CPEB Interacts with an Ovary-specific eIF4E and 4E-T in Early Xenopus Oocytes

Received for publication, June 5, 2007, and in revised form, October 16, 2007 Published, JBC Papers in Press, October 17, 2007, DOI 10.1074/jbc.M704629200

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CPEB (cytoplasmic polyadenylation element-binding protein) is an important regulator of translation in oocytes and neurons. Although previous studies of CPEB in late Xenopus oocytes involve the elf4E-binding protein maskin as the key factor for the repression of maternal mRNA, a second mechanism must exist, since maskin is absent earlier in oogenesis. Using co-immunoprecipitation and gel filtration assays, we show that CPEB specifically interacts, via protein/protein interactions, with the RNA helicase Xp54, the RNA-binding proteins P100(Pat1) and RAP55, the elf4E-binding protein 4E-T, and an elf4E protein. Remarkably, these CPEB complex proteins have been characterized, in one or more organism, as P-body, maternal, or neuronal granule components. We do not detect interactions with elf4E1a, the canonical cap-binding factor, elf4G, or elf4A or with proteins expressed late in oogenesis, including maskin, PARN, and 4E-BP1. The elf4E protein was identified as elf4E1b, a close homolog of elf4E1a, whose expression is restricted to oocytes and early embryos. Although elf4E1b possesses all residues required for cap and elf4G binding, it binds m7GTP weakly, and in pull-down assays, rather than binding elf4G, it binds 4E-T, in a manner independent of the consensus elf4E-binding site, YSKELL. Wild type and Y-A mutant 4E-T (which binds elf4E1b but not elf4E1a), when tethered to a reporter mRNA, represses its translation in a cap-dependent manner, and injection of elf4E1b antibody accelerates meiotic maturation. Altogether, our data suggest that CPEB, partnered with several highly conserved RNA-binding partners, inhibits protein synthesis in oocytes using a novel pairing of 4E-T and elf4E1b.

Selective protein synthesis in oocytes, eggs, and early embryos of many organisms drive several critical aspects of early development, including meiotic maturation and entry into mitosis, establishment of embryonic axes, and cell fate determination. Protein synthesis is usually regulated at the initiation stage, mediated by the 5′ m7GpppN mRNA cap structure bound by the translation initiation complex elf4F, composed of elf4E, the cap-binding protein, the RNA helicase elf4A, and the large scaffold protein elf4G, which has a consensus binding site YXXXXXLΦ for elf4E, and additional sites for elf3 and the poly(A)-binding protein. elf3 recruits the small ribosomal subunit, whereas the elf4E elf4G-poly(A)-binding protein relay results in the so-called “closed loop” model, responsible for the synergistic enhancement of translation by capped and polyadenylated mRNAs. A hallmark of translational control mechanisms is the role of mRNA-binding proteins, which recognize specific (and usually) 3′-UTR cis-elements and influence the recruitment of the small ribosomal subunit to the 5′ cap (reviewed in Refs. 1–3). Probably, the best studied mRNA-binding protein is CPEB1 (cytoplasmic polyadenylation-binding protein 1), characterized in flies, worms, clams, Aplysia, Xenopus, and mammals, which, in its conserved C terminus, contains a many RNA recognition motif domains followed by two zinc finger domains, responsible for binding 3′-UTR cytoplasmic polyadenylation elements, consensus U4→A1→3U (reviewed in Refs. 1–3). The related proteins CPEB2–CPEB4, found in mammals, have different RNA-binding preferences and function in neurons (4, 5).

CPEB1 (hereafter CPEB) in clams and Xenopus performs a dual role; it represses cap-dependent translation in the oocyte and activates translation, via cytoplasmic polyadenylation, in meiotically maturing eggs and early embryos (6, 7). Activated maternal mRNAs contain one or more cytoplasmic polyadenylation elements near the nuclear polyadenylation hexanucleotide, AAUAAA, whereas mRNAs lacking cytoplasmic polyadenylation elements are deadenylated upon meiotic maturation and concomitantly exit from polysomes. Unusually, deadenylated mRNAs are stable in eggs and early embryos, reflecting the absence of decapping activity. In Xenopus, cytoplasmic polyadenylation elements and hexanucleotides elements mediate poly(A) length control by a complex, including CPEB and the recently identified cytoplasmic poly(A) polymerase, GLD-2, as well as cleavage and polyadenylation specificity factor, symplekin, and the deadenylase PARN (8–12). The complex between CPEB and cleavage and polyadenylation specificity factor/GLD-2 is stabilized in response to progesterone-stimulated signaling during meiotic maturation, which leads to translational activation (12, 13), and additional elements, including polyadenylation response element and its trans-acting factor Musashi, control temporal aspects of translational activation of CPE-containing mRNAs (14). When CPEB functions as a

* This work was supported by the Wellcome Trust and the Biotechnology and Biological Sciences Research Council (to N. S.) and La Ligue Nationale contre le Cancer (to D. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. S1–S4.

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2 The abbreviations used are: UTR, untranslated region; MS, mass spectrometry; GST, glutathione S-transferase; RNP, ribonucleoprotein.
CPEB RNP in Early Oocytes

repressor, on the other hand, it has been reported to interact with maskin (TACC3), an eIF4E-binding protein, an interaction that precludes the productive binding of eIF4E to eIF4G (15). Maskin is phosphorylated in response to progesterone, a modification that releases eIF4E (16). This paradigm example of a repressed “closed loop” form of mRNA may, however, be confined to late stage Xenopus laevis oocytes, since maskin is absent early in oogenesis (17, 18) (this study), and its nonconsensus eIF4E-binding site is not conserved among vertebrates (2, 15). Nevertheless, maskin serves as a prototype of a protein that bridges an RNA-binding protein bound to the 3′-UTR and eIF4E bound to the cap to inhibit translation initiation. Other examples include two regulators of posterior patterning in the Drosophila oocyte and embryo, Bruno and Smaug, which repress oskar and nanos mRNAs respectively, and the eIF4E-binding protein Cup (19–22). Alternatively, a repressive “closed loop” form of mRNA may form directly, without an intermediary protein such as maskin or Cup, using a homolog of the canonical eIF4E1a (class I) initiation factor. Thus caudal and hunchback mRNAs, which establish opposing morphogen gradients in Drosophila embryos, are repressed by Bicoid and the Nos-Pum-Brat NRE complex, respectively, in conjunction with 4E-HP, a class II eIF4E, which binds the cap but not eIF4G (23–26). Intriguingly, recent studies indicate that 4E-HP has a considerably lower affinity for the cap than eIF4E1a (27, 28). Given that Drosophila possesses seven genes encoding eight eIF4E-related proteins (26) and that vertebrates contain 4–6 such proteins, spread among three distinct classes (25, 29), which vary in their abilities to interact with the cap, eIF4G, and the 4E-BP family of proteins (which regulate general translation by competing for eIF4G using the shared eIF4E-binding consensus XXXXXLdb) (25, 26), it seems likely that additional noncanonical eIF4E proteins will be found that regulate translation.

