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Embryonic Poly(A)-Binding Protein Stimulates Translation in Germ Cells

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The function of poly(A)-binding protein 1 (PABP1) in poly(A)-mediated translation has been extensively characterized. Recently, *Xenopus laevis* oocytes and early embryos were shown to contain a novel poly(A)-binding protein, ePABP, which has not been described in other organisms. ePABP was identified as a protein that binds AU-rich sequences and prevents shortening of poly(A) tails. Here, we show that ePABP is also expressed in *X. laevis* testis, suggesting a more general role for ePABP in gametogenesis. We find that ePABP is conserved throughout vertebrates and that mouse and *X. laevis* cells have similar tissue-specific ePABP expression patterns. Furthermore, we directly assess the role of ePABP in translation. We show that ePABP is associated with polysomes and can activate the translation of reporter mRNAs in vivo. Despite its relative divergence from PABP1, we find that ePABP has similar functional domains and can bind to several PABP1 partners, suggesting that they may use similar mechanisms to activate translation. In addition, we find that PABP1 and ePABP can interact, suggesting that these proteins may be bound simultaneously to the same mRNA. Finally, we show that the activity of both PABP1 and ePABP increases during oocyte maturation, when many mRNAs undergo polyadenylation.

During its lifetime in the cytoplasm, an mRNA normally undergoes multiple rounds of translation, and its poly(A) tail is gradually shortened, eventually leading to degradation. However, it is increasingly being recognized that regulated changes in poly(A) tail length can form a significant mechanism for controlling gene expression. While most vertebrate mRNAs exit the nucleus with a poly(A) tail of 200 to 250 nucleotides (48), dramatic changes in polyadenylation can occur in the cytoplasm. These can lead to changes in mRNA translation, with increases in poly(A) tail length being associated with translational activation and shortening with silencing (22, 46). Cytoplasmic polyadenylation is essential during male and female gametogenesis, and in the early embryo, where transcription is often quiescent and changes in the pattern of protein synthesis rely on the activation, repression, or destruction of stored mRNAs (30, 41, 56). It is also thought to play a role in other cell types, including neurons, where at least one transcript important in learning and memory appears to be regulated by changes in poly(A) tail length (29).

It is widely accepted that an important function of the poly(A) tail is to recruit poly(A)-binding proteins (PABPs) to mRNAs, and PABP1 can functionally replace the poly(A) tail when tethered (8, 21). There are two distinct branches of PABP proteins, based on their sequence and intracellular location. The structural organization of different PABP proteins is summarized in Fig. 1. Nuclear PABPs function in the polyadenylation and maturation of pre-mRNAs and are only distantly related to cytoplasmic PABPs (reviewed in references 34 and 39). While *Saccharomyces cerevisiae* has a single gene for cytoplasmic PABP (*pab1*), higher eukaryotes typically contain multiple cytoplasmic PABPs.

In addition to PABP1, three additional cytoplasmic PABP members have been identified as expressed genes in humans and mice. Testis PABP (also known as PABPC3 in humans and PABPC2 in mice) and inducible PABP (also known as PABPC4) are both highly related to PABP1 (Fig. 1). Whereas PABP1 is considered ubiquitous, testis PABP is expressed only in subsets of male germ cells, suggesting a specific role in spermatogenesis (16, 31). Levels of inducible PABP mRNA are upregulated during T-cell activation, although its mRNA is found in a wide variety of tissues (27, 62). In contrast, PABP5 (also known as PABPC5) appears to be a truncated protein which lacks the C-terminal domain of other cytoplasmic PABPs (Fig. 1). PABP5 is expressed in a few adult tissues, including the ovary (3).

Recently, a new PABP protein, ePABP2, was identified in *Xenopus laevis*, mice, and humans. Curiously, it is closely related to nuclear PABP (PABPN1) in structure (Fig. 1) but is predominantly localized in the cytoplasm in *X. laevis* (19). In *X. laevis* and mouse, ePABP2 is expressed predominantly in oocytes and early embryos, although its function remains unclear (10, 19).

While cytoplasmic PABPs are thought to be central to mRNA regulation, surprisingly, the roles and contributions of different PABP family members to controlling gene expression are not yet fully understood. Only PABP1 has been studied extensively and shown to have important roles in mRNA stability and translation (reviewed in reference 20), although testis PABP, inducible PABP, and ePABP2 have all also been shown to specifically bind poly(A) RNA. The PABP1 gene is highly conserved among eukaryotes and is essential for viability in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* (reviewed in reference 20).

PABP1 is composed of four nonidentical RNA recognition

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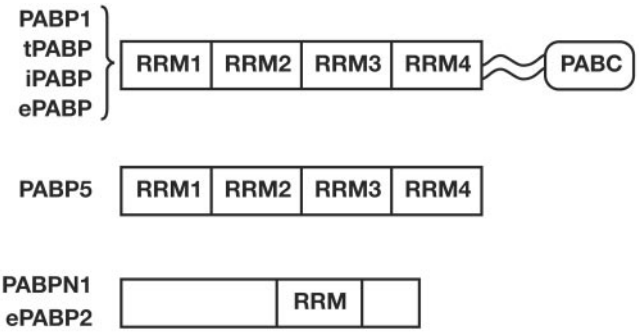


FIG. 1. Structures of vertebrate poly(A)-binding protein (PABP) family members. PABP1 consists of four nonidentical RNA recognition motifs (RRMs), linked by an unstructured proline-rich region (represented by a wavy line) to a globular C terminus (PABC). This structure is shared by other cytoplasmic PABPs, including testis PABP (tPABP, PABP2 and -3), inducible PABP (iPABP, PABP4) and embryonic PABP (ePABP). PABP5 also has four nonidentical RRMs but lacks the proline-rich region and C-terminal domains. By contrast, nuclear PABP (PABPN1, PAB2, PABPII) and ePABP2 have a different structure, being composed of a relatively long acidic N terminus, a single RRM, and a short arginine-rich C terminus.

motifs (RRMs) and a C-terminal domain that does not bind RNA (see Fig. 1 and 6A). Poly(A) binding is essentially conferred by the first two RRMs (RRMs 1 and 2), which, when bound to poly(A), provide a hydrophobic surface that is available to interact with translation initiation factors (14). In *X. laevis* PABP1, this domain is capable of stimulating translation to the same extent as full-length PABP when tethered (21), underlying the importance of these interactions. RRMs 3 and 4 of *X. laevis* PABP1 are also capable of stimulating translation when tethered (21), although functional protein partners of this domain await identification. This domain has a low affinity for poly(A), and its RNA-binding properties are often described as nonspecific (5, 13, 33, 45), but it may stabilize or increase PABP-poly(A) binding. Interestingly, it has recently been reported that RRMs 3 and 4 preferentially bind AU-rich sequences (4, 54), raising the possibility that this domain might enable PABP to bind specifically to non-poly(A) sequence elements.

The C terminus of PABP1 is composed of a poorly conserved proline-rich linker region that is thought to be relatively unstructured and a globular carboxyl-terminal domain (sometimes referred to as the PABC domain) and has only limited capacity to stimulate translation when tethered (21). The proline-rich region is implicated in PABP1 self-association, which is thought to facilitate cooperative binding of multiple PABP molecules to long poly(A) tails (33) and is required for the ability of PABP1 to regulate its own expression (40). The PABC domain is thought to be particularly important in promoting protein-protein interactions, and a PABP-interacting motif (PAM2) has been defined which is found in many PABP1 partners that bind to this domain (32).

The current model for how PABP1 stimulates translation posits that it forms complexes with initiation factors bound to the 5′ end, effectively circularizing the mRNA (28, 59). It is thought that formation of these end-to-end complexes aids the recruitment of ribosomal subunits, and positive effects of PABP1 on both 40S and 60S ribosome recruitment have been shown (44, 52, 55). One important interaction in this complex appears to be between RRMs 1 and 2 of PABP1 and eIF4G (22, 34, 39, 59), a scaffolding protein in the trimeric eIF4F complex that is bound directly to the cap via eIF4E. The PABP1-eIF4G interaction may stimulate translation by stabilizing or increasing poly(A) binding by PABP1 and/or enhancing the affinity of eIF4F for the m^7G cap (reviewed in reference 59). Increased eIF4F binding may facilitate 40S recruitment via the interaction of eIF4G with eIF3 on the 40S subunit. The eIF4F complex also contains eIF4A, an RNA-dependent helicase whose activity is stimulated by eIF4B. Helicase activity is proposed to unwind secondary structures in the 5′ untranslated region, aiding recruitment of small ribosomal subunits (23).

