 and Dhh1 are essential for sexual reproduction but not for growth (Maekawa et al., 1994; Moriya & Isono, 1999). Significantly, Xp54, the *Xenopus* homolog of human RNA helicase p54, is an abundant and integral component of stored mRNP in oocytes and possesses ATP-dependent helicase activity (Ladomery et al., 1997).

Coimmunoprecipitation of p82/CPEB and p47 helicase

To further characterize the p47 helicase, we prepared a rabbit antibody against bacterially expressed full-length clam p47. Only one protein, of 47 kDa, was recognized on western blots of total clam oocyte and egg proteins (Fig. 3A). Immunoprecipitations were carried out as described in Figure 1, and the protein-A Sepharose bead-bound proteins were analyzed by silver staining as well as western blotting with both p82 and p47 antibodies (Fig. 3B). First, as noted earlier, both p82 and its phosphorylated p92 forms are present in the CPEB precipitates as shown by western blotting, though p92 is barely discernible by silver staining. Secondly, the identification of clam p47 as a RCK helicase is confirmed by western blotting of the CPEB precipitates with the p47 antibody. This protein coimmunoprecipitates with p82 in untreated oocyte lysates, but is absent from RNAse A-treated oocyte lysates and from both egg lysates. Moreover, in the reciprocal precipitations, p47 antibody brought down p82/CPEB in oocyte lysates, but not in egg lysates. The interaction between p47 and p82 in these oocyte complexes was partially sensitive to RNAse A. The difference in nuclease sensitivity between the two sets of precipitates may arise from varying protection of RNA by the bound antibodies.

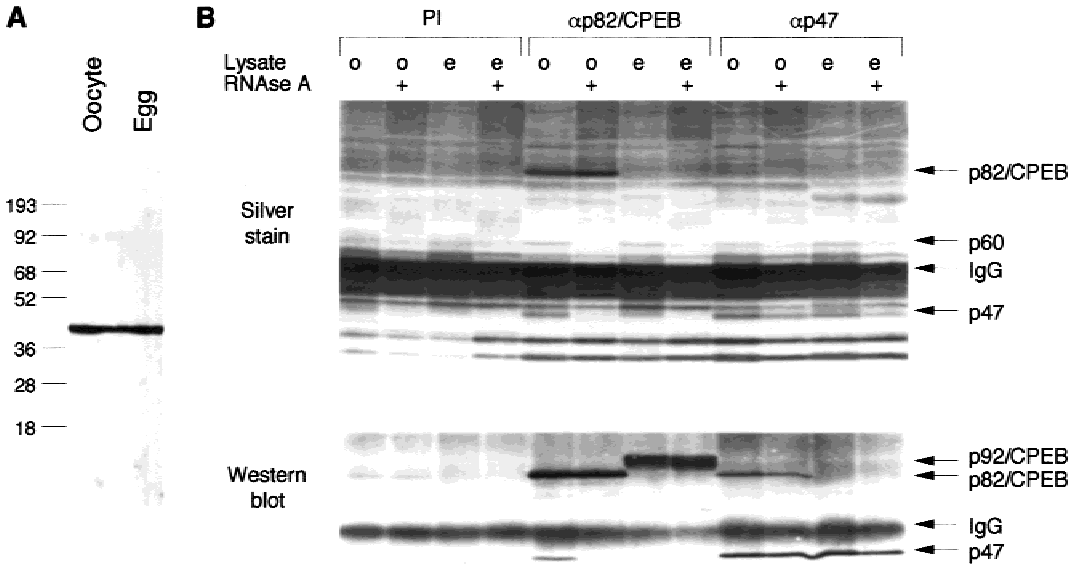

FIGURE 3. Verification of CPEB and p47 helicase coimmunoprecipitation in reciprocal antibody pull downs. **A:** Immunoblot of total clam oocyte and egg lysate proteins developed with anti-p47 antibody. Sizes, in kilodaltons, of molecular weight standards are indicated. **B:** Proteins that coimmunoprecipitate with preimmune (PI), anti-p82/CPEB, and anti-p47 antisera covalently bound to protein A-Sepharose, from clam oocyte (o) and egg (e) lysates. In indicated lanes, extracts were preincubated with RNAse A prior to immunoprecipitation (+). Proteins were separated on a 15% polyacrylamide gel and detected by silver nitrate staining (top panel) and by western blotting with a mixture of p82/CPEB and p47 antibodies (bottom panel). The positions of p82/p92/CPEB, p47, p60, and IgG are shown.

Altogether, these data show that p82/CPEB and p47 helicase copurify in oocytes in reciprocal antibody pull-down experiments and that the helicase no longer interacts with phosphorylated CPEB in eggs. We estimate by western blotting, using serial dilutions of lysates and known amounts of recombinant proteins, that the clam S10 oocyte lysate contains about 100 μg/mL of p82/CPEB and 50 μg/mL of p47 helicase (data not shown). In other words, in oocytes, these two proteins are approximately equimolar. Last, a close examination of the silver stain gel reveals that p60 is present in both p82 and p47 precipitates of untreated oocyte and egg lysates, but not in control IgG-bound beads, suggesting that this protein is an additional component of the CPEB/p47 complex.

Biochemical copurification of p47 and p82 in oocyte mRNP

Clam oocyte lysates were fractionated by large pore gel filtration on Sepharose CL6B in low and high (0.5 M KCl) salt-containing buffers. Previously we showed that masked mRNP and ribosomes separate from cytosolic proteins in low salt conditions. In high salt conditions, the mRNP were translationally activated concomitant with the release of salt-labile proteins including p82 (Walker et al., 1996). Here we show that both clam CPEB and p47 helicase are exclusively present in heavy complexes and are released from them by salt treatment. In contrast, another RNA-binding protein, clam PABP, which is present in excess over mRNA in oocytes (de Melo Neto et al., 2000), is largely free in the cytosol (Fig. 4).

In 15–50% sucrose gradients of oocyte lysates (Fig. 4), CPEB and p47 show a similar broad distribution, with the majority sedimenting in the 80S region. In contrast, in egg lysates, whereas the distribution of p47 in the gradient is unaffected, CPEB is released from the large RNP and migrates towards the top of the gradient (in these fractions it appears also to be partially dephosphorylated). Neither CPEB nor p47 helicase appear to be associated with polysomes, though it is important to note that very few ribosomes are translationally active in either stage. Together these observations suggest that meiotic maturation-induced phosphorylation releases CPEB from mRNP, though not p47; very likely explaining why CPEB and p47 helicase do not coimmunoprecipitate in eggs.