Previously, we showed that in clam and Xenopus oocytes, CPEB interacts with a DDX6 RNA helicase called Xp54 and that Xp54 tethered to the 3′-UTR of a reporter RNA represses its translation (30, 31). A role in translation repression has also been reported for other members of this highly conserved helicase family, including Saccharomyces cerevisiae Dhh1 (32), Plasmodium DOZI (33), Drosophila Me31B (34), and mammalian RCK/p54 (35) (reviewed in Refs. 1 and 36). p54 helicases are mediators of both RNA storage and of RNA decay in yeast and in mammalian cells and are composed of mRNA and factors mediating both RNA degradation and translational repression (37–42), as well as in maternal and neuronal granules in flies and worms (43–45). Here we report an extensive analysis of proteins that interact with CPEB in Xenopus oocytes, enabled by a recently described monoclonal CPEB antibody (40). Co-immunoprecipitation and gel filtration analyses show that in early stage oocytes, CPEB interacts with Xp54, P100 (S. cerevisiae Pat1, Drosophila melanogaster HPat), RAP55B (S. cerevisiae Scd6, D. melanogaster Trailer Hitch, Caenorhabditis elegans CAR-1, and Lsm14), eIF4E-Transporter (4E-T, D. melanogaster Cup), and an eIF4E protein, all P-body components, in an RNA-independent manner. The eIF4E protein was identified as eIF4E1b, a close homolog of the canonical eIF4E1a cap-binding protein. Although eIF4E1b possesses all residues known to be required for cap- and eIF4G-binding, it binds m7GTP weakly, and rather than binding eIF4G, it binds 4E-T, in a YSKEELL-independent manner. Wild type and Y-A mutant 4E-T tethered to a reporter mRNA represses its translation in a cap-dependent manner, and injection of eIF4E1b antibody accelerates meiotic maturation. Our data point to a conserved translational complex operating in early Xenopus oocytes, which represses cap-dependent translation not by severing the eIF4E1a–eIF4G link but by using an alternative eIF4E-binding protein and an alternative eIF4E protein.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—All plasmids, including IMAGE expressed sequence tag clones, subcloning, and mutagenesis are described in the supplemental materials.

In Vitro Transcription/Translation—In vitro transcription was previously described (31, 46). Translations were performed in reticulocyte lysate also containing 100 mM KCl, 0.5 mM MgCl2, 10 mM creatine phosphate, a 2 mM concentration of each amino acid, and mRNA. In eIF4E mRNA translations, Met and Cys were substituted by [35S]Met and [35S]Cys. For all other translations, only Met was substituted. Translation was allowed to proceed for 90 min at 30 °C before RNAse A was added for termination. Samples were analyzed by SDS-PAGE and autoradiography.

Xenopus Oocyte Lysate Preparation and Gel Filtration—Isolation, staging, handling, lysate preparation, and enucleation of Xenopus oocytes was as previously described (31, 46). Stage I/II oocyte lysate was gel-filtered using a Superose 6 HR 10/30 column (GE Healthcare) in buffer containing 34.2 mM Na2HPO4, 15.8 mM NaH2PO4, 150 mM NaCl. To calibrate the column, a protein standard sample was used containing thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), ovalbumin (43 kDa), and chymotrypsinogen (25 kDa) (31, 46). RNase treatment was performed as previously (31). Alternate fractions were separated by SDS-PAGE and visualized by Western blot.

Immunoprecipitation—Immunoprecipitation experiments using lysates prepared from oocytes injected with MS2 fusion protein mRNAs with mouse monoclonal MS2 antibodies and protein G-Sepharose were described previously (31). Immunoprecipitation of endogenous proteins was performed in a similar manner, using mouse monoclonal CPEB antibodies (40) or rabbit eIF4E1b antibody, 40 μl of stage I–V lysate prepared in 1 ml of NET buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, pH 8.0, 0.25% gelatin, 0.02% NaN3) was incubated, with gentle mixing, with 1 μl of CPEB antibody, 0.2 μl control MS2 antibody, or 0.5 μl of eIF4E1b/preimmune control antibodies for 2 h at 4 °C prior to the addition of 10 μl of protein G-Sepharose (mouse monoclonal antibodies) or protein A-Sepharose beads (rabbit polyclonal antibodies) for 2 h at 4 °C. Beads were washed in NET buffer, and bound proteins were eluted in 20 μl of protein sample buffer. Ten μl of each sample was separated by SDS-PAGE, and proteins were detected by silver staining or Western blot. When RNase A-treated, Xenopus extracts were supplemented with RNAse A to 20 pg/μl extract and incubated at 20 °C for 20 min prior to...
clarification by centrifugation at 10,000 \( \times g \) for 10 min at 4 °C. Immunoprecipitation reactions for the isolation of proteins for peptide sequencing were performed essentially as described above by scaling up reaction volumes 10–25-fold. RNA was extracted from scaled up immunoprecipitation reactions and aliquots of whole cell lysate as follows. Bound beads or extracts were incubated in 500 \( \mu l \) of TNES (0.1 M Tris, pH 7.5, 0.3 M NaCl, 5 mM EDTA, 2% SDS) with the addition of 200 \( \mu g/ml \) proteinase K at 50 °C for 30 min with intermittent vortexing. RNA was purified by phenol/chloroform extraction followed by ethanol precipitation in the presence of tRNA and was resuspended in 12.5 \( \mu l \) of \( H_2O \).

Reverse Transcription-PCR—Reverse transcription-PCRs were performed using 1 \( \mu l \) of purified RNA and 0.5 \( \mu l \) of random hexamers (Promega) in a 20-\( \mu l \) reaction using Superscript II (Invitrogen) and following the manufacturer’s instructions. PCRs were performed using 1 \( \mu l \) of cDNA template in a 20-\( \mu l \) reaction volume with the following oligonucleotide pairs: actin (1:2) and cyclin B1 (3:4; see supplemental Table 1) with Taq DNA polymerase.