PABP1 also interacts with eIF4B (6, 37) and with PABP-interacting protein 1 (Paip1) (21, 47), a protein with homology to eIF4G, but the relative contribution of these proteins to PABP1-mediated translation is less clear. Lastly, PABP1 interacts with a translation termination factor, eRF3 (9, 26, 57), which may facilitate the recycling of terminating ribosomes to the 5′ end (57) or link translational termination to mRNA turnover (26). The mechanism by which cytoplasmic polyadenylation promotes translation is less clear, but it is generally considered that binding of additional PABP1 molecules to newly extended poly(A) tails increases end-to-end complex formation.

Recently, a novel poly(A)-binding protein was identified as a *Xenopus*-specific protein that binds AU-rich sequences (58). Embryonic PABP (ePABP) was shown to share PABP1's ability to bind poly(A) (11) and to protect mRNAs from deadenylation (58). ePABP maintains the same general structure as PABP1 (see Fig. 1 and 6A) but shows most divergence in RRM 3 and the proline-rich linker region (58). ePABP is present at higher levels than PABP1 during most of oogenesis and early embryogenesis (11, 58), with its levels decreasing as PABP1 levels increase at the onset of zygotic transcription (11). Thus, it appears to play a specific developmental role in protecting mRNA from deadenylation.

Here we examine the potential of this protein to stimulate translation and explore its mechanism of action. We find that PABP1 and ePABP can interact, suggesting that mRNAs may be simultaneously bound by both proteins. Additionally, we find that the activity of both proteins is developmentally regulated and that ePABP is conserved in vertebrates. Our expression analysis shows that ePABP is also expressed in testis, suggesting a wider role in the regulation of mRNAs required for gametogenesis.

MATERIALS AND METHODS

Plasmids. pMSP, pMS2-U1A, pMS2-PABP, pMS2 1-2, pMS2 3-4, pMS2-Ct, pLG-MS2 (21), and pJK350 (15) have all been described previously. pMSPN was created from pET-MS2 (8, 21) by insertion of annealed oligonucleotides into the BamHI site in pMSP. The sequences of the oligonucleotides used were 5′-GATCGGATCCAATTGACTAGT-3′ and 5′-GATCACTAGTCAATTGGATCC-3′. pMS2-ePABP was generated by PCR with primers 5′-CAGTCAGCTAGCATGAATGC AACCGGAGCC G-3′ and 5′-TTTGGAGGCT GCGGCAT G-3′ from IMAGE clone 3437819. The PCR product and MS2-eCt were cut with NheI prior to ligation.

pMS2 e1-2 was generated by reverse transcription (RT)-PCR from RNA from stage VI oocytes with primers 5′-CAGTCACAAT TGATGAATGC AACCG GAGCC G-3′ and 5′-CAGTCAACTA GTTTATAATT CTCTTTCCCT CCGTG-3′. The product was digested with MfeI and SpeI and ligated into MSPN digested with MfeI and SpeI. pMS2 e3-4 was generated by RT-PCR from

RNA from stage VI oocytes with primers 5′-CAGTCAGAAT TCGAATATGG GGCAAAAGTT ATGGAATTTA CCAACGTGTA C-3′ and 5′-CAGTCA CCTA GGTTAAAGGA GAGGACCAGG CATCGCCCTC ATAGTGGCTA G-3′. The PCR product was digested with AvrII, treated with Klenow, digested with EcoRI, and ligated into MSPN digested with BlpI treated with Klenow and then digested with MfeI. pMS2-eCt was generated by RT-PCR from RNA from stage VI oocytes with primers 5′-CATGCAGAAT TCATGCTAGC CACTAT GAGG GCGATGCCTG GTCCTCTC-3′zsqx and 5′-CAGTCACTCG AGTC AGATCA AAGATGGTTG GGCACTTTTT TGAGC-3′. The PCR product was digested with XhoI, Klenow treated, digested with EcoRI, and ligated into MSPN digested with BlpI, Klenow treated, and then MfeI digested.

pLexA-MS2, pACT-IRP (53), PAB 1-2, PAB 3-4, PAB-Ct, and pACT-4GNt have all been described previously (21). pXl-Paip1 was isolated in a yeast two-hybrid screen with PAB 1-2 with a library prepared from *X. laevis* oocytes (D. Lawson and N. K. Gray, unpublished data). BTM e1-2 was generated by digesting and ligating the MfeI-SpeI RT-PCR fragment (see above) into BTMKnDB digested with EcoRI and AvrII. BTM e3-4 was generated by RT-PCR from RNA from stage VI oocytes with primers 5′-CAGTCAGAAT TCGAATATGG GGCAAAAGTT ATGGAATTTA CCAACGTGTA C-3′ and 5′-CAGTCA CCTA GGTTAAAGGA GAGGACCAGG CATCGCCCTC ATAGTGGCTA G-3′. The PCR product and BTMKnDB were digested with EcoRI and AvrII prior to ligation. BTM-eCt was created by RT-PCR of RNA from stage VI oocytes with primers 5′-CATGCAGAAT TCATGCTAGC CACTATGAGG GCGATGCCTG GTCCTCTC-3′ and 5′-CAGTCACTCG AGTCAGATCA AAGATGGTTG GGCACTTTTT TGAGC-3′. The PCR product was digested with EcoRI and XhoI and ligated into BTMKnDB digested with EcoRI and SalI.

Tethered function assays. Tethered function assays and isotopic labeling and analysis were performed as described previously (21). Briefly, in vitro-transcribed mRNAs encoding MS2 fusion proteins were microinjected into stage VI *X. laevis* oocytes. Following 6 h of incubation, the luciferase-MS2 reporter RNA was coinjected with a polyadenylated β-galactosidase reporter RNA that does not contain MS2 binding sites and acts as a control for variations in injection efficiency or translational activity between oocytes. Oocytes were then incubated overnight. At least three groups of five oocytes were harvested per point, and levels of luciferase and β-galactosidase activity were assayed in duplicate. Expression of MS2 fusion proteins was confirmed by [^{35}S]methionine labeling and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in all cases (data not shown). Oocytes were matured by the addition of 10 μg of progesterone (Sigma) per ml and scored by the appearance of a white spot on the animal pole. Error bars on graphs represent standard error in all cases.

RNA stability analysis. Total RNA was extracted from injected oocytes (21) and first-strand cDNA was synthesized from total RNA with a transcriptor kit (Roche) according to the manufacturer's instructions, except that reverse transcription was carried out at 42°C. Quantitative RT-PCR analysis was performed in a LightCycler (Roche) by SYBR I green incorporation with primers 5′-GGCGCGGTCG GTAAAGTT-3′ and 5′-AGCGTTTTCC CGGTATCCA-3′ for luciferase and 5′-TCACGAGCAT CATCCTCTGC-3′ and 5′-CAGCGG ATGG TTCGGATAATGC-3′ for β-galactosidase. Data analysis was performed with LightCycler software (Roche), and standard curves generated from luciferase and β-galactosidase DNA standards were used to determine concentrations in the test samples. All samples were analyzed in duplicate.

RNA expression analysis. Tissues were homogenized with an UltraTurrax (IKA Labortechnik), and total RNA was extracted with Tri reagent (Sigma) according to the manufacturer's instructions; 30 μg of total RNA from each tissue (normalized by measuring the intensity of rRNA bands) was electrophoresed in denaturing formaldehyde gels and subjected to Northern blotting with Hybond N$^+$ membranes (Amersham Biosciences). Specific antisense RNA probes were generated by runoff transcription with [α-^{32}P]UTP. Hybridization was carried out overnight in Ultrahyb (Ambion) according to the manufacturer's instructions.

RT-PCR analysis was performed on 0.5 μg of total mRNA with the Titan one-tube RT-PCR kit (Roche) according to the manufacturer's instructions.