Developmental expression and localization of clam p47 helicase and p82/CPEB

Next, we examined the expression levels and localization of clam p47 helicase and p82/CPEB. The levels of clam p47 and p82/CPEB in developing oocytes were examined by western blotting of samples taken at 10-min intervals from a fertilized culture. The same samples were also analyzed with cyclin A antibodies (Fig. 5A). The initial rise in cyclin A levels is due to the translational activation of masked cyclin A mRNA, and its periodic decreases mark the meiotic and mitotic cell divisions in the developing embryos (Hunt et al., 1992). CPEB undergoes a complex set of phosphorylation-induced mobility shifts, initiated at GVBD, around 10 min postfertilization, and culminating in a 92-kDa polypeptide at 30 min, p92/CPEB then undergoes two waves

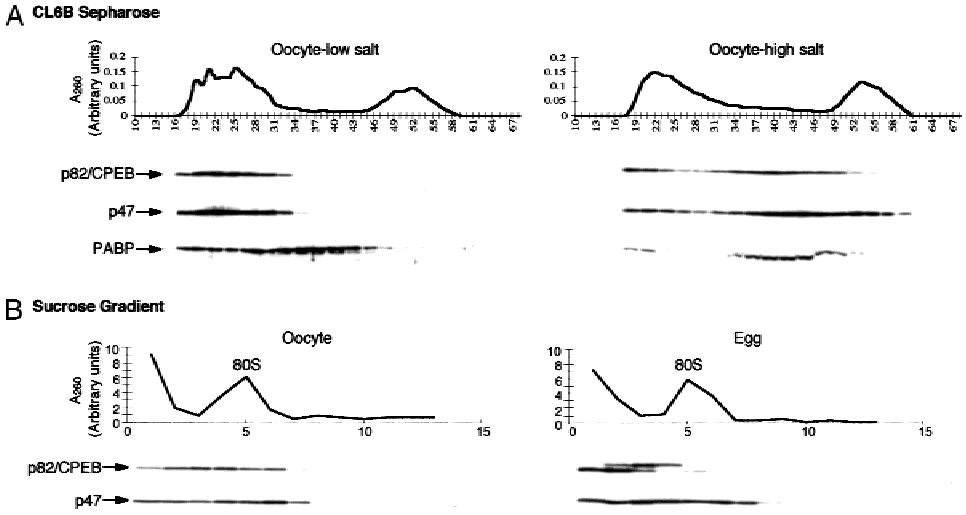

FIGURE 4. Biochemical copurification of p47 helicase and CPEB in mRNP. **A:** Immunoblots of fractions obtained by Sepharose-CL6B gel filtration of oocyte lysates in low salt- (left panel) and high salt- (right panel) containing buffers developed with p82/CPEB, p47, and PABP antibodies as indicated. **B:** Sedimentation of CPEB and p47 from oocyte (left panel) and egg (right panel) extracts through a 15–50% sucrose gradient. Immunoblots of fractions were developed with CPEB and p47 helicase antibodies.

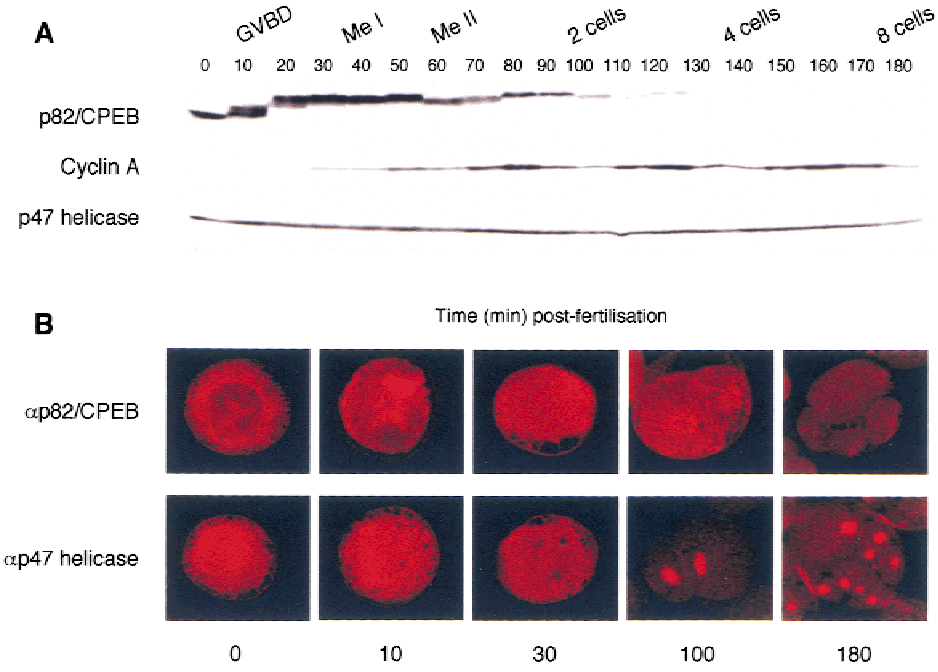

FIGURE 5. Clam helicase levels and subcellular localization during meiotic maturation. **A:** Developmental expression pattern of p82/CPEB and p47/helicase. A culture of fertilized oocytes was sampled at 10-min intervals for 3 h at 17–18 °C and total cell protein from each sample was analyzed by western blotting with p82/CPEB, p47, and cyclin A antibodies. The levels of cyclin A show that in this culture meiotic Ana I and II occurred at around 35 and 60 min and the first and second mitotic cleavages at 90 and 130 min postfertilization, respectively. **B:** Clam p82/CPEB and p47/helicase subcellular localization. Paraformaldehyde-fixed cells withdrawn at 0, 10, 30, 100, and 180 min postfertilization were stained with p47 and p82 antibodies and subsequently with rhodamine Alexa 568-conjugated secondary antibodies, and visualized in optical sections in a confocal microscope.

of degradation at around the time of the second meiotic anaphase and the first mitotic anaphase, concomitant with apparent dephosphorylation, and is barely detectable after 2 h. In contrast to these modifications of CPEB, the apparent size and level of clam p47 remains unchanged, at least until 3 h postfertilization (Fig. 5A).