Mass Spectrometry Analysis—Proteins within the gel-excised bands were sequenced by MS/MS in the Cambridge Centre for Proteomics, as described previously (31). Fragmentation data were used to search the National Center for Biotechnology Information data base using the MASCOT search engine (available on the World Wide Web). Probability-based MASCOT scores were used to evaluate identifications. Only matches with \( p < 0.05 \) for random occurrence were considered significant. 4E-T peptides were YDSDGVDWDEPK, ATGR, and VISVEDLEYR, and the P100(Pat1) peptides were WTDTVTFLVAK, LSEEELLGER, EEEPEALQPVK, VSTYATGQILEDK, AIDAYSVYAMPDEAIK, and VFLMFEVEELAR; the eIF4E1a peptide was VSYAMPDEAIK, and the eIF4E1b peptide was VFLMFLEVEELAR; the eIF4G peptide pairs: actin (1:2) and cyclin B1 (3:4; see supplemental Table 1).

Preparation of Antibodies—The P100(Pat1) antibody was raised against an N-terminal peptide DSQDDEVPKLEDDC, and the elf4E1b antibody was raised against an N-terminal peptide LSREKLDNEKKRC (Sigma). 4E-T antibody was raised against recombinant Xenopus 4E-T (Eurogentec). The \( X. laevis \) 4E-T expression plasmid is described in the supplemental materials. A culture of transformed BL21-CodonPlus-RIL cells was induced at 37 °C using an Overnight Express autoinduction system (Novagen) following the manufacturer’s instructions. Cells were harvested and resuspended in nitriilotriacetic acid buffer (300 mM NaCl, 1% (v/v) Triton-X-100, 50 mM sodium phosphate buffer, pH 7.8). Following French pressing, pellets were resuspended in nitriilotriacetic acid buffer, and aliquots were run on SDS-PAGE preparative gels. Coomassie-stained 4E-T containing gel slices were excised and used for immunization. Antiserum was subsequently purified against 10 \( \mu g \) of 4E-T recombinant protein isolated by SDS-PAGE and Western blot analysis.

Protein Gel Electrophoresis and Western Blot Analysis—Protein samples were separated by SDS-PAGE on either 10 or 15% polyacrylamide gels and then used for Coomassie Blue staining, silver staining, or Western blot analysis using ECL (31, 46). We used mouse monocular CPEB (1:5000 (40)), guinea pig FRYG2 (1:10,000 (47)), goat TIAR (1:2000, C-18; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and goat Rsk1/p90 (4:1000, 1:1000, C-21-G; Santa Cruz Biotechnology) antibodies and the following rabbit antibodies raised against Xenopus CPEB (1:12,000 (48): Xp54 (1:1000) (31), human elf4E1 (1:12,000), elf4G (1:30,000), 4E-BP1 (1:800), elf4A (1:1000), CBP80 (1:5000) (49, 50), Xenopus maskin (1:1000) (15, 51, 52), PARN (1:2000) (53), RAP55/Trailer Hitch (1:2000) (54), Erk1/MAPK (1:25,000, K-23; Santa Cruz Biotechnology), elf4E1b (this work; 1:10,000), P100(Pat1) (this work; 1:2000), 4E-T (this work; 1:100) (affinity-purified).

m7GTP-Sepharose Binding Assays—Oocyte lysate (stage III/IV) was applied to 7-methyl GTP-Sepharose beads (Amersham Biosciences) equilibrated with HKE buffer (50 mM HEPES (NaOH), pH 7.4, 150 mM KCl, 0.5 mM EDTA, 0.5 mM EGTA, 0.1% (v/v) β-mercaptoethanol, 1× Complete EDTA-free protease inhibitors (Roche Applied Science)). The columns were incubated at 4 °C for 1 h with agitation and then washed with HKE buffer. Proteins were eluted from the resin with 0.1 mM GTP and then with 70 \( \mu M \) m7GpppG in HKE buffer and finally with 1× SB (SDS). For peptide sequencing, 1 ml of stage III/IV lysate was applied to 100 \( \mu l \) of cap-Sepharose beads in a 15-ml final volume. Columns were processed as above. Bound fractions were subjected to SDS-PAGE and Coomassie Blue staining, and the appropriate bands were excised for sequencing.

For binding of \textit{in vitro} translated elf4E1a or elf4E1b, 100 \( \mu l \) of translation mix was applied to 10 \( \mu l \) of cap-Sepharose beads equilibrated with HKE buffer. After binding and elutions as above, the samples were analyzed by SDS-PAGE and autoradiography.

GST Binding Assays—GST-elf4E1a- and GST-elf4E1b-carrying plasmids were amplified in \textit{Escherichia coli} and transformed into expression-competent BL21 CodonPlus-RIL cells. Proteins were expressed as described for recombinant X4E-T and purified from the insoluble fraction essentially following the protocol described by Joshi \textit{et al.} (25). Control GST proteins were expressed as described above but were present in the soluble fraction following French pressure cell lysis and centrifugation.

GST proteins were purified by affinity chromatography with glutathione-Sepharose (GE Healthcare). 1-ml aliquots of beads were incubated with the GST or GST fusion proteins for 1 h at 4 °C with gentle mixing. Beads were washed with buffer B (50 mM HEPES, pH 7.2, 100 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 2 mM benzamidine, 1% Triton X-100) and finally in buffer C (50 mM Tris, pH 8.0, 100 mM NaCl, 2 mM dithiothreitol, 2 mM benzamidine) followed by elution in 1 ml of buffer C supplemented with 10 mM glutathione.

Three \( \mu g \) of GST or 6 \( \mu g \) GST-elf4E1a or elf4E1b was bound to 25 \( \mu l \) of glutathione-Sepharose for 1 h at 4 °C in buffer BB (20 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, and 0.5% (v/v) Nonidet P-40). 50 \( \mu l \) of reticulocyte lysate containing \textit{in vitro} translated \textsuperscript{35}S Met-labeled protein was added and allowed to bind for 2 h at 25 °C. Beads were washed in BB, and bound proteins were eluted in SDS-containing buffer before separation by SDS-PAGE and autoradiography.

MS2 Tethering—The MSP vector, the firefly Luc-MS2, and the CSFV-firefly Luc-MS2 reporter cDNAs were supplied by Nicola Gray (55, 56). cDNAs encoding MS2-Xp54 were described previously (30). MS2-Xp54N and MS2-4E-T wild

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type and mutant constructs are described in the supplemental materials. MS2 tethering using nonadenylated firefly luciferase-MS2 reporter mRNA and a control Renilla luciferase mRNA was performed essentially as described (30, 31).

**Xenopus Oocyte Antibody Injection and Maturation**—eIF4E1b and preimmune serum were purified by binding to protein A-Sepharose in 100 mM Tris (pH 8.0) for 1 h at 20 °C. Beads were washed with 100 mM Tris (pH 8.0) and then with 10 mM Tris (pH 8.0) prior to elution with 100 mM glycine (pH 3.0) and dialysis against phosphate-buffered saline. Pools of 120 stage VI oocytes were injected with 50 nl of purified antibody (1/250 IgG). Oocytes were incubated at 20 °C in MBS with progesterone added to 0.1 μg/ml as required and scored for germinal vesicle breakdown (GVBD) every 2 h. Random samples of 10 oocytes were taken at each time point for SDS-PAGE and Western blot analysis.