Primers used for RT-PCR analysis. The primers used for RT-PCR analysis were as follows: for mouse ePABP, 5′-CACCTTCTCT ACTTTTGGGA GC-3′ and 5′-ACATAGAGCG GTTTCGTGCC-3′; for mouse β-Actin, 5′-GGTCAG AAGG ACTCCTATGT GG-3′ and 5′-TCTCAGCTGT GGTGGTGAAG-3′; for *X. laevis* ePABP, 5′-ATGCAGAGGC TAGCCACTAT GAGGGCGATG-3′ and 5′-GCATTTTCCT TGGCTTGGTG GGCTTGCAGG-3′; and for *X. laevis* glyceraldehyde-3-phosphate dehydrogenase, 5′-CAAGTCATCA CCGTCT TCCA GG-3′ and 5′-CTGGTCTTGT GTGTATCCC AGG-3′.

Yeast two-hybrid analysis. Yeast two-hybrid analysis was performed with strain L40ura$^-$ as described (63).

Antibody production. Antibodies were raised in New Zealand White rabbits with the PABP1- and ePABP-specific peptides CLAQRKEERQAHLTN and CLMRAVQPRRMSSN, respectively (Sigma), conjugated to keyhole limpet hemocyanin. Bleeds were checked for reactivity, and antiserum from bleed 6 was affinity purified with antigenic peptides (CovalAb). The RED2 anti-PABP1 antibody was a kind gift of Simon Morley (University of Sussex, Brighton, United Kingdom).

Western blot analysis. Protein was extracted from tissues by homogenizing in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) followed by sonication to fragment genomic DNA and centrifugation to pellet cell debris. Equal amounts of protein (determined by Bradford assay and Coomassie staining) were separated on SDS–10% PAGE gels, transferred to polyvinylidene fluoride membranes (Millipore), and probed with anti-ePABP (1:2,000), anti-PABP (1:1,000), or RED2 anti-PABP (1:2,500) antibodies. Goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (1:10,000) was used as a secondary antibody, and signals were detected by enhanced chemiluminescence (Amersham).

Immunoprecipitation. Where indicated, stage VI *X. laevis* oocytes were incubated for 6 h with [^{35}S]methionine (ICN) to label newly synthesized proteins. Labeled or unlabeled oocytes were lysed in immunoprecipitation lysis buffer (10 μl/oocyte) (20 mM HEPES [pH 7.6], 10 mM KCl, 1.5 mM $MgCl_2$, 100 mM NaCl, 0.5% Triton X-100, 0.5 mM dithiothreitol, Roche protease inhibitors). The oocyte lysate was cleared by centrifugation. The volume was increased to 1 ml with immunoprecipitation lysis buffer and mixed for 1 h or overnight at 4°C with an anti-PABP antibody or anti-ePABP antibody; 30 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech) was added to the lysate and mixed for 60 to 90 min at 4°C. The beads were washed three times with immunoprecipitation lysis buffer. Where indicated, 200 U of RNase I (Ambion) was added to the last wash and incubated at 37°C for 15 min. Bound material was eluted in 30 μl of SDS gel loading dye prior to SDS-PAGE and Western analysis.

Sucrose gradient analysis. We subjected 30 stage VI oocytes to sucrose gradient analysis through 10 to 50% sucrose and fractionated them as described (18). Protein was extracted from fractions by precipitation with 10% trichloroacetic acid prior to Western blot analysis.

Bioinformatic analysis. The genome databases at http://www.ensembl.org (human NCBI34, mouse NCBIm33, zebra fish WTSI Zv4, chicken WASHUC1; FUGU v2.0) were searched with BLAST software (1). The *Xenopus tropicalis* genomic sequence was assembled from the JGI website (http://genome.jgi-psf.org/xenopus0/xenopus0.home.html). When no satisfying gene predictions were found in Ensembl, gene structure was determined with Genewise software (Birney et al., unpublished data; http://www.ebi.ac.uk/Wise2/doc_wise2.html). ClustalW (version 1.82 with default settings) (24) was used to generate final alignments for protein sequences. Phylogenetic trees were constructed by the neighbor-joining method (49) with MEGA (version 2.1; http://www.megasoftware.net/) (35). Positions in alignments containing gaps were omitted from subsequent analyses. The reliability of each interior branch of a given topology was assessed with the bootstrap interior branch test with 1,000 bootstraps.

The sequences used included human PABP1 (NP_002559), mouse PABP1 (NP_032800), *X. laevis* PABP1 (P20965), chicken PABP1 (Genewise predictions from the Ensembl genomic sequence chromosome (chr) unknown, 157909142 to 157922934), human PABP3 (NP_112241), zefra fish PABP1 (ENSDARP00000011650), zebra fish PABP2 (Genewise prediction from Ensembl genomic sequence Chr2 2046830 to 2062302), mouse PABP2 (NP_035163), zebrafish ePABP (NP_956133; misannotated *pabpc1*), *X. laevis* ePABP (AAK29408), chicken ePABP (Genewise prediction from Ensembl genomic sequence Chr20, 4966678 to 4974824), human ePABP (Ensembl Vega transcript C20orf119-001 translated, OTTHUMP00000031080), mouse ePABP (Genewise prediction from Ensembl genomic sequence Chr2, 164088831 to 164113369), human PABP4 (NP_003810), mouse PABP4 (NP_570951), chicken PABP4 (ENSGALP00000006018), zebra fish PABP4 (ENSDARP00000021064), *Xenopus tropicalis* PABP4 (Genewise prediction from genomic sequence), human PABP5 (NP_543022), mouse PABP5 (NP_444344), and *X. laevis* ePABP kidney expressed sequence tag clones BX849575, BU904844, and CB559228.

RESULTS

Expression of ePABP in testis. ePABP was first described as a protein that is present throughout *Xenopus* oogenesis and early embryogenesis with levels decreasing at the onset of zygotic transcription (11, 58) and was previously reported to be

absent in adult *Xenopus* tissues (11). However, given the importance of cytoplasmic polyadenylation in spermatogenesis as well as oogenesis, we examined several adult tissues, including testis, for expression of ePABP. Northern blot analysis for selected tissues is shown in Fig. 2A. This analysis demonstrates that ePABP mRNA (≈2,400 nucleotides) is present in testis at approximately the same levels as in stage VI oocytes. This result was confirmed by RT-PCR, which also revealed low levels of expression in kidney and heart (Fig. 2B). This is in keeping with the presence of expressed sequence tag clones encoding ePABP from kidney libraries. RT-PCR fails to detect ePABP mRNA in all other tissues tested, including brain (Fig. 2B).

Selected tissues were analyzed by Western blot with ePABP-specific antibodies (Fig. 2C). This revealed that ePABP protein (≈70 kDa) can be detected in testis but at a reduced level compared to stage VI oocytes, despite the presence of approximately equal amounts of mRNA in both tissues. Stage I oocytes show strong ePABP expression, as previously reported (11). PABP1 has previously been shown to be present in most tissues at various levels. Its levels decrease after early oogenesis and then increase following oocyte maturation and are most abundant after the onset of zygotic transcription (11, 58). Both PABP1 and ePABP were readily detectable by immunoprecipitation from stage VI oocytes labeled with [^{35}S]methionine (Fig. 2D), demonstrating that both proteins are actively translated in this tissue.

ePABP is conserved throughout vertebrate species. Steitz and colleagues (58) initially reported ePABP as a *Xenopus*-specific protein that was not conserved in other species. Identification of PABP genes in mammals is complicated by the presence of multiple pseudogenes. However bioinformatic analysis based on homology to *Xenopus* ePABP allowed us to identify a mammalian ePABP gene (Fig. 3), as ePABP is significantly different from the other PABP genes. Phylogenetic analysis of different PABP proteins from available genomes (Fig. 3A) clearly illustrates that ePABP is present in mammals, birds, frogs, and fish. However, no clear homologue is found in flies, worms, or sea squirts, suggesting that it arose during evolution of the vertebrate lineage. Mouse ePABP is located on chromosome 2 (bp 164639456 to 164664014), and its human homologue is on chromosome 20q13.12. An alignment of *Xenopus* ePABP with its predicted orthologues from other vertebrate species is shown in Fig. 3B. The *X. laevis* and mouse ePABP proteins are 65% identical and have 76% similar residues.