Oocytes and embryos were briefly fixed in paraformaldehyde and extracted with 0.1% Triton-X100 (Wu & Palazzo, 1998), before being processed for immunofluorescence with p82/CPEB and p47/helicase antibodies (Fig. 5B). Embryos were harvested at 10, 30, 100, and 180 min postfertilization, corresponding approximately to GVBD, metaphase I, two cells and eight cells, respectively. CPEB staining observed in the laser-scanning confocal microscope is largely cytoplasmic and granular, with hints of tubular networks outside the GV, and with the most intense staining surrounding the nucleus. At GVBD, this intense staining surrounds the disintegrating germinal vesicle, and subsequently the granular staining is distributed homogeneously throughout the cytoplasm (30 and 100 min). By 180 min, the eight-cell stage, the CPEB signal is essentially absent (see Fig. 5A). p47 in oocytes and eggs shows a similar granular staining to that of CPEB though not as punctate; the major difference being the presence of p47 in both the nucleus and cytoplasm. In two- and eight-cell-stage embryos, p47 undergoes a dramatic and complete nuclear translocation (Fig. 5B).

Clam p82 coprecipitates with Xp54 in *Xenopus* oocytes

To assess the conservation of the helicase-CPEB interaction, mRNA encoding HA-tagged p82/CPEB was injected into *Xenopus* oocytes, and anti-HA immunoprecipitates were analyzed by western blotting using anti-Xp54 antibodies. Xp54 coprecipitated with clam p82/CPEB in *Xenopus* oocytes (data not shown). However, this interaction was not reproducibly observed in repeated experiments, possibly reflecting the higher endogenous content of *Xenopus* CPEB (Hake & Richter, 1994) relative to the protein synthesized from the microinjected construct and/or low affinity interactions between vertebrate and invertebrate RNP components. To pursue functional studies, we therefore turned to analysis of the Xp54 helicase in *Xenopus* oocytes.

Tethered Xp54 represses translation in *Xenopus* oocytes

To investigate the role of this class of helicases in mRNA masking, we adopted the tethered approach. Here, pro-

teins suspected of affecting RNA metabolism, be it at the level of stability (Coller et al., 1998) or translation (Gray et al., 2000), are tethered to the 3′ UTR of reporter mRNA. Tethering is achieved via an MS2–protein fusion and a tandem pair of MS2-binding sites placed downstream of firefly luciferase mRNA (Fig. 6A). The advantages of this approach are that knowledge of the protein's own RNA-binding specificity is not required, and that high levels of endogenous protein are not an obstacle to its analysis. *Xenopus* oocytes were injected with mRNAs encoding the following fusion proteins (Fig. 6B): as negative controls we used MS2 alone and MS2 fused with U1A, a spliceosomal RNA-binding protein; as positive control, MS2-PABP (Gray et al., 2000), wild-type MS2-Xp54, and two inactivating mutant forms, MS2-Xp54-DQAD and MS2-Xp54-HRIGQ (see below). Synthesis of the fusion proteins was routinely monitored by incubating injected oocytes in ^{35}S Trans-label followed by SDS-PAGE and autoradiography, and by western blotting with anti-MS2 antibodies. Similar levels of all fusion proteins were expressed in oocytes (Fig. 6C). The small MS2 protein was only visualized by western, as it has too few methionines to be readily detected by autoradiography.

Fusion protein mRNAs were injected into oocytes, which were then incubated for 6 h to allow protein production prior to a second injection of firefly luciferase mRNA-MS2 3′ UTR. Initially, the reporter RNA was capped, but nonadenylated. The lack of a poly(A) tail approximates the state of masked *Xenopus* mRNAs, and allowed the use of the positive control MS2-PABP fusion protein. Firefly luciferase-MS2 3′ UTR mRNA was coinjected with an internal control mRNA encoding *Renilla* luciferase, which lacked any regulatory sequences in its 3′ UTR. After a further 18-h incubation, lysates were prepared, and both luciferase activities determined.

In these experiments, translational activity was quantified as firefly luciferase activity normalized to *Renilla* luciferase activity. Variations in *Renilla* luciferase were