**RESULTS**

**Characterization of the CPEB RNP Complex in Xenopus Oocytes**—Previously, we showed that MS2-tagged Xp54 helicase interacts with CPEB and eIF4E in oocytes, in the absence of RNA, and with endogenous Xp54, in an RNA-dependent manner (31) (Fig. 1A). To extend the identification of Xp54-binding proteins, further MS2 immunoprecipitations using lysates from stage VI oocytes expressing MS2-Xp54 mRNA were carried out. A prominent ~100-kDa protein that specifically interacted with MS2-Xp54 in an RNA-independent manner but not with MS2 protein (Fig. 1A) was sequenced. Two sets of peptides were obtained, both corresponding to a 100-kDa protein. Three peptides corresponded to the Xenopus homolog of human eIF4E-Transporter (4E-T) (57), and seven peptides corresponded to the egg-specific protein P100 (58), a homolog of yeast Pat1 (32). Subsequently, the co-immunoprecipitation of 4E-T and P100(Pat1) with MS2-Xp54 was verified by Western blot analysis (Fig. 1F).

Since our Xp54 antibody did not precipitate efficiently, we used a mouse monoclonal antibody raised against human CPEB1 (40) to verify endogenous interactions. The mouse antibody detects a single band in Xenopus oocytes, corresponding in size to the 58-kDa CPEB (59) (Fig. 1B). The CPEB antibody, alongside a control mouse monoclonal antibody and a bead only control, was used to immunoprecipitate CPEB from mixed stage oocyte lysates. Prior to immunoprecipitation, the lysates were untreated or were digested with RNase that was sufficient to degrade the endogenous RNA (Fig. 1C and D). Protein G-Sepharose-bound proteins were analyzed by silver staining, which showed the co-precipitation of several prominent and specific proteins with CPEB, most of them in an RNA-inde-
pendent manner (Fig. 1C). We also examined the mRNAs precipitated by CPEB and control antibodies by semiquantitative reverse transcription-PCR, using primers corresponding to actin and cyclin B1 mRNA. Although both transcripts were maternally expressed, the CPE-containing cyclin B1 mRNA was enriched in CPEB-bound material relative to actin CPE-lacking mRNA (Fig. 1E). These data confirm that the monoclonal antibody immunoprecipitates a specific CPEB RNP complex in *Xenopus* oocytes.

We then investigated several known and potential partners of CPEB by Western blot analysis. Each antibody was also used to probe a Western blot containing two cell equivalents of each of the six stages of oogenesis (I–VI), and of progesterone-matured eggs (E) to monitor the expression of each protein during oogenesis, and upon meiotic maturation (Fig. 1, F and G). Strong interactions, corresponding to ~5–10% of input, were observed in the case of Xp54, Pat1, 4E-T, and elf4E, supporting data obtained with MS2-tagged Xp54 (31) (Figs. 1A and 7). An additional strong (>10% of input) interaction was observed between CPEB and RAP55B, detected by an antibody generated against the *Drosophila* Trailer Hitch (Tral) Sm domain that has been shown to recognize the maternal mouse paralog (54). (We note that both *Xenopus* RAP55A (~62 kDa) and RAP55B proteins (~54 kDa) (36) are detected by this antibody in oocytes and that the levels of RAP55A increase during oogenesis as reported previously (60), whereas those of RAP55B, which uniquely co-immunoprecipitates with CPEB (Fig. 1G), decline during this period.) These interactions did not depend on RNA, except in the case of Xp54, whose binding to CPEB was partially reduced by RNase treatment. This reduction is probably due to RNA-mediated Xp54 oligomerization (31). A second, apparently entirely, RNA-dependent and slightly weaker (~2–3% of input) interaction was noted between CPEB and FRGY2 (Fig. 1G).

To examine further the significance of 4E-T binding to CPEB, we also probed Western blots with other elf4E-binding protein antibodies but failed to observe significant binding of elf4G or 4E-BP. Moreover, elf4A, a component of elf4F, similarly failed to efficiently co-precipitate with CPEB, attesting to the specificity of the interactions described above. We also probed with antibodies raised against *Xenopus* maskin (TACC3), obtained from three laboratories (15, 51, 52), and the deadenylase PARN (53) but did not observe significant binding (Fig. 1G) (see “Discussion”).

All examined proteins were expressed during oogenesis, and with the exception of maskin, PARN and 4E-BP were present at high levels in early stages (Fig. 1F). In particular, eIF4E appears as three different sized proteins, of about 24, 26, and 30 kDa, as detected using a rabbit polyclonal antibody raised against the C-terminal peptide of human eIF4E, TATKSGSTTKNRFVV (49) (Fig. 3), conserved among vertebrate Class I elf4E proteins (25, 29). These proteins are differentially expressed during oogenesis, with the smallest protein being most abundant in early oogenesis and the two larger proteins only detectable from middle to late oogenesis and in eggs (Fig. 1F). Significantly, the smallest eIF4E protein was preferentially found in CPEB immunoprecipitates (Figs. 1, F and G and 4B), an observation also made recently using the same eIF4E antibody and an independent, polyclonal CPEB antibody (61). We conclude that in oocytes CPEB interacts with Xp54, Pat1, RAP55B, a 24-kDa elf4E protein, and only one known elf4E-binding protein, 4E-T. These CPEB partners as well as CPEB itself are more abundant in early stage oocytes in contrast to maskin and PARN, which accumulate late in oogenesis (Fig. 1F) (17, 18, 53) and may thus represent an early oocyte complex.

Supporting evidence for such an early CPEB RNP complex comes from Superose 6 HR 10/30 FPLC gel filtration of stage I/II oocyte lysates, in which Xp54, Pat1, RAP55B, FRGY2, 4E-T,
CPEB RNP in Early Oocytes

FIGURE 3. Identification of eIF4E1a(L), eIF4E1a(S), and eIF4E1b cap-binding proteins. A, m7GTP-Sepharose chromatography was performed using stage III/IV oocyte lysate. Following binding, the beads were washed and then eluted with GTP- and mGpppG-containing buffer and finally with SDS buffer. Aliquots of the indicated fractions (load (L), flow-through (FT), wash, and elution fractions) were analyzed by silver staining (A) and Western blot analysis (B, top). mRNAs encoding eIF4E1a and eIF4E1b were translated in vitro and analyzed by m7GTP-Sepharose chromatography as in A and by SDS-PAGE and autoradiography (B, bottom). C, alignment of X. laevis eIF4E1a(L), eIF4E1a(S) (two allelic variants), and eIF4E1b. *, residues involved in cap binding; +, residues involved in eIF4G binding (65, 89). Peptides obtained by mass spectrometry sequencing of the indicated bands in A are shaded. The conserved C-terminal peptide used to generate eIF4E1a antibodies is boxed.