To determine the expression pattern of mouse ePABP, RT-PCR analysis was performed on selected mouse tissues. RT-PCR analysis revealed that ePABP is expressed at high levels in mouse ovary and is also present in embryos (14.5 days postcoitum) and testis (Fig. 3C). Expression of ePABP was not detected in any other adult tissue tested, including brain (data not shown). Levels of ePABP protein could not be directly determined in the mouse, as the antibodies generated do not recognize the mouse protein. Nevertheless, these results support the idea that ePABP may play a conserved role in both male and female gametogenesis in the mouse as well as in *X. laevis*. Furthermore, bioinformatic analysis indicates that zebrafish ePABP expressed sequence tag clones are present in embryo, kidney, ovary, and testis libraries (P. Gautier, unpub-

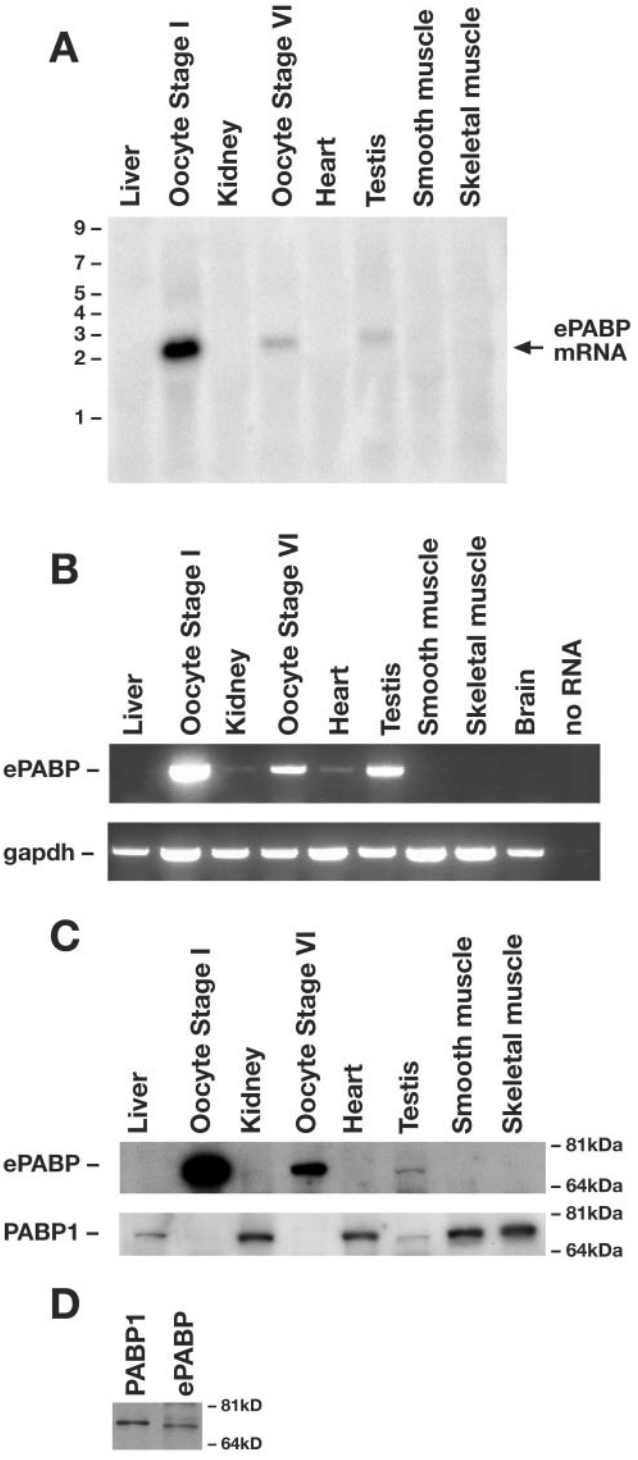
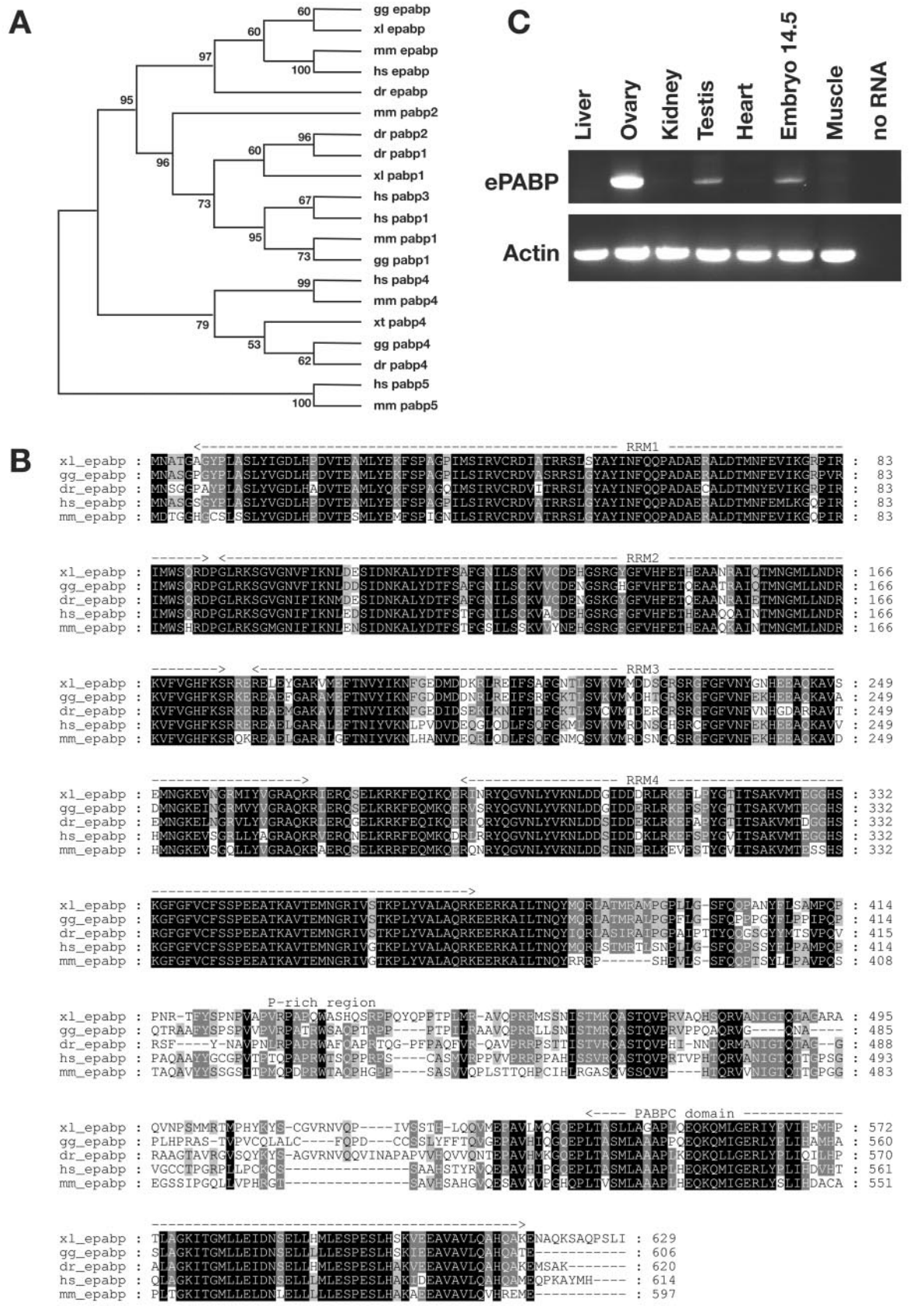


FIG. 2. Expression analysis of *Xenopus* ePABP. (A) Northern analysis of total RNA, normalized for the amounts of rRNA extracted from the indicated tissues. A specific ePABP antisense RNA probe was used. (B) RT-PCR analysis of total RNA from the indicated tissues, normalized for detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. (C) Western analysis of proteins extracted from the indicated tissues with antibodies specific for ePABP and PABP1. Equal protein loading was assessed by the Bradford assay and Coomassie staining. (D) Immunoprecipitation of PABP1 and ePABP from stage VI oocytes after 6 h of incubation in [^{35}S]methionine, which labels actively translated proteins.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gg pabp4
dr pabp4
hs pabp5
mm pabp5
C
Liver
Ovary
Kidney
Testis
Heart
Embryo 14.5
Muscle
no RNA
ePABP
Actin
B
RRM1
RRM2
RRM3
RRM4
P-rich region
PABPC domain

lished data), strongly suggesting that the expression pattern of ePABP may be conserved in many species.

ePABP is associated with polysomes. The abundance of ePABP prior to the onset of zygotic transcription, and its presence in testis suggest that this protein could play a role in poly(A)-mediated translation. As an initial approach to determining whether ePABP has a role in translation, its association with polyribosomes (polysomes) was tested by sucrose gradient analysis of stage VI *Xenopus* oocytes.