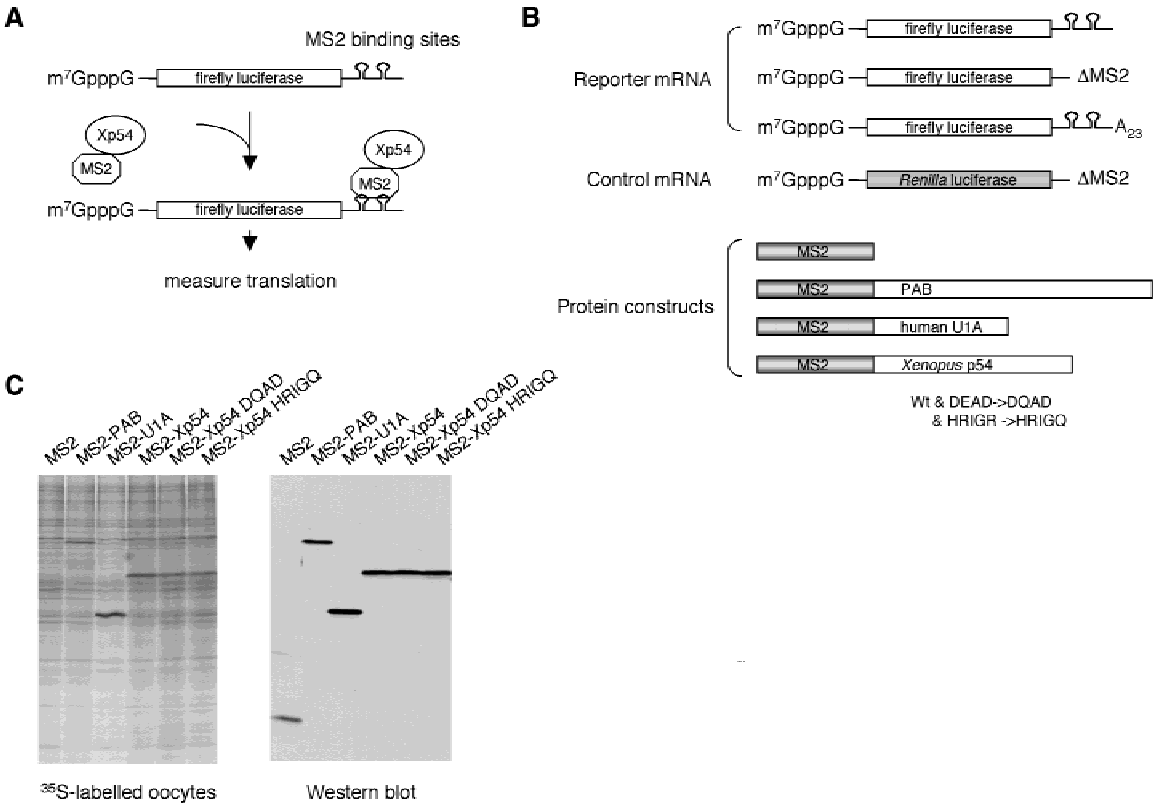

FIGURE 6. The "tethered function" approach for assaying translational effects of Xp54 on firefly luciferase mRNA. **A:** Tethering strategy. **B:** Reporter and protein constructs. *Xenopus* oocytes are first injected with mRNAs encoding MS2-protein fusions as indicated. Following 6 h incubation to allow expression, two luciferase mRNAs are coinjected, encoding control *Renilla* luciferase that lacks 3′ UTR regulatory sequences, and firefly luciferase mRNA containing two MS2-binding sites (luc-MS2), firefly luciferase mRNA lacking MS2-binding sites (luc-ΔMS2), or polyadenylated firefly luciferase mRNA (luc-MS2$^+$). Activities of both luciferases are then assayed after a further 18 h. **C:** Monitoring MS2-fusion protein expression in ^{35}S Trans-labeled oocytes (left) and in western blots with MS2 antibody (right). mRNAs encoding fusion protein were injected into oocytes, which were incubated for 22 h to allow expression and lysates were prepared for gel electrophoresis, autoradiography, and western blotting.

<10%, showing that the control mRNA was insensitive to the fusion proteins (see Fig. 7 legend). The ratios of firefly/*Renilla* luciferases activities obtained in the presence of the fusion proteins are shown relative to the value observed with the MS2 protein alone, set at 1 (Fig. 7A). The control MS2-U1A fusion protein does not have a significant effect on firefly luciferase, whereas MS2-PABP activates translation almost sixfold (Fig.7A, right panel), entirely in agreement with the results of Gray et al. (2000). In contrast, MS2-Xp54 represses firefly luciferase expression three- to fivefold (Fig. 7A, left panel).

To test whether the inhibitory effect of Xp54 helicase was *cis* dependent, we assayed the effects of the fusion proteins on a firefly luciferase mRNA that lacked MS2-binding sites. None of the fusion proteins, including the stimulatory PABP and the inhibitory Xp54, affected the expression of firefly luciferase DMS2 mRNA (Fig. 7B), indicating that such effects required the MS2-binding sites in the reporter 3′ UTR. Moreover, as noted