CPEB Interacts with eIF4E1b, Not the Canonical eIF4E1a Cap-binding Protein—To identify maternal X. laevis eIF4E proteins, m7GTP-Sepharose affinity chromatography was performed using lysates prepared from stage III/IV oocytes, in which the three proteins (Fig. 1F) are roughly equivalent in abundance. Following the binding of the lysate proteins to m7GTP-Sepharose, the beads were washed several times and then consecutively eluted with GTP- and cap-analog-containing buffer, and any proteins remaining bound to the beads were removed with SDS-containing buffer. The samples were separated by SDS-PAGE, and one gel was stained with silver, whereas a duplicate gel was analyzed by Western blot to reveal the binding of the eIF4E proteins (Fig. 3, A and B). The silver-stained gel shows three prominent bands ranging from 20 to 30 kDa in the cap-analog elutions, corresponding in size to the eIF4E proteins detected in Western blots (Fig. 1). Comparing the load and flow-through fractions (Fig. 3B) shows that, although the two larger proteins quantitatively bound to the cap-Sepharose beads, the fastest migrating form bound more weakly, whereas the GTP and cap elutions show that all three proteins bound preferentially to m7GpppG (Fig. 3A).

Mass spectrometry sequencing identified the 30-kDa cap-binding protein as X. laevis eIF4E, with seven matching peptides (Fig. 3C). In particular, the N-terminal peptide ETGQEIENTNPQSTEEEK indicated the protein to be the long isoform of eIF4E, with a duplicated insert of 18 amino acids, presumably due to alternative splicing (62). With nine matching peptides, the 26 kDa band was identified as the eIF4E isoform of eIF4E, with seven matching peptides, and the 24 kDa band was identified as the eIF4E isoform that lacks the 18-amino acid insert. Single amino acid substitutions in some peptides indicated the existence of two allelic variants of this protein (Fig. 3C). Both the short and the long eIF4E proteins have been previously identified as members of the class I eIF4E group and have been assigned to the subgroup eIF4E1a (29). The least abundant, 24-kDa protein was assigned as eIF4E1b based on the sequences of four of its five peptides, which distinguish eIF4E1b from eIF4E1a, with the remaining peptide being common to both proteins (Fig. 3C) (29). eIF4E1b, a class I eIF4E, has been characterized in zebrafish oocytes (63) and is conserved in mammals (29, 64). eIF4E1b proteins are highly related to the canonical cap-binding translation initiation factor; e.g. over the length of the whole
protein, *Xenopus* elF4E1a(S) and elF4E1b are 69% identical (83% similar). The vertebrate elF4E1b proteins possess all of the known residues required for interaction with the cap structure, elF4G and 4E-BP, defined in their elF4E1a counterparts (Fig. 3C) (29) (see "Discussion"). Nevertheless, *Danio rerio* elF4E1b does not bind the cap nor elF4G or 4E-BP1 (63).

To verify the difference in cap-binding between *Xenopus* elF4E1a and elF4E1b, their mRNAs were translated in rabbit reticulocyte lysate, and the products were analyzed by m7GTP-Sepharose chromatography (Fig. 3B). elF4E1b binding to m7GTP was considerably weaker than that of elF4E1a, when comparing both the amount bound (load and flow-through fractions) and the amount eluted with cap analog (m7GpppG). These experiments confirm the initial mass spectrometry data and indicate that *Xenopus* elF4E1b, in contrast to elF4E1a, binds m7GTP only weakly (Fig. 3, A and B), like its zebrafish counterpart (63).

Taking advantage of the unique basic N terminus of elF4E1b, an antibody was raised against the peptide LSREKLDNEKRRKK (Fig. 3C). The specificity of this antibody was verified by Western blot analysis of GST-elF4E1a, GST-elF4E1b, GST, and total oocyte lysate proteins. The elF4E1b antibody specifically recognizes GST-elF4E1b and the smallest, 24-kDa, elF4E protein detected by the C-terminal peptide elF4E1 antibody (Fig. 4A). Use of this elF4E1b antibody confirms the identification of elF4E1b as the elF4E1 protein that co-immunoprecipitates with CPEB (Fig. 4B). Furthermore, in a reciprocal experiment, elF4E1b antibody, but not preimmune antibody, immunoprecipitates CPEB in an RNA-independent manner (Fig. 4C). Altogether, these experiments provide evidence that CPEB interacts with elF4E1b, rather than elF4E1a, in line with the gel filtration data (Fig. 2).

*elF4E1b Is Cytoplasmic and Its Expression Is Limited to Oocytes and Early Embryos—* A distinguishing feature of elF4E1b proteins is the presence of several tandem basic residues in their N termini, reminiscent of proteins that undergo nuclear import (29, 64) (Fig. 3C). To ascertain to what extent elF4E1b is cytoplasmic, *Xenopus* oocytes were fractionated into nuclear and cytoplasmic fractions. All three elF4E1 proteins were exclusively cytoplasmic (Fig. 5A), with CBP80 attesting to the content of the nuclear fraction.

The levels of elF4E1b, per oocyte, slowly decline during oogenesis, whereas the expression of elF4E1a(L) and elF4E1a(S) increases (Figs. 1F and S1). To examine their expression during embryogenesis, samples of equal numbers of staged embryos were analyzed by Western blot with the elF4E1 antibody. The levels of both elF4E1a(L) and elF4E1a(S) remain approximately constant, at least until stage 42 (tadpole-like), whereas those of elF4E1b decline to undetectable levels after stage 9, corresponding to midblastula (Fig. 5B). In the tested adult tissues, elF4E1b is only detectable in the ovary (Fig. 5C). *Xenopus* elF4E1b expression mirrors that of its zebrafish counterpart; *D. rerio* elF4E1b mRNA is not detectable after the 10-somite stage, and the protein is largely restricted to the ovary, although low amounts are found in the testis and muscle (63). In mice, elF4E1b (Elf4e1oo) mRNA is very abundant in the oocyte, in contrast to elf4E1a mRNA, but undetectable at the late two-cell stage (64). Together, these data show...
that the expression of eIF4E1b is restricted to the oocyte, egg, and early embryo.