Figure 4 shows that ePABP is found in the messenger ribonic leoprotein (mRNP) fractions but importantly is also present in the polysomal region of the gradient which sediments below the 80S monosome peak (Fig. 4A and B). Addition of EDTA causes the release of ribosomes from mRNA and their dissociation into 60S and 40S subunits (Fig. 4A, dotted line). This results in a redistribution of ePABP from heavier to lighter complexes in the mRNP region (Fig. 4C), supporting the conclusion that its presence in heavier fractions was due to its association with polysomes. Polysomes represent actively translated mRNAs, indicating that ePABP is likely to have a positive role in translation. PABP1 has previously been shown to be present in both mRNP and polysomal fractions (50, 60).

ePABP can stimulate translation in *X. laevis* oocytes. The potential of ePABP to regulate translation was tested directly by the tethered function assay (21) in stage VI oocytes. This assay allows the role of ePABP in translation to be examined without the need to deplete PABP proteins in vivo. Moreover, it liberates analysis of its role in translation from its other functions (21). This assay has two components: a luciferase reporter RNA containing binding sites for the bacteriophage MS2 coat protein within its 3′ untranslated region, and a fusion protein composed of MS2 coat protein and the protein of interest. The interaction of the MS2 coat protein with its target RNA binding sites brings the protein of interest to the reporter mRNA (Fig. 5A).

Reporter mRNAs were injected into the cytoplasm of stage VI oocytes expressing either the MS2 tether protein alone, tethered PABP1 (MS2-PABP1), or tethered ePABP (MS2-ePABP). Throughout this work, luciferase activity was normalized against β-galactosidase activity by coinjection of a β-galactosidase reporter mRNA which lacks MS2 sites and provides an internal standard for variations in injection efficiency or translational activity between oocytes. MS2-PABP1 was used as a positive control as it has previously been shown to activate translation in these oocytes (21) and its effects on translation can be clearly seen (Fig. 5B) when compared to the tether protein alone (MS2).

Significantly, when ePABP was tethered (MS2-ePABP) it stimulated luciferase expression to a level just below that of PABP1 (Fig. 5B). Typically, stimulation was eightfold, seldom being below fivefold or above 11-fold, and was dependent on the presence of the MS2 binding sites, as a luciferase mRNA that lacks MS2 binding sites did not show stimulation (data not

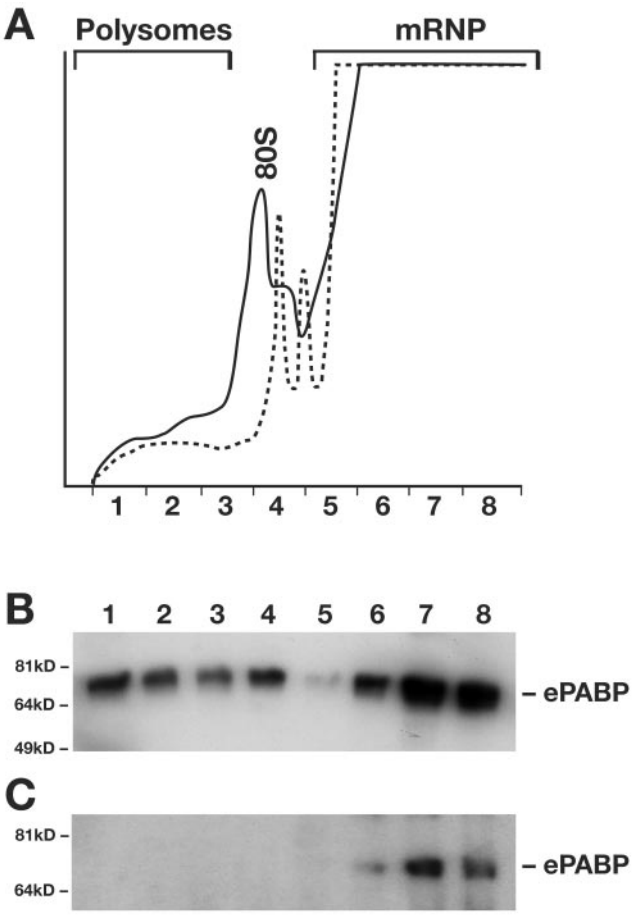

FIG. 4. ePABP is associated with polyribosomes. (A) Cleared extracts of stage VI *Xenopus laevis* oocytes were subjected to sucrose gradient analysis with 10 to 50% sucrose. The UV absorbance profile (254 nm) of untreated (solid line) and EDTA-treated (dotted line) extracts is shown, and the positions of polyribosomes, the 80S monosome, and mRNP fractions are indicated. EDTA treatment causes release of ribosomes from mRNA and their dissociation into faster-sedimenting 60S and 40S subunits. Fractions 1 to 8 of untreated oocyte extract (B) and EDTA-treated extract (C) were subjected to Western blotting with an anti-ePABP antibody; 7.5-fold more fraction was loaded in lanes 1 to 4 than in lanes 5 to 8 in both panels B and C due to the high total protein content in fractions 5 to 8.

FIG. 3. ePABP is present in other vertebrates. (A) A phylogenetic comparison of vertebrate PABP proteins. PABP2 and -3 are expressed in testis (tPABP) and are highly related to PABP1, while PABP4 corresponds to inducible PABP (iPABP), which is upregulated in activated T cells. PABP5 lack the entire C-terminal domain of other cytoplasmic PABPs and is expressed in a few adult tissues. The ePABP, PABP4, and PABP5 genes form groups that are clearly distinct from the PABP1/testis PABP group. PABPs from *Gallus gallus* (gg), *Homo sapiens* (hs), *Mus musculus* (mm), *Danio rerio* (dr), *Xenopus laevis* (xl), and *Xenopus tropicalis* (xt) were compared. (B) The amino acid sequence of *Xenopus laevis* ePABP is compared to that of the predicted ePABPs from other vertebrates (species abbreviations defined above). Shaded regions denote identical or similar residues in three or more ePABPs in the alignment. The positions of the RRMs and PABC domain are indicated. RRM 4 and the PABC domain are linked by a relatively unstructured proline-rich region, which is highly divergent in different PABPs. (C) RT-PCR analysis of ePABP (upper panel), normalized for β-actin (lower panel) mRNA expression from the indicated mouse tissues.