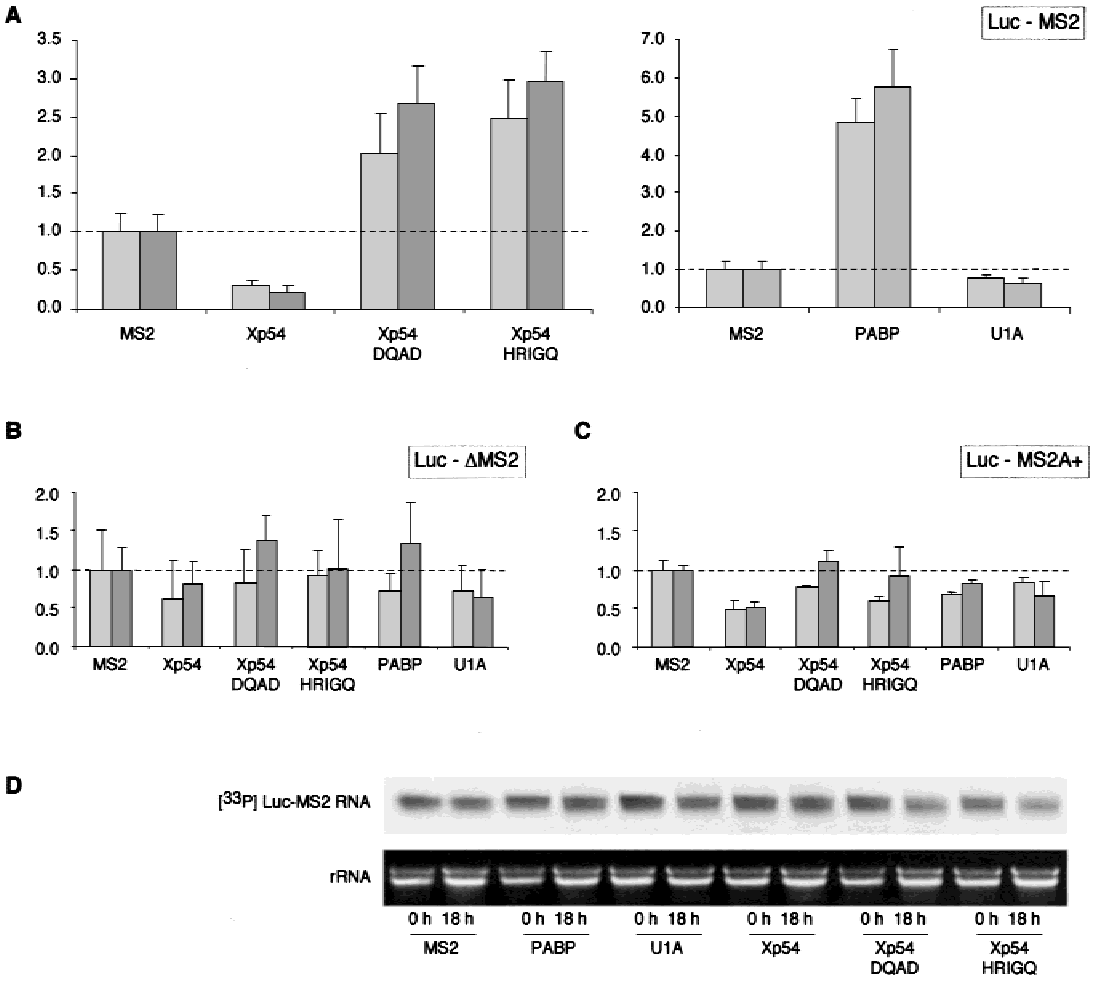

FIGURE 7. Tethered Xp54 represses translation. **A:** Xp54 represses translation of firefly luciferase-MS2 reporter mRNA. Two representative experiments are shown. Three to five pools each containing three to five oocytes were assayed per experimental point, and mean values and standard deviations were determined. The ratio of firefly to *Renilla* luciferase activities was set to 1 in the case of MS2 protein. (Relative *Renilla* luciferase activities, in the presence of the fusion proteins were MS2 100%, PABP 109%, U1A 89%, Xp54 89%, Xp54 DQAD 108%, Xp54 HRIGQ 103%.) The left panel reports the data obtained with Xp54 fusion proteins, and the right panel shows the data observed with the control fusion proteins, PABP and U1A (note the twofold change in scale). **B:** Effects of wild-type and mutant Xp54 helicase on firefly luciferase mRNA are *cis*-dependent. Two representative experiments are shown. Following injection of fusion protein mRNAs as in **A**, firefly luciferase mRNA lacking MS2-binding sites (luc-ΔMS2) was injected and the activities of both luciferases determined. **C:** None of the fusion proteins appreciably affect polyadenylated firefly luciferase mRNA (luc-MS2$^+$). Two representative experiments are shown. See **A** and **B** for further details. **D:** The stability of ^{33}P-labeled firefly luciferase mRNA (luc-MS2) after incubation for 18 h in oocytes expressing MS2, U1A, PABP, Xp54, Xp54 DQAD, and Xp54 HRIGQ (18 h lanes). RNA was also harvested from oocytes immediately following injection of luciferase mRNA (0 h lanes). 28S and 18S rRNA (lower panel) served as recovery and loading controls.

above, *Renilla* luciferase mRNA, also lacking MS2-binding sites, was unaffected by the fusion proteins.

^{33}P-labeled reporter RNA was equally stable in oocytes expressing MS2 and MS2–fusion proteins over the course of the experiment, strongly suggesting that the helicase fusion protein affected translational activity rather than the integrity of the reporter RNA (Fig. 7D).

To determine whether repression required the helicase activity of Xp54, mutations were made in two of the conserved helicase motifs of Xp54, DEAD → DQAD (Pause & Sonenberg, 1992) and HRIGR → HRIGQ (Pause et al., 1993). The DEAD region (motif II) represents the Walker B motif of ATP-binding proteins. The highly conserved glutamic residue is required for ATP hydrolysis; DQAD or DAAD mutants of eIF4A, Dbp5, and Ded1 can bind ATP and RNA, but lack ATPase activity (Pause & Sonenberg, 1992; Iost et al., 1999; Schmitt et al., 1999). In contrast, the HRIGRXXR (motif VI) region mediates RNA binding. The HRIGQ mutants of eIF4A and Dbp5 do not bind RNA, whereas their ATPase activity is only partially reduced (Pause et al., 1993; Schmitt et al., 1999). None of these mutants possess helicase activity. Moreover, mutants in these motifs of the *S. pombe* homolog of Xp54, Ste13, could not complement the *ste13* disruptant strain (Strahl-Bolsinger & Tanner, 1993). Strikingly, mutations in the Xp54 helicase motifs not only alleviated repression, but in fact led to an approximately threefold increased expression relative to MS2. In other words, two single amino acid residue mutations enhance expression by approximately 10- to 15-fold, relative to the wild-type helicase (Fig. 7A). The Xp54 mutant fusion proteins required MS2 sites *in cis* (Fig. 7B), and did not affect reporter RNA stability (Fig. 7D). These experiments indicated that repression relied on an active Xp54 helicase, and, moreover, that abrogation of the putative helicase activity resulted in translational activation.

Last, we tested whether Xp54 could repress translation of a polyadenylated reporter RNA. When the luc-MS2 reporter RNA was transcribed with an A23 tail, a similar general enhancement of translation (5–10-fold) was seen, irrespective of the identity of the fusion protein (Fig. 7C). Here again, the ratios of firefly/*Renilla* luciferases activities obtained in the presence of the fusion proteins are shown relative to the value observed with the MS2 protein alone, set at 1. Overexpressed MS2-PABP does not further stimulate the translation of polyadenylated RNA, and, conversely, Xp54 does not repress polyadenylated RNA as efficiently as nonadenylated RNA. Furthermore, Xp54 mutants do not appreciably affect the translation of luc-MS2A$^+$ RNA, in contrast to their stimulation of nonadenylated luc-MS2 RNA (contrast Fig. 7C with 7A). These data lead us to conclude that the presence of a poly(A) tail prevents Xp54-mediated repression.

DISCUSSION

RCK/p54 helicase in oocyte RNP

We used coimmunoprecipitation to identify stage-regulated partners of clam p82/CPEB. Several polypeptides that interact with p82/CPEB and/or p92/CPEB, including p47, p60, and p105, were sequenced (Fig. 1). The peptide sequences obtained from p60 and p105 have not yet allowed their identification, whereas p47 contained four peptides, placing it within the RCK subfamily of DEAD box RNA helicases (Fig. 2). Partners of *Xenopus* CPEB in oocytes include the 150-kDa maskin, an eIF4E-sequestering protein (Stebbins-Boaz et al., 1999), and the 137-kDa XPum, a homolog of *Drosophila* Pumilio that represses *hunchback* mRNA translation (Nakahata et al., 2001). The direct interaction between CPEB and Pumilio is conserved in *C. elegans*, between CPB-1 and FBF, to control spermatogenesis (Luitjens et al., 2000). None of the peptides we obtained corresponded to these polypeptides, which may reflect the size range of our selected proteins and/or their abundance. This may also explain why we did not detect proteins in eggs that showed homology to potential cytoplasmic polyadenylation machinery partners of p82/CPEB, such as poly(A) polymerase and CPSF subunits characterized in *Xenopus* (Ballantyne et al., 1995; Gebauer & Richter, 1995; Dickson et al., 1999; Mendez et al., 2000b).