Characterization of Maternal Xenopus 4E-T—Human 4E-T was characterized as a large protein that binds eIF4E via an N-terminal peptide YTKEELL, very similar to eIF4E-binding sites in eIF4G and eIF4E-BP proteins (57). In HeLa cells, 4E-T is a largely cytoplasmic, shuttling protein, which can import eIF4E into nuclei via NLS and NES sequences (57). Xenopus 4E-T was characterized as described in detail in the legend to Fig. S2. Briefly, we noted the presence of two splice isoforms, differing in the inclusion or exclusion of a 546-nucleotide-long exon at codon 317. The X4E-T short isoform is 67% identical (79% similar) to human 4E-T and contains all three sequences that have been reported to promote nuclear import/export (57), whereas the short form lacks the N-terminal NES. Both the long and short forms of X4E-T contain a potential consensus eIF4E-binding site YSKEELL at a very similar N-terminal location to the human protein. The X4E-T short isoform is more abundant in oocytes than the longer form, and both forms are cytoplasmic (Fig. S2). The full-length insert-lacking clone, used in most subsequent experiments, corresponded to NCBI accession number BC077338.

eIF4E1b Binds 4E-T But Not eIF4G in Vitro—To examine the binding of the eIF4E1 proteins to eIF4E-binding proteins, GST-tagged eIF4E1a and eIF4E1b proteins, as well as GST alone, were purified from E. coli, and pull downs were performed with equimolar amounts of recombinant proteins and in vitro translated eIF4G, 4E-T, and maskin (Fig. 6). In the case of 4E-T, the wild type protein was compared with a mutant protein, in which Tyr28 alone or in combination with Leu33 and Leu34 residues of the eIF4E-binding site YSKEELL was mutated to alanine to abrogate eIF4E binding (57). As shown in Fig. 6B, eIF4E1a bound 4E-T and eIF4G with high affinity and specificity, and the binding to 4E-T was largely YSKEELL-dependent, as expected from previous studies (57), both single and triple mutations behaving the same (data not shown). In contrast, eIF4E1b showed significantly reduced binding to eIF4G, the same as background binding by GST, although it possessed all residues known to be necessary for eIF4G binding (Fig. 3C) (65). In similar assays, zebrafish eIF4E1b, in contrast to its eIF4E1a paralog, also did not bind eIF4G or 4E-BP (63). However, X. laevis eIF4E1b bound 4E-T to nearly the same extent as eIF4E1a and had approximately the same affinity for both wild type and mutant proteins, indicating that eIF4E1b probably binds 4E-T at a different site from eIF4E1a. Binding of the in vitro translated X4E-T long isoform to eIF4E1a and eIF4E1b proteins was indistinguishable from that of the X4E-T short isoform, demonstrating that the alternative 182-amino acid insert (Fig. S2) did not influence binding to eIF4E proteins, at least in vitro (data not shown). Last, we noted that neither eIF4E protein had very significant affinity in vitro for maskin, in line with the observation that in the yeast two-hybrid system, maskin and eIF4E interact weakly (15). We conclude that in vitro, Xenopus eIF4E1b binds preferentially to 4E-T, rather than eIF4G, in agreement with immunoprecipitation and gel filtration data (Figs. 1 and 2). Moreover we note that this binding was insensitive to mutation of the eIF4E1a-binding site, YSKEELL, consistent with the absence of significant binding to eIF4G and 4E-BP1 (Fig. 6B) (63).

Tethered 4E-T Represses Translation—To assess its possible role in translational regulation, 4E-T was tethered to the 3′-UTR of firefly luciferase mRNA via MS2 binding sites, as previously described for Xp54 (30, 31). mRNAs encoding MS2 fusion proteins were injected into stage VI oocytes first, followed by a second injection of (nonadenylated) firefly luciferase (Fluc) mRNA alongside an internal control Renilla mRNA. X4E-T was observed to repress firefly luciferase expression in four experiments ~4-fold (Figs. 7A and S3), in a manner requiring MS2 binding sites (Fig. 7B) and independently of similar changes in Fluc mRNA levels (Fig. 7D). The degree of repression by 4E-T was comparable with that seen with full-length Xp54, whereas a truncated version of Xp54 (Xp54N), comprising the N-terminal domain (residues 1–302) alone, was inactive (Fig. 7A).

To assess the dependence of the repression mechanism on the 5′ cap structure, we first compared the level of translation of (nonadenylated) Fluc RNAs bearing m7GpppG or ApppG caps or the CSFV IRES (56). The IRES reporter RNA was capped with ApppG to avoid degradation. Translation of the CSFV IRES-led reporter RNA in oocytes was found to be as robust as that mediated by the m7GpppG cap, whereas A-capped mRNA was very poorly translated (Fig. 7, A and C) (data not shown). Translational repression by 4E-T required the reporter RNA translation initiation to be cap-dependent, rather than mediated by the CSFV IRES, which does not require eIF4F or eIF3 (66).

Binding to MS2-tagged proteins expressed in oocytes was determined using a monoclonal MS2 antibody, as previously described (30, 31). According to the silver-stained gel of immunoprecipitated samples, both MS2-Xp54 proteins are efficiently synthesized in oocytes, whereas the two 4E-T proteins are less well expressed (Fig. 7E). As shown in Fig. 7F, no significant binding was observed to the two negative controls, MS2
alone and MS2-Xp54N. MS2-Xp54 co-immunoprecipitated with Pat1 and 4E-T, in line with the previously described mass spectrometry peptide data (Fig. 1A; see “Experimental Procedures”). Moreover, MS2-Xp54, like endogenous CPEB, co-immunoprecipitated with elf4E1b rather than elf4E1a. (Indeed, when we originally described the binding of elf4E to MS2-Xp54 (31), using the same elf4E antibody, we did not appreciate the heterogeneity of maternal elf4E1 proteins and that only the smallest elf4E, now identified as elf4E1b, binds Xp54). In contrast, ectopically expressed 4E-T binds both elf4E1a and elf4E1b, unlike endogenous 4E-T, which is found in complexes and gel filtration fractions that only contain elf4E1b (Figs. 1 and 2). Binding of elf4E1a to MS2–4E-T could be eliminated by mutation of the elf4E-binding site, Y28A (Fig. 7F). Such a mutation in MS2–4E-T only partially reduced binding of elf4E1b, in line with the in vitro binding data (Fig. 6), and only partially alleviated its translational repression (Fig. 7A).

Both in vitro and in vivo binding experiments (Figs. 6 and 7) indicate that elf4E1b binds 4E-T at a separate site to elf4E1a. Preliminary attempts to delineate this second site suggest that it also lies in the N terminus, corresponding to 4E-T (residues 1–380), which retains full binding of elf4E1b and full translational repression (data not shown). We conclude that MS2-Xp54 and MS2–4E-T(Y-A) repress translation of reporter RNA while specifically interacting with elf4E1b, rather than elf4E1a. This finding is suggestive of a role for elf4E1b as a co-repressor of cap-dependent translation, for which more direct evidence is given in Fig. 8.