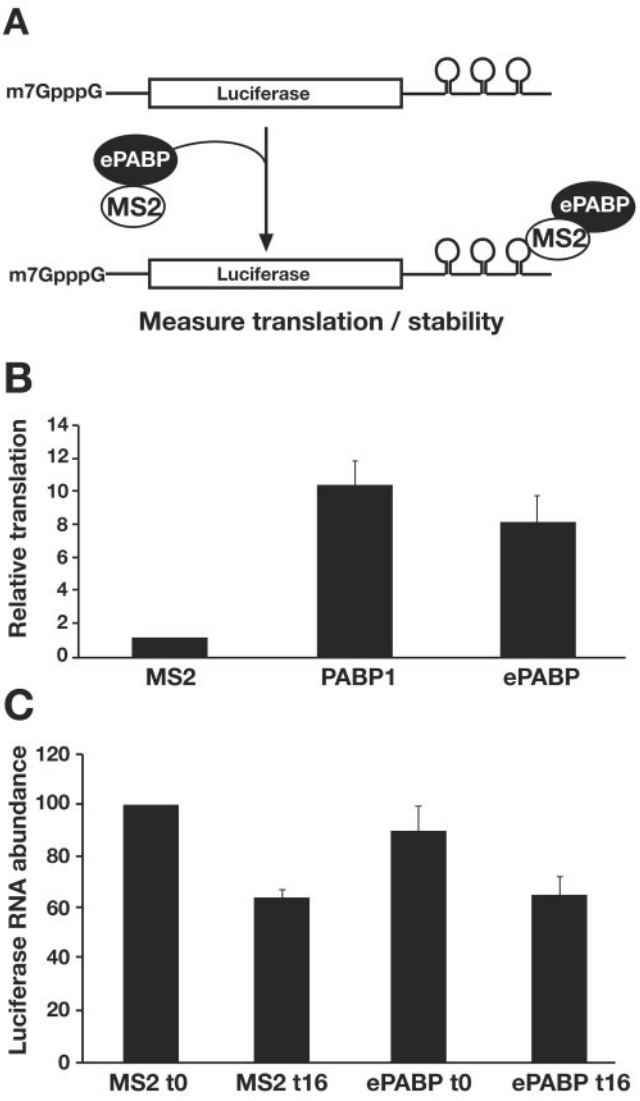

FIG. 5. ePABP can stimulate translation when tethered. (A) The tethered function assay has two components: a luciferase reporter mRNA with binding sites for the MS2 coat protein within its 3′ untranslated region (Luc-MS2), and a fusion protein between MS2 coat protein and ePABP. Binding of the coat protein tethers ePABP to the mRNA. The effects of fusion proteins on translation are measured by the luciferase assay, normalized to a coinjected β-galactosidase mRNA (not shown) that contains no MS2 sites. (B) Oocytes expressing the MS2 tether protein alone, MS2-PABP, or MS2-ePABP were coinjected with Luc-MS2 and β-galactosidase mRNAs. Normalized luciferase activity is plotted, where the value of MS2 was set to 1. The average of seven independent experiments is shown. (C) Injections were performed as in B, and total RNA was extracted from injected oocytes expressing the indicated fusion proteins after 0 h (t0) and 16 h (t16) of incubation. The levels of luciferase and β-galactosidase activity were analyzed by quantitative RT-PCR, and normalized values are plotted.

shown). Quantitative RT-PCR of the injected mRNAs indicated that Luc-MS2 was not stabilized by the presence of MS2-ePABP over the time course of the experiment (Fig. 5C). These results demonstrate that the increase in luciferase activity is due to differences in the translation and not in the stability of Luc-MS2 mRNA, indicating that ePABP can stimulate the translation of mRNAs to which it is bound. The effects of ePABP are specific, as we have previously shown that other RRM proteins, which do not function in translation, do not elicit this effect (21). Thus, it appears that ePABP can stimulate the translation of mRNAs in *X. laevis* oocytes in addition to its role in protecting mRNAs from deadenylation (58).

PABP1 and ePABP have similar functional domains. The conservation between PABP1 and ePABP is not evenly distributed, with RRM 3 and the C terminus being more divergent (Fig. 6A). To address whether PABP1 and ePABP use the same functional domains, three domains of ePABP, corresponding to those previously defined for PABP1 (21), were tethered and assessed for their ability to stimulate translation (Fig. 6B).

The most highly conserved region between PABP1 and ePABP is RRMs 1 and 2 (Fig. 6A). When tethered, RRMs 1 and 2 of ePABP were found to stimulate translation to roughly the same extent as full-length ePABP (compare Fig. 5B and 6B) and to a level just below that of RRMs 1 and 2 of PABP1 (Fig. 6B), suggesting that the function of this domain in stimulating translation is conserved. RRMs 3 and 4 of ePABP were also found to stimulate translation to a similar level as full-length ePABP and to be as active as RRMs 3 and 4 of PABP1 (Fig. 6B). Thus, the ability of RRMs 3 and 4 to stimulate translation is conserved between PABP1 and ePABP despite the lower homology within RRM 3 (Fig. 6A).

The C-terminal domains (Ct) of PABP1 and ePABP are quite divergent (56% identity, compared to 91% between human and *X. laevis* PABP1), with homology in the proline-rich linker region being particularly poor. Tethering of the C terminus of ePABP (Fig. 6B) revealed that this region had a small but significant ability to stimulate translation that was consistently higher than the stimulation seen with the corresponding domain in PABP1. Nonetheless, the ability of ePABP-Ct to stimulate translation was comparatively small compared to that of ePABP RRMs 1 and 2 and of 3 and 4. Taken together these data indicate that ePABP, like PABP1, has two domains that can stimulate translation to a level similar to that stimulated by the full-length protein.

ePABP maintains interactions with PABP1-interacting proteins. The conservation of functional domains suggests that ePABP and PABP1 may utilize some of the same factors to stimulate translation. An interaction between ePABP and eRF3 was demonstrated previously (11), which may contribute to the ability of the C terminus of ePABP to stimulate translation or to link translation to mRNA decay (reviewed in reference 20). *X. laevis* PABP1, like mammalian PABP1, has previously been shown to interact with eIF4G and Paip-1 (21). Therefore, to address whether ePABP maintained similar interactions, directed yeast two-hybrid analyses were undertaken (Fig. 6C). This analysis revealed that RRMs 1 and 2 of ePABP maintain interactions with eIF4G and Paip1 and that the C-terminal interaction with Paip1 is also conserved (Fig. 6C). These interactions are specific, as the LacZ reporter was only activated when both ePABP and eIF4G or Paip1 were expressed. PABP1 was previously reported to bind directly to eIF4B (6). However, we were unable to detect significant interaction between either *X. laevis* PABP1 or ePABP with eIF4B in directed two-hybrid assays (data not shown), although this does not rule out an interaction between these

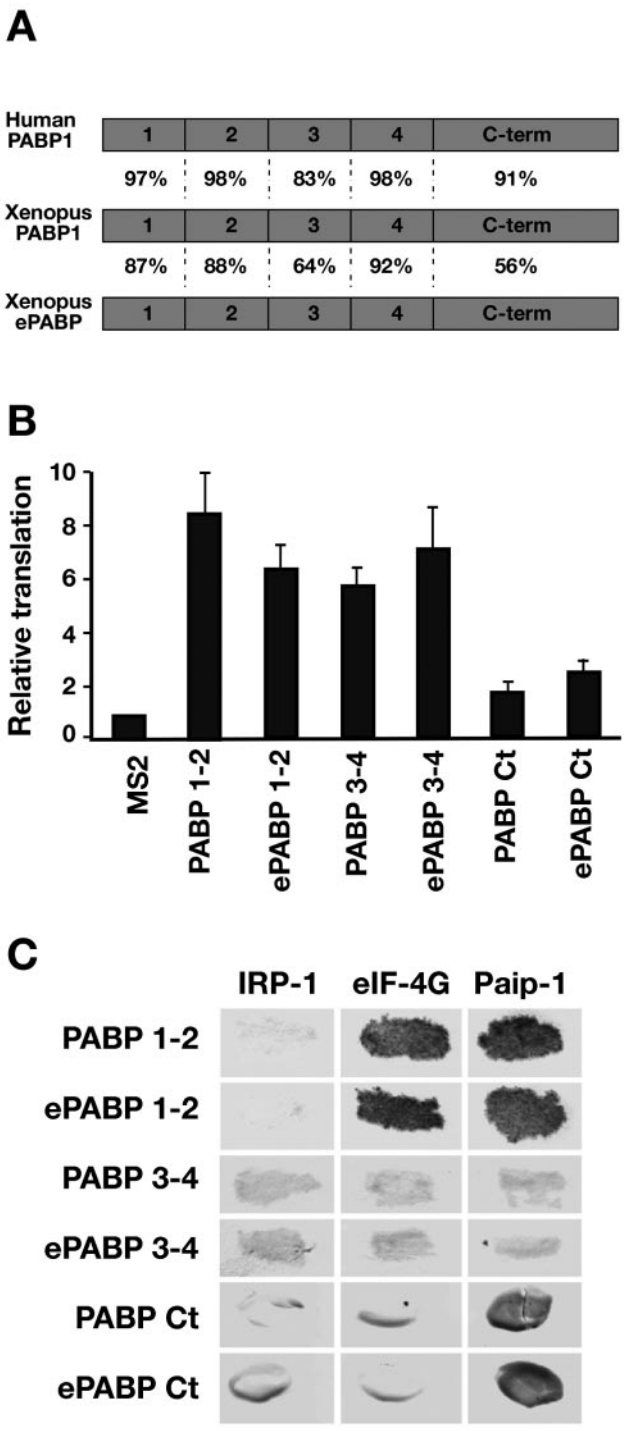

FIG. 6. ePABP shows functional conservation of domains with PABP1, (A) Cartoon of the domains of *Xenopus* PABP1 versus human PABP1 and *Xenopus* ePABP, showing the percentage of identical residues. All three proteins have the same structural organization, with four RNA recognition motifs (RRMs) and a C-terminal domain which does not bind RNA. The overall identity between *Xenopus* and human PABP1 is 93% and is evenly spread throughout the proteins. In contrast, *Xenopus* PABP1 and ePABP have 71% identity, with the C terminus being the most divergent domain. (B) Stage VI oocytes expressing MS2 alone, MS2-PABP 1-2, MS2-ePABP 1-2, MS2-PABP 3-4, MS2-ePABP 3-4, MS2-PABP-Ct, and MS2-ePABP-Ct were coinjected with Luc-MS2 and β-galactosidase mRNAs. Normalized luciferase activity is plotted, where the value of MS2 was set to 1. The average of five independent experiments is shown. (C) Yeast two hybrid analysis with the indicated domains of PABP1 and ePABP against Paip-1 and eIF4G. IRP-1, an RNA-binding protein, was used as a negative control. Expression of β-galactosidase (dark colonies) indicates protein-protein interaction.