We showed that the clam p47-CPEB interaction is RNAse sensitive (Figs. 1 and 3), implying the existence of RNA(s) that bridge both proteins. *Spisula* CPEB, like its counterparts in *Xenopus* and mouse, binds U-rich tracts in the 3′ UTRs of translationally regulated mRNAs (Minshall et al., 1999); we have so far been unable to ascribe these or any other specific RNA targets to clam p47 helicase. *Drosophila* Me31B, a maternal protein, participates in an RNP complex during oogenesis with Exuperantia and Yps, a cold-shock domain protein, in an RNA-dependent manner and with oocyte-localizing RNAs (Wilhelm et al., 2000; Nakamura et al., 2001). CGH-1 (conserved germline RNA helicase), the *C. elegans* ortholog, is expressed specifically in the germline and early embryo and is localized to P granules and other possible mRNA–protein particles (Navarro et al., 2001). Whereas both the clam helicase and CPEB are part of a large complex in oocytes, in eggs, phosphorylated CPEB (but not the helicase) is released from the RNP complex (Fig. 4), very likely explaining the absence of CPEB/helicase coimmunoprecipitation in mature eggs (Fig. 3). The release of phosphorylated p92/CPEB from RNP probably reflects a late stage of maturation in these lysates. Immunostaining revealed that the clam helicase is distributed throughout the oocyte, in a granular and fibrilar pattern, similar to that observed in *Xenopus* (Ladomery et al., 1997) and *Drosophila* (Nakamura et al., 2001).

The developmental profiles of these two clam proteins (Fig. 5) bear striking similarities to those of their vertebrate counterparts. *Xenopus* CPEB is phosphorylated in maturing oocytes and largely degraded shortly after GVBD (Hake & Richter, 1994; Reverte et al., 2001). In mouse oocytes, CPEB becomes phosphorylated at metaphase I and is not detectable in one- and two-cell embryos (Tay et al., 2000), whereas zebrafish CPEB (zorba) is present in oocytes, but not after fertilization (Bally-Cuif et al., 1998). As shown by Ladomery et al. (1997), the levels of *Xenopus* p54 helicase are constant throughout oogenesis and following fertilization up to blastula. Thereafter its levels decline, but remain discernible through to the swimming tadpole stage. The persistence of the clam and *Xenopus* helicases in embryogenesis indicates possible additional roles of the RCK helicase that do not involve CPEB. In this respect, it is interesting to note that the clam helicase undergoes a striking translocation to nuclei at the two-cell stage, at about the time CPEB levels decline. The timing of p47 helicase import into nuclei also coincides approximately with zygotic transcription, suggesting that this protein is a developmentally regulated shuttling protein with nuclear functions (Fig. 5). Indeed, it has recently been reported that Xp54 is a shuttling protein, recruited to nascent transcripts in the nucleus (Smillie & Sommerville, 2001).

Role(s) of RCK/p54 helicases

When tethered to the 3′ UTR of a reporter RNA, Xp54 represses translation (Figs. 6 and 7). Purified Xp54 displays ATP/GTP-dependent RNA duplex unwindase activity (Ladomery et al., 1997). We found that two independent point mutations, DEAD-DQAD and HRIGR-HRIGQ, which abrogate helicase activity (Pause & Sonenberg, 1992; Pause et al., 1993; Iost et al., 1999; Schmitt et al., 1999) not only overcome the inhibitory effects of the wild-type protein, but result in significant translational activation. We conclude that the activity of Xp54 helicase is required for masking. Moreover, as the mutants activate translation, we speculate that this helicase interacts with a coactivator. In the absence of repression imposed by the helicase, this coactivator can exert its (unknown) functions. When tethered, clam p47 did not significantly repress firefly luciferase translation, though mutation of the conserved DEAD motif resulted in a two- to threefold activation relative to MS2 (data not shown). Although the reason for the observed difference between Xp54 and clam p47 is not immediately obvious, we assume that clam p47 is unable to completely substitute for the *Xenopus* homolog in *Xenopus* oocytes in forming a repressed RNP on firefly luciferase mRNA, but is still capable of binding the potential coactivator. We have so far been unable to prepare sufficient soluble recombinant forms of either the *Xenopus* or the clam protein to provide the tools with which to explore their different behavior (data not shown, J. Sommerville, pers. comm.).

Our data showing that Xp54 helicase represses translation may relate to the observation that translational masking by a *Xenopus* oocyte 320-kDa particle, which contains FRGY2, CPEB, and Xp54, in the reticulocyte lysate cell-free system required a prior particle–mRNA binding step in the presence of ATP. Omitting the binding step or ATP resulted in active translation (Yurkova & Murray, 1997; Mary Murray, pers. comm.).

Remarkably, the fly ortholog Me31B is also involved in translational silencing of maternal mRNAs. Loss of Me31B causes premature translation of the oskar and BicD RNAs during their transport from nurse cells to the oocyte in an RNP complex. It has been proposed that the complex links localization and translational control of maternal RNAs during oogenesis, and may be related to the stored maternal RNP particle in amphibian oocytes (Nakamura et al., 2001). In *S. cerevisiae*, Dhh1 stimulates mRNA decapping by physical association with the decapping enzyme Dcp1 (Uetz et al., 2000; Coller et al., 2001). mRNA turnover in eukaryotes is initiated by deadenylation, which leads to decapping and then 5′-to-3′ exonuclease digestion. As Dhh1 also interacts with Caf1, a subunit of the mRNA deadenylase, it has been suggested that Dhh1 links the processes of deadenylation and decapping (Coller et al., 2001).

Role of RCK helicases in translational repression

A unifying feature that may bridge the role of Dhh1 in yeast mRNA turnover and the roles of the *Xenopus* p54 and *Drosophila* Me31B orthologs in translational silencing of stored maternal mRNAs is the deadenylated state of the target RNAs. Coller et al. (2001) propose that in yeast, Dhh1 promotes decapping by allowing access of Dcp1 to the cap structure by dissociation of the cap binding protein, eIF4E (Schwartz & Parker, 2000). Removal of eIF4E from the cap would also, of course, inhibit translation initiation. In *Xenopus* oocytes or early embryos, translationally silent mRNAs are stable in a nonadenylated state (Voeltz & Steitz, 1998). The unusual stability of deadenylated RNAs in *Xenopus* is very likely due to the absence of decapping activity until midblastula (Zhang et al., 1999). In combination, these data lead us to speculate that Xp54 helicase in oocytes inhibits the formation of an active initiation complex by preventing the association of cap-binding factors with, or displacing them from, mRNA.