**Injection of elf4E1b Antibody Accelerates Meiotic Maturation**—The addition of progesterone to stage VI oocytes, arrested in prophase of meiosis I, triggers their completion of meiosis I, marked by nuclear breakdown (or GVBD), and entry into meiosis II, where they arrest in metaphase awaiting fertilization. GVBD results from the translational activation of CPE-containing mRNAs, including c-mos mRNAs. Synthesis of c-mos, a MAPKKK, leads to the activation of MAPK, one of whose targets is p90MK (reviewed in Ref. 67). To assess elf4E1b function in this process, protein A-Sepharose-purified antibody was injected into stage VI oocytes to neutralize protein function. Similarly purified preimmune serum was also injected into a separate batch of oocytes, and all were stimulated with progesterone to undergo meiotic maturation. In contrast to preimmune control antibody-injected oocytes, elf4E1b antibody-injected oocytes showed accelerated maturation, as determined by timing of germinal vesicle breakdown (percentage of GVBD), MAPK/ERK kinase, and p90 activation (Fig. 8).

Acceleration of meiotic maturation by injection of elf4E1b was relatively modest although consistently noted in three separate experiments (data not shown). It was not detectable in the absence of progesterone, showing that elf4E1b neutralization is not sufficient to cause GVBD and that other changes in the messenger RNP complex are required and are promoted by
progesterone signaling (Fig. 8). Acceleration of GVBD by antibody neutralization demonstrates that normally eIF4E1b represses translation, and the previously described partners maskin and PARN, identified as such in late stage oocytes, when they are most abundant (11, 12, 15, 17, 18, 53) (Fig. 1). This may reflect the use of different antibodies, which could preferentially target CPEB in separate complexes differentially expressed during oogenesis.

In this study, we have exploited, for the first time, the availability of mouse monoclonal CPEB antibodies (40), which recognize the Xenopus protein specifically in oocyte lysates and enable the ready detection of co-immunoprecipitating proteins using rabbit antibodies by Western blot analysis. The existence of two CPEB-containing complexes in stage VI Xenopus oocytes, reflecting (at least in part) its dual role as a translational repressor and a cytoplasmic polyadenylation factor, has been previously described (11). The early oocyte CPEB complex we describe remains to be fully characterized, both in terms of protein and RNA components. However, our data provide a possible mechanism for the repression of CPEB target mRNAs early in oogenesis, by the use of an alternative eIF4E-binding protein, 4E-T, in combination with eIF4E1b and a set of conserved RNA-binding proteins.

We cannot exclude the possibility that this complex functions also later in oogenesis, since we continue to detect interactions between CPEB, Xp54, 4E-T, and eIF4E1b in stage VI oocytes (Figs. 7F and S4), whereas we fail to observe binding of maskin to CPEB (Fig. S4). Others have also noted some of the interactions reported in this study in stage VI Xenopus oocytes. Tanaka et al. reported that FLAG-tagged FRGY2 interacts in an RNA-dependent manner with Xp54, whereas Xp54 binds with Rap55A in a largely RNA-independent manner. Moreover, tethered Rap55A represses translation in oocytes, mediated by the Lsm domain shared with Rap55B (60). Our data extend these observations considerably by showing that endogenous RNP complexes, abundant in early oocytes, contain CPEB, in addition to Xp54, Rap55B, Pat1, 4E-T, and an ovary-specific eIF4E1b, all interacting via protein-protein interactions, and that tethered 4E-T represses translation in conjunction with eIF4E1b. We also find the association between CPEB and FRGY2 to require RNA. The two studies differ in the identification of the Rap55 protein that binds Xp54 in stage VI oocytes (Rap55A)3 and the one that co-purifies with CPEB in early oocytes (Rap55B) (Figs. 1 and 2). We find that the ratio between Rap55A and -B proteins changes dramatically during oogenesis (Fig. 1), possibly at least partly explaining the varying results, although the existence of two Xp54-Rap55 complexes cannot be excluded.

Co-immunoprecipitation and pull-down assays have revealed interactions between paralogs of p54 and Rap55 proteins in C. elegans embryos (Cgh-1 and CAR-1) (44, 68) and Drosophila oocytes (Me31B and Tral) (69). The Xp54-P100/Pat1 interaction is conserved in yeast (Dhh1 and Pat1) (70) and Drosophila Schneider cells (Me31B and Hpat) (71). Xenopus P100 is the first vertebrate member of the Pat1 family to be characterized. The sequences of P100 and its highly conserved mammalian counterparts do not provide any hint regarding function. P100 probably binds RNA, it is present in a P-body like complex in oocytes, and its expression is restricted to the ovary (58) (this study). Interestingly, data base searches suggest the presence of two related Pat1 proteins in vertebrates, one of

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3 N. Minshall, M. H. Reiter, D. Weil, and N. Standart, unpublished observations.

4 Dr. K. Matsumoto, personal communication.
which is equivalent to P100 and the other possibly more widely expressed. In yeast, Dhh1 and Pat1 have both been characterized as translational repressors and decapping enhancers (32, 70). Homologs of FRGY2, considered to be nonspecific cap-dependent translational repressors (72), interact with p54/RAP55 proteins in flies (Yps) (19, 20, 69) and worms (Cey) (44, 68), and Drosophila Orb (CPEB) co-immunoprecipitates with Yps (73). Usually, where tested, interactions between Yps/Cey proteins and other complex proteins have been noted to require RNA (19, 20, 44, 68, 69), similarly to FRGY2 in Xenopus oocytes (60) (this study). RNA is not required between Dhh1 and Pat1 (70), nor between Tral and Me31B, Yps, and Cup (69), also in line with Xenopus oocyte studies (60) (this study).