factors. Taken together, these results suggest that ePABP and PABP1 may utilize at least some of the same factors to stimulate translation.

ePABP and PABP1 can interact. PABP1 has been shown to self-associate. This is suggested to aid the binding of PABP1 to poly(A) and to be important for its ability to regulate its expression by binding to the 5′ untranslated region of its own mRNA (33, 40). Self-association of PABP1 is dependent on the proline-rich region within the C-terminal domain of PABP1, which is particularly poorly conserved in ePABP (43% identical), although this region of ePABP maintains a high proline content (see Fig. 3B). Thus, we tested the ability of ePABP to self-associate by directed yeast two-hybrid analysis with the entire C-terminal domain of ePABP (Fig. 7A). This assay revealed that ePABP C termini can interact, showing that this feature is conserved.

This result raised the possibility that ePABP is also able to interact with PABP1. To address this, the C termini of both ePABP and PABP1 (Fig. 7B) were tested by directed yeast two-hybrid analysis. This analysis demonstrated self-association between PABP1 C termini, as previously shown (33). Moreover, a strong interaction between the PABP1 and ePABP C termini was observed, suggesting that these proteins can interact (Fig. 7B). Neither of these regions can bind RNA, and thus this interaction is RNA independent. Coimmunoprecipitation of endogenous PABP1 with an anti-ePABP antibody from mature oocytes (Fig. 7C, upper panel) shows that these proteins can also associate in vivo. RNase treatment does not disrupt this interaction, confirming that endogenous PABP1 and ePABP can interact independently of RNA.

Regulation of PABP activity during oocyte maturation. Poly(A) tail length changes are known to occur during oogenesis and early embryogenesis. However, to date the ability of PABP1 (21) or ePABP (Fig. 5) to stimulate translation has only been directly addressed in stage VI oocytes. To address whether translational stimulation by PABP1 or ePABP is altered during development, tethered function assays were performed in mature oocytes. Maturation following progesterone treatment was scored by the appearance of a white spot on the animal pole, indicating germinal vesicle breakdown.

In stage VI oocytes, PABP1 and ePABP stimulated translation with approximately equal efficiency (Fig. 5B and 8). Following maturation, the relative stimulation by both PABP1 and ePABP was significantly increased, with stimulation rising by a factor of 1.5- and 2.3-fold, respectively (Fig. 8). This suggests that the activity of these proteins or their partners is increased at a time when many mRNAs become polyadenylated and translationally active. As these proteins are tethered, it is unlikely that this is due to a modification that affects their ability to bind mRNA. It is unclear whether the apparently larger increase in ePABP-mediated stimulation is physiologically significant, but the higher abundance of ePABP in stage VI and mature oocytes (11) strongly suggests that ePABP is the main

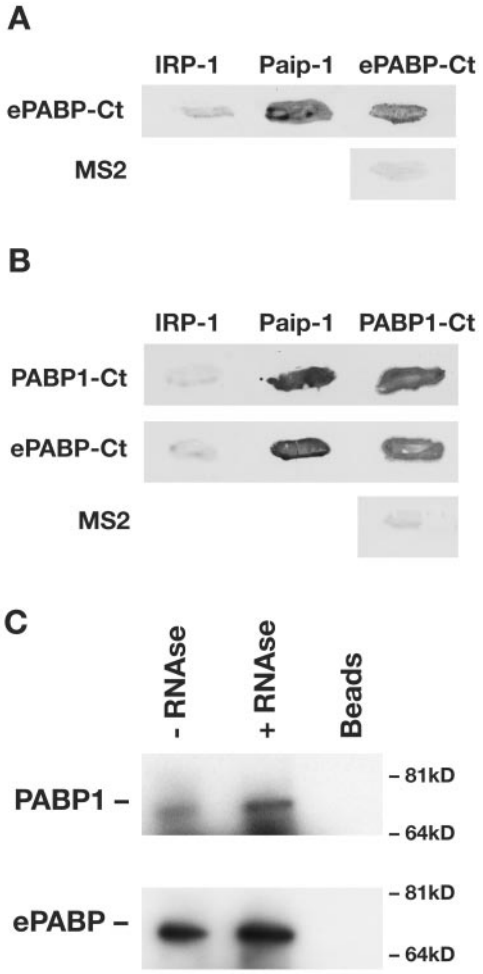

FIG. 7. ePABP can self-associate and interact with PABP1 through its C terminus. (A) Yeast two-hybrid analysis with the C terminus (Ct) of ePABP. (B) Yeast two-hybrid analysis with PABP1 or ePABP C termini (Ct) against the PABP1 C terminus. In A and B, IRP and MS2 are negative controls and Paip-1 serves as a positive control. Expression of β-galactosidase (dark colonies) indicates protein-protein interaction. (B) Western analysis with anti-PABP1 (upper panel) and anti-ePABP (lower panel) of extracts from matured oocytes immunoprecipitated with an anti-ePABP antibody (lanes 1 and 2). RNase I was included where indicated (+). Lane 3, bead-only control.

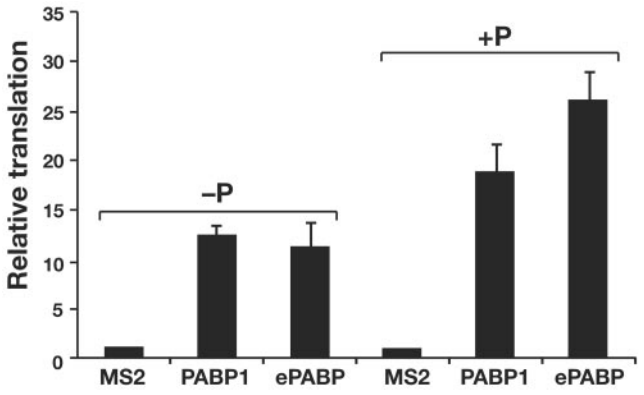

FIG. 8. Activities of ePABP and PABP1 increase during oocyte maturation. Stage VI oocytes expressing MS2 alone, MS2-PABP1, or MS2-ePABP were coinjected with Luc-MS2 and β-galactosidase reporter mRNAs, and oocytes were either untreated (−P) or treated with progesterone (+P) to induce maturation. Matured oocytes were scored by the appearance of a white spot on the animal pole, indicating germinal vesicle breakdown. Normalized luciferase activity is plotted, where the value of MS2 was set to 1. The average of three independent experiments is shown.

effector of poly(A)-mediated translation at this stage of development.

DISCUSSION

Here we show that ePABP is conserved in vertebrates and that its expression pattern supports a role in male and female gametogenesis. We also find that it is associated with polysomes and can stimulate translation of reporter mRNAs in vivo. Thus, ePABP is the second member of the PABP family shown to directly activate translation. Given the greater abundance of ePABP than of PABP1 during oogenesis and early development (11, 58), it is likely that this protein plays a predominant role in poly(A)-mediated translation during this developmental time frame. Surprisingly, while ePABP and PABP1 show low conservation over certain domains, we show that they maintain similar interactions. Moreover, we show that PABP1 and ePABP are coexpressed in *X. laevis* oocytes and testis and can interact with each other.

Mechanism of ePABP stimulation. Our results demonstrate that PABP1 is not unique in its ability to stimulate translation and that ePABP can activate translation with efficiency similar to that of PABP1 (Fig. 5). This is in keeping with previous work that suggested a role for *Xenopus* ePABP in translation, since it can interact with translation factors (11) and restore a block in cyclin B synthesis in *Xenopus* oocytes injected with excess polyadenylated RNA (7). Given that ePABP is the predominant PABP protein in certain cells (11, 58) (Fig. 2), it is clearly important that its mechanism of action be understood. In order to address this, we systematically compared different domains of PABP1 and ePABP for their ability to stimulate (Fig. 6B). Perhaps surprisingly, given the divergence in certain regions, ePABP maintains similar functional domains (Fig. 6B) and shares with PABP1 the ability to interact with the same translation factors (Fig. 6C).