Efficient inhibition by wild-type Xp54 helicase and stimulation by helicase mutants was only observed with nonadenylated firefly-MS2 reporter RNA, implying that polyadenylation overcomes repression. Because mutations in Xp54 lead to translational activation of nonadenylated RNAs, the putative helicase activity

may be attenuated by modification or loss of a cofactor (e.g., eIF4B and eIF4A) prior to progesterone-induced cytoplasmic polyadenylation in vivo. In summary then, the data are compatible with a model that posits that in oocytes, CPE-containing mRNAs are repressed by Xp54 bound to CPEB. Early during maturation, the helicase activity of Xp54 is abrogated, leading to translational activation, which is further enhanced by polyadenylation. Determination of the relative contributions to repression of CPE-containing mRNAs by maskin sequestration of eIF4E, and Xp54 helicase's proposed role in eIF4E displacement from mRNA are challenges for the future. It is interesting to note that maskin levels are regulated during oogenesis, and significant amounts are not expressed until stage V/VI (Groisman et al., 2000), suggesting that the helicase may be responsible for repression in earlier stages.

Clam p47 helicase was identified as a polypeptide that coimmunoprecipitated with p82/CPEB. This association was RNA dependent, and only observed in oocytes, not in eggs. Future work will be directed toward exploring the regulation of the association of Xp54 and CPEB in *Xenopus* oocytes, and the regulation of Xp54 activity during meiotic maturation.

MATERIALS AND METHODS

Preparation of clam oocyte and egg lysates

Mature *Spisula solidissima* were obtained from the Marine Resource Center of the Marine Biological Laboratory (Woods Hole, Massachusetts). Oocyte and egg lysates were prepared as previously described (Katsu et al., 1999; Minshall et al., 1999; Walker et al., 1999).

Immunoprecipitation

Polyclonal antibodies were coupled to protein A-Sepharose 6 MB beads (Pharmacia) prior to their use in immunoprecipitations: 100 μL of protein A-Sepharose beads were incubated with 200 μL whole antiserum in 200 μL NET buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, pH 8.0, 0.25% gelatin, 0.02% NaN_3) plus 2% BSA for 2 h at 4 °C with gentle mixing. Beads were then washed with PBS, coupling buffer (27 mM sodium tetraborate, 73 mM boric acid), and 1% (w/v) dimethyl pimelimidate, 100 mM sodium tetraborate. Beads were incubated in the final wash for 16 h at 4 °C with gentle mixing prior to washing with coupling buffer and 1 M Tris-HCl, pH 9.0. The reaction was stopped by incubation in 1 M Tris-HCl, pH 9.0, for 10 min at 25 °C. Five microliters of coupled beads were gently mixed with 10 μL clam extract in 200 μL NET buffer + 2% BSA for 2 h at 4 °C. Beads were washed in NET buffer and bound proteins eluted in protein sample buffer. Samples were separated by SDS-PAGE and proteins detected by silver staining. When required, lysates were supplemented with RNAse A to 375 pg/μL extract and incubated at 10 °C for 20 min prior to their use. This treatment was sufficient to degrade mRNA, as judged by in vitro translation of phenol-extracted RNA. For peptide sequencing, the reactions were scaled up 50-fold. Peptide sequencing was performed at the W.M. Keck Biomedical Mass Spectroscopy Laboratory, University of Virginia.

Immunoblotting

Protein samples were separated by SDS-PAGE on 15% gels and transferred onto Immobilon P membrane (Millipore) using a semidry blotting apparatus. Rabbit anti-clam p82/CPEB antibody (Minshall et al., 1999) was used at a dilution of 1:25,000; rabbit anti-Xp54 antibody at 1:5,000; rabbit anti-eIF4A antibody at 1:1,000, rat anti-HA antibody at 1:1,000; and rabbit anti-MS2 antibody at 1:2,000. Anti-p47 antibody was obtained from rabbits immunized with His-p47 (Eurogentec), and used at 1:2,000. Western blots were subsequently detected by ECL.

Cloning of clam p47

PCR was performed with degenerate primers based on the conserved motifs GNEFED (5′ GGN AAY GAR TTY GAR GA) and INFDFP (5′ GGR AAR TCR AAR TTD AT) present in the p54/RCK subfamily of RNA helicases. Template DNA was produced by random hexamer-primed reverse transcription of total clam oocyte RNA using the First Strand cDNA Synthesis Kit (Boehringer Mannheim). A 1-kbp p47 RT-PCR fragment was labeled using an Oligolabelling Kit (Pharmacia) and used to screen a *Spisula* ovarian cDNA library in λgt22. Three positive plaques were isolated from first round screens and used in PCR reactions using oligo pairs 5′ CTG TCT CAA AAG AGA TTT AC (p47 base 173–192) and 5′ GGC GGC CGC CAG ACC AAC TGG TAA TG (λ reverse primer); 5′ GGC GGC CGC GAC TCC TGG AGC CCG TG (λ forward primer) and 5′ AAC GTT CAC TGC TTG GAT GT (p47 base 1082–1063). Sequence data from these PCR reactions enabled the design of oligos to the 5′ (5′ TTA GAA GCT TTC AAC GTG TT) and 3′ (5′ ATG GAA TTC GGT GTG ATT AT) ends containing *Hin*dIII and *Eco*RI sites, respectively (underlined). PCR reactions from phage eluted from first round screens were performed and the resulting DNA cloned into *Hin*dIII, *Eco*RI-restricted pGEM 1. All three clones were verified by sequencing.

Recombinant His-tagged p47 and preparation of anti-p47 antibody

The p47 expression plasmid was constructed by inserting the open reading frame of p47, as an *Nde*I-*Xho*I fragment into pET21b (Novagen). The cDNA was produced by PCR from the above clones using oligonucleotides 5′ GTA CAT ATG GCT TCA GTG AA and 5′ ATT GCT CGA GTG CTT TTG GT. The amino acid sequence of the bacterially expressed protein differed from the endogenous clam protein by the addition of a C-terminal extension with the sequence LEHHHHHH. A culture of transformed BL21 (DE3) was grown to an A_{600} of ~0.5 at 37 °C and 300 rpm and induced at 20 °C at 16 h by addition of 0.5 mM IPTG. Cells were harvested by centrifugation, resuspended in 300 mM NaCl, 50 mM sodium phosphate buffer, pH 7.8, and lysed using a French pressure cell

and subsequent addition of 1% Triton-X100. The cell debris, including the predominantly insoluble His-tagged p47, was pelleted at 20,000 × *g* for 20 min and resuspended in SDS sample buffer. Aliquots were run on SDS polyacrylamide gels and stained with Coomassie blue dye. Gel slices containing p47-His were excised and used to immunize rabbits.