Moreover, the identified CPEB RNP proteins have been reported to be enriched in P bodies, maternal “germinal granules,” and neuronal granules; these various forms of visible RNP granules contain translationally inactive mRNA, which may be destined for eventual decay, transport to a particular location, or translational activation. Yeast Dhh1, Pat1, Scdh6, and elf4E and human CPEB, RCK/p54, RAP55, YB-1, 4E-T, and elf4E are found in P-bodies, Drosophila Orb, Me31B, Tral, HPat, Yps, Cup, and elf4E in P bodies in tissue culture cells and in germinal granules and C. elegans Cgh-1, CAR-1, and Cey proteins in embryo P granules (reviewed in Refs. 41, 42, and 71). For example, Xp54 (Dhh1, Me31B, Cgh-1, and RCK) and RAP55 (Scdh6, Tral, and CAR-1) proteins localize to P bodies in yeast and mammalian cells (37, 40, 74), in Drosophila S2 cells (71), and neuronal granules (45), in germinal granules in Drosophila oocytes (69, 75), and in P granules in C. elegans embryos (44, 68, 76). In Drosophila, Orb, Me31B, Tral, and Cup localize within the future oocyte in developing regions 2 and 3 of the germarium, composed of germ line cysts, and are associated with the Balbiani body (69, 75, 77). The Balbiani body is a collection of organelles, including mitochondria, endoplasmic reticulum, and granulofibrillar material, located adjacent to the nucleus in young oocytes of diverse species. In Xenopus, granulofibrillar material has been shown to contain germinal granule proteins and RNAs that are incorporated into germ cells. Molecular studies of germinal granules strongly implicate them in regulated utilization of mRNA, but their precise composition and role of components remains to be determined (reviewed in Refs. 78 and 79). Although a systematic assessment of the localization of CPEB RNP proteins requires extensive future investigation, we note that Xp54 and RAP55 proteins are found in the Balbiani body in early Xenopus oocytes and in mouse oocytes within germ cell cysts, respectively (43, 54). It is tempting to speculate on the basis of these studies that the CPEB complex functions in the differentiation of the oocyte in the Xenopus germ line cyst. Such a hypothesis is supported by the report that adult female CPEB knock-out mice contained vestigial ovaries devoid of oocytes, and ovaries from midgestation embryos contained oocytes arrested at the pachytene stage (80).

The remarkable retention in multicellular organisms of RNP complexes composed of a set of RNA-binding proteins (CPEB, Xp54, RAP55, Pat1, and FRGY2) as well as elf4E and the elf4E-binding protein 4E-T to repress mRNAs demonstrates their fundamental roles in translational control. And yet, we barely understand how these proteins effect their control, providing considerable scope for future investigations. In somatic cells, in addition to P bodies, under certain conditions, stress granules are generated that also contain untranslated mRNA, RNA-binding proteins, and initiation factors. Although some components of P bodies and stress granules are common or at least can be detected in close proximity, by and large they have distinct components. For example, stress granules contain elf4F4G, elf4A4, and elf3, absent from P bodies, as are the proteins TIAR and ribosomal proteins, found in stress granules. Common to both are untranslated RNAs, which can reenter polysomes (reviewed in Refs. 41 and 81). Although some reports have shown that micro-RNA-mediated repression occurs in P bodies (35, 82) (reviewed in Ref. 83), a recent study demonstrates that P body formation is a consequence rather than a cause of repression (35, 71). Determination of the precise architecture of the RNP complexes and the contribution of individual components to translational repression is a major future goal. It will be of particular interest to understand the relationship between P bodies in somatic cells, with a rapidly changing mRNA population transcribed in response to cellular needs, and where some mRNAs decay whereas others return to translation, with that of the P-body-like CPEB RNP in Xenopus oocytes, containing stably stored maternal mRNAs, destined only for translational activation.

This study reports on Xenopus 4E-T for the first time. In HeLa cells, 4E-T (where “T” represents transporter) was initially characterized as an elf4E-binding protein that is capable of importing elf4E into nuclei in the presence of leptomycin B (57). At steady state, human 4E-T localizes to P bodies. To date, 4E-T is the only elf4E-binding protein detected in P bodies; elf4G (and elf4A) is distributed homogenously in the cytoplasm in unstimulated cells (39, 84). 4E-T is required for P body formation and for the localization of elf4E in P bodies (39, 84). When overexpressed, human 4E-T represses cap-dependent reporter mRNA translation in a YTKEELL-dependent manner (84). In Drosophila, the characterized paralog of 4E-T is Cup, which binds elf4E and mediates translational repression by the 3'-UTR-binding proteins Bruno and Smaug (19–22). Besides the elf4E-binding sites, vertebrate 4E-T and Cup are most similar in a short 25-amino acid region, unique to this family of proteins, whose role remains to be determined. The mouse paralog, Clast4, is maternally expressed and is phosphorylated during meiotic maturation (85). Interestingly, phosphorylation of human 4E-T during mitosis releases elf4E (86).

We show that Xenopus 4E-T, but not elf4G or 4E-BP1, is found in the CPEB RNP complex in early oocytes and that the only elf4E1 protein in this complex is elf4E1b, rather than the canonical cap-binding factor elf4E1a. elf4E1b binds the cap very weakly and interacts with 4E-T, rather than elf4G, at a separate site from elf4E1a. Preliminary experiments indicate that the potential separate elf4E1b binding site lies within the first 329 amino acids of X4E-T but does not require the 25-amino acid-long “Cup-homology” domain (Fig. S2) (data not shown). Although the existence of an alternative binding site is supported by the observation that Cup has two distinct binding sites for elf4E1b (21) (Fig. S2), we found that mutation of the
CPEB RNP in Early Oocytes

sequence in X4E-T resembling the second site in Cup had no effect on elf4E1a or elf4E1b binding (data not shown).

Paralogs of elf4E1b are conserved in vertebrates but are not discernible in flies or worms. These invertebrates contain 5–8 elf4E-like proteins, and possibly one may fulfill the role of elf4E1b. elf4E1b expression is confined to oocytes, eggs, and early embryos in Xenopus, zebrafish, and mice. The participation of a weak cap-binding protein in the CPEB complex is at first sight perplexing in view of its proposed role in translational repression. Nevertheless, alongside recently described paralogs, it is possible to propose a model for CPEB. 4E-HP, a class II elf4E, represses caudal and hunchback mRNAs by binding the cap and Bicoid and Nason/Pum/Brat, respectively, which interact with the mRNAs directly via particular 3′-UTR cis-elements (23, 24). Subsequent studies of 4E-HP have shown that it binds the m^GpppG 30-fold less well than elf4E1a (27, 28). Interestingly, it has also been shown that Argonaute 2, tethered to the target mRNA 3′-UTR by micro-RNA (83), also possesses weak cap-binding activity, responsible for repression of translation initiation by micro-RNAs (87). A similar scenario may underlie the repression of CPE-RNAs in Xenopus oocytes by the elf4E1b-4E-T-CPEB complex. According to this model, weak cap binding by 3′UTR-tethered proteins would be sufficient for inhibition of translation of target mRNAs, could in principle be readily reversed by altering 3′-UTR RNP dynamics, and would avoid inhibition of general protein synthesis (Fig. 9).

The structure of XelF4E1b, with and without a cap, was homology-modeled using the respective solved structures of mouse elf4E1a. The structures of mouse and Xenopus pro-

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