Previous work has shown that RRMs 1 and 2 of PABP1 can interact with eIF4G and Paip1, and the majority of work to date supports a dominant role for eIF4G in the action of this domain (reviewed in reference 20). Here, we find that ePABP RRMs 1 and 2 can interact with both eIF4G and Paip1 (Fig. 6C). Our results, coupled with observations that ePABP is associated with eIF4F complexes and can disrupt Maskin-mediated repression (7, 11), strongly suggest that an interaction with eIF4G is important for the function of ePABP RRMs 1 and 2.

Our finding that ePABP RRMs 3 and 4 can stimulate translation to a level resembling that of full-length ePABP and slightly more than that of PABP1 RRMs 3 and 4 extends our previous observations that multiple domains of PABP1 can function independently to activate translation (21). This result also highlights the immediate importance of identifying factors

that interact with RRMs 3 and 4, which may be important partners used by both PABP1 and ePABP.

The C terminus of ePABP, which is the least conserved domain, was found to have a relatively small ability to stimulate translation (Fig. 6B). However, this was consistently greater than that of the corresponding domain of PABP1. Given the ability of this domain to interact with eRF3 (11) and Paip1 (Fig. 6C), either of these factors could contribute to its limited ability to stimulate translation. However, our observation that ePABP-Ct is capable of mediating interactions with PABP1 (Fig. 7) means that we cannot formally rule out the possibility that ePABP-Ct may function at least in part by recruiting full-length PABP proteins.

A more detailed understanding of the mechanisms of action of PABP1 and ePABP, in particular identification of the partners of RRMs 3 and 4, may be required to explain why ePABP is expressed at higher levels than PABP1 during early development.

Regulation of PABP activity. As many mRNAs are polyadenylated during oocyte maturation (22), it was of interest to determine whether the activity of PABP1 or ePABP may be altered during this developmental step. To examine this possibility, we analyzed the activity of tethered PABP1 and ePABP during maturation (Fig. 8). Interestingly, we found that the activity of both proteins was significantly upregulated. This observation raises the possibility that these proteins are subject to differential regulation in response to various signals. This could underlie the reason for expressing multiple PABP proteins with similar functions. In this regard, the apparently higher activity of ePABP in mature oocytes is interesting (Fig. 8).

It is unclear how the activities of these proteins are regulated. Plant PABP has been shown to be phosphorylated (17), but neither *Xenopus* PABP1 nor ePABP appears to be phosphorylated following maturation (11), suggesting that this modification cannot account for their increased activity. Mammalian PABP1 is also subject to cleavage (6, 36), which could potentially regulate its activity in a manner similar to eIF4G. However, we did not detect significant or differential cleavage of PABP1 or ePABP in stage VI versus mature oocytes (data not shown). Lastly, PABP1 can be modified by methylation, at least in HeLa cells (38), but the functional consequences of this are unknown. We therefore hypothesize that the activities of PABP1 and ePABP may be regulated by protein factors, similar to the regulation of eIF4E by eIF4EBP, or by the increased level or activity of a partner that is required for stimulation. Many of the kinase pathways that modify translation factors are activated during this period (42, 51). This will be an avenue for future investigation.

PABP1 and ePABP can interact. The importance of PABP1 self-association for its function is established (40). Despite poor homology in the region implicated in this process, our results indicate that ePABP can self-associate and interact with PABP1 (Fig. 7). Given that PABP and ePABP are coexpressed in oocytes, early embryos, and testis (Fig. 2B, C, and D) (11), this may enable mRNAs to be bound by PABP1 and ePABP simultaneously. This raises the possibility that the poly(A) tails of particular mRNAs may be preferentially bound by PABP1 or ePABP exclusively or by a complex containing both proteins. It will be of interest to determine whether other divergent PABPs such as PABP5 and ePABP2 can also self-associate or interact with other PABPs. This may allow complex regulation of mRNAs by PABPs that may vary in their capacity to bind poly(A), stimulate translation, or interact with different factors.

Expression of ePABP proteins. Our analysis of ePABP expression levels in *Xenopus laevis* extend previous studies that showed the protein was detectable until 72 h after fertilization and was absent from adult tissues (11). While our results show that ePABP mRNA is barely detectable by RT-PCR in most adult *Xenopus* tissues (Fig. 2B), it appears to be expressed at low levels in the kidney and heart. In contrast, ePABP is clearly detectable by RT-PCR and Northern and Western blots in oocytes and in adult testis (Fig. 2A, B, and C). ePABP mRNA is also expressed at significantly high levels in both ovary and testis from juvenile frogs (G. S. Wilkie, unpublished data). This suggests that ePABP may have a specific role in tissues associated with gametogenesis.

In keeping with this idea, we see that ePABP shows a similar expression pattern in mice. It is currently unclear whether expression in 14.5-day-postcoitum mouse embryos is restricted to gonadal tissue. Interestingly, there is an apparent discrepancy between the levels of ePABP mRNA and protein in *Xenopus* stage VI oocytes and testis (compare Fig. 2A and B with Fig. 2C). This may reflect an inherent stability of ePABP protein during the months required to complete oogenesis, although it is turned over relatively quickly following fertilization (11). Alternatively, it is interesting to speculate that ePABP mRNA itself is under translational control, with its translation in testis being repressed in comparison to stage VI oocytes. Conversely, translation of ePABP mRNA could be activated more effectively in stage VI oocytes by a factor that is not present in testis.

PABP1 mRNA is known to be autoregulated and to be regulated by 5′-terminal oligopyrimidine tracts (12, 25, 61). However, the expression patterns of ePABP and PABP1 (Fig. 2C) suggest that they should not be regulated by the same mechanism. In keeping with this, extension by 5′ rapid amplification of cDNA ends has failed to reveal either regulatory element in the ePABP mRNA (G. S. Wilkie, unpublished data). The absence of an autoregulatory element in ePABP may allow higher levels of expression in oocytes, where the effects of poly(A) are profound. However, it is not currently possible to exclude the presence of a 5′-terminal oligopyrimidine tract, as we cannot be certain that our sequence of the 5′ untranslated region is complete. Thus, the potential regulation of ePABP mRNA will be a future avenue of research.

Role of multiple PABP proteins. The recent identification of ePABP2, which displays an expression pattern similar to that of ePABP (10, 19), indicates that three PABPs are present during oogenesis and early embyogenesis in *X. laevis*. The simultaneous expression of more than one PABP in a particular cell type has been demonstrated in plants (2) and may also occur in mammals, as both PABP1 and testis PABP mRNAs are expressed during the later stages of human spermatogenesis (16). Similarly, T cells contain both PABP1 and inducible PABP, but only inducible PABP mRNA levels are increased upon T-cell activation (62). Thus, it appears that vertebrates have multiple PABP genes that may play redundant, overlapping, or distinct roles in mRNA regulation. Structural differ-

ences between PABPs suggest that some may have very different functions, e.g., ePABP2, whereas others may display more subtle differences. Our results to date support the idea that PABP1 and ePABP have similar basic activities despite their relatively divergent sequences.

In conclusion, our results reveal that a second PABP is able to stimulate translation and functions, at least in part, through similar partners. Given the divergence of ePABP from PABP1, these results make it likely that testis PABP and inducible PABP may also share this ability. Our results raise the interesting question of why two PABP proteins with similar basal activities are expressed in the same cell and why ePABP is predominant until zygotic transcription. Given the similar activities of these proteins, it is interesting to hypothesize that ePABP and PABP may regulate specific subsets of mRNAs by binding to other RNA sequence elements in addition to directing poly(A)-mediated translation. In support of this idea, ePABP was first identified as an AU-rich-sequence-binding protein (58), and several recent reports suggest that PABP1 can bind to non-poly(A) sequences via RRMs 3 and 4 (4, 43, 54). Indeed, several mRNAs have already been identified which are regulated by the interaction of PABP1 not with the poly(A) tail, but with sequences in either the 5′ or 3′ untranslated region (reviewed in reference 20). Moreover, PABP1 can be recruited to mRNAs by interaction with specific RNA-binding proteins, providing more potential for specific regulation of mRNAs by individual PABPs (B. Collier et al., submitted for publication). Thus, the potential for PABP and ePABP to regulate specific subsets of mRNAs will be the target of future study.

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