Biochemical fractionation studies

Clarified clam S10 extracts were fractionated on Sepharose-CL6B (Pharmacia) columns in LSB (25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10 mM HEPES, pH 7.2), or HSB (LSB plus 0.5 M NaCl) (Walker et al., 1996). The A_{260} of each fraction was determined and protein content analyzed by SDS-PAGE and subsequent western blot analysis.

Samples (0.1 mL) of clarified clam S10 extract were diluted fivefold in buffer G (10 mM NaCl, 150 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 10 mM HEPES, pH 7.0, 0.5 mM DTT) and loaded onto 12.5-mL 15–50% sucrose gradients in buffer G. Samples were centrifuged at 40,000 rpm for 1 h 15 min at 4 °C in a Beckman SW40 rotor prior to harvesting 1 mL fractions. Each fraction was analyzed by measurement of the A_{260} and for protein content by precipitation with TCA. Proteins were pelleted by microfugation, washed with acetone, and resuspended in protein sample buffer. Aliquots were separated by SDS-PAGE and proteins detected by western blot analysis.

Immunolocalization

At different time points after fertilization, two to three drops of the embryo seawater suspension were loaded onto poly-L-lysine coated coverslips. The embryos were allowed to attach for 5 min, fixed in 6.4% paraformaldehyde, and washed with PBS. The fixed cells were extracted with 0.1% Triton-X100 in PBS before being processed for immunofluorescence (Wu & Palazzo, 1998) using primary antibodies (1:2,000) and secondary antibodies (goat rhodamine Alexa 568-conjugated anti-rabbit secondary antibodies; Molecular Probes, 1:400). The coverslips were observed with a Zeiss Axiovert inverted microscope or a laser-scanning confocal microscope.

Xenopus oocyte preparation and microinjection

Ovarian lobes were removed from *Xenopus laevis* females. Stage VI oocytes were isolated and stored at all times in modified Barth's solution (8.8 mM NaCl, 1 mM KCl, 330 μM $Ca(NO_3)_2$, 410 μM $CaCl_2$, 820 μM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES-NaOH, pH 7.4). Oocytes were injected with 10–15 ng capped, 3×HA-tagged clam p82/CPEB RNA and incubated at 20 °C for 16 h prior to homogenization in 4 μL modified Barth's solution/oocyte. Extracts were clarified by centrifugation at 8,500 × *g* at 4 °C. When ^{35}S labeling was required, five oocytes were transferred, 3 h postinjection, into 50 μL modified Barth's solution containing ^{35}S-Translabel (ICN) at 1 μCi/μL and incubated for 16 h at 20 °C. After removing excess label, oocytes were processed as normal.

Tethering

Control plasmids and the Luc-MS2 reporter were supplied by N.K. Gray (Gray et al., 2000). cDNAs encoding Xp54 (clone B2) and p47 were cloned as PCR products using oligo pairs 5′-GTTGCTAGCATGGCTTCAGTGAAAG and 5′-TCACTCGAGCTATGCTTTTGGTGCA or 5′-CGCGCTAGCATGAGCACCGCCAGAA and 5′-ACGGTCGACTTAAGGTTTGTCTTCC, respectively, containing *Nde*I and *Xho*I/*Sal*I restriction sites (underlined), into *Nde*I-*Xho*I restricted MSP vector. To enhance expression, fusion proteins were subsequently modified by addition of an ~250-nt *Bgl*II-*Bam*HI fragment encoding a β-globin 3′-UTR-$A_{23}C_{30}$ (from pS664TEN), cloned into *Bam*HI restricted plasmids. cDNA encoding U1A (from clone MS2-U1A) was cloned as a PCR product using oligos 5′-CGCTAGCATGGCAGTTCCCGAGACCCG and 5′-CCTCGAGCTACTTCTTGGCAAAGGAGA into *Nde*I-*Xho*I restricted MSP vector, previously modified by addition of a 3′ UTR, as described above. All PCR reactions were performed under standard conditions using pfu DNA polymerase (Stratagene). 3′ UTR modification of the MSP vector resulted in deletion of its stop codon. A new stop codon was inserted over the *Bam*HI site at codon 158 by insertion of a G-to-T point mutation. Xp54 DEAD → DQAD (E246Q) and HRIGR → HRIGQ (R423Q) mutants were cloned by insertion of G-to-C and AG-to-CA mutations, respectively. All mutagenesis was performed using a Stratagene QuikChange site-directed mutagenesis kit and verified by sequencing.

Plasmids encoding fusion proteins were linearized with *Hin*dIII. Luc-MS2 was linearized with *Bgl*II and to obtain Luc-ΔMS2 mRNA, Luc-MS2 was linearized with *Spe*I; prior to transcription with T7 RNA polymerase. Capped ^{33}P-labeled Luc-MS2 RNAs were transcribed in the presence of [$\alpha^{33}P$]-UTP (25 μCi, ≥2500 Ci/mmol, Amersham).

Oocyte micromanipulation and microinjection were performed essentially as described above. Fifty nanoliters (at ~500 ng/μL) of mRNA encoding fusion protein were injected 6 h prior to injection of 10 nl of a Luc-MS2 reporter mRNA (10 ng/μL) and *Renilla* luciferase control mRNA (0.35 ng/μL). Incubation was continued overnight before harvesting. Three to five pools each containing three to five oocytes were assayed per experimental point. Oocytes were homogenized in lysis buffer (50 μL/oocyte; Promega) and 10 μL samples assayed for firefly and *Renilla* luciferase activities using a Dual-Luciferase assay system (Promega) in a TD20/20 Turner Designs luminometer. Where RNA extraction was required, 10 oocytes were lysed in 200 μL TNES (0.1 M Tris-HCl, pH 7.5, 0.3 M NaCl, 5 mM EDTA, 2% SDS, 200 μg/mL proteinase K) at 50 °C for 30 min prior to phenol/chloroform extraction and ethanol precipitation.

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