

## Identification and Characterization of the Poly(A)-Binding Proteins from the Sea Urchin: a Quantitative Analysis

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**Poly(A)-binding proteins (PABPs) are the best characterized messenger RNA-binding proteins of eucaryotic cells and have been identified in diverse organisms such as mammals and yeasts. The *in vitro* poly(A)-binding properties of these proteins have been studied intensively; however, little is known about their function in cells. In this report, we show that sea urchin eggs have two molecular weight forms of PABP (molecular weights of 66,000 and 80,000). Each of these has at least five posttranslationally modified forms. Both sea urchin PABPs are found in approximately 1:1 ratios in both cytoplasmic and nuclear fractions of embryonic cells. Quantification in eggs and embryos revealed that sea urchin PABPs are surprisingly abundant, composing about 0.6% of total cellular protein. This is 50 times more than required to bind all the poly(A) in the egg based on the binding stoichiometry of 1 PABP per 27 adenosine residues. We found that density gradient centrifugation strips PABP from poly(A) and therefore underestimates the amount of PABP complexed to poly(A)<sup>+</sup> RNA in cell homogenates. However, large-pore gel filtration chromatography could be used to separate intact poly(A)-PABP complexes from free PABP. Using the gel filtration method, we found that the threefold increase in poly(A) content of the egg after fertilization is paralleled by an approximate fivefold increase in the amount of bound PABP. Furthermore, both translated and nontranslated poly(A)<sup>+</sup> RNAs appear to be complexed to PABP. As expected from the observation that PABPs are so abundant, greater than 95% of the PABP of the cell is uncomplexed protein.**

In eucaryotic cells, mRNA is associated with specific proteins in complexes termed messenger ribonucleoprotein particles (mRNPs) (50). Although mRNP proteins are hypothesized to perform roles in translational regulation, the function of most of them remain unknown (18, 31, 32, 37, 43, 54–56). To date, the four cap-binding proteins (eIF-4E and the three proteins of the eIF-4F complex) and the poly(A)-binding proteins (PABPs) are the only mRNP proteins that have been identified across species boundaries. Cap-binding proteins bind the 5' 7-methylguanosine cap of eucaryotic mRNAs and participate in the binding of the 40S ribosomal subunit to mRNA (51). PABPs bind the 3' poly(A) tail of eucaryotic mRNAs (8). Although they have been the subject of much study, the exact functions of both the poly(A) tail and its associated PABPs are not yet understood.

PABPs have been isolated from the cells of phylogenetically diverse organisms and appear to be as ubiquitous as the 3' poly(A) tail. The PABPs of birds and mammals range in molecular weight from approximately 70,000 to 80,000 (5, 8, 24, 26, 30, 46, 54, 56). The PABP of the yeast *Saccharomyces cerevisiae* has a molecular weight of 68,000 (1, 41), while those of other lower eucaryotes are generally smaller proteins ranging in molecular weight from 18,000 to 60,000 (2, 31, 33, 47). Protein and DNA sequencing of yeast, human, and *Xenopus* PABPs reveals that these proteins are members of a family of RNA-binding proteins that contain the RNP octapeptide consensus sequence—Lys/Arg Gly Phe/Tyr Gly/Ala Phe/Tyr Val X Phe/Tyr (1, 19, 39, 59). PABPs also contain four highly conserved RNA-binding domains within the N-terminal region of the protein (1, 19, 39, 40, 59).

Construction of mutants in yeasts has revealed that a 66-amino-acid polypeptide containing only one-half of one RNA-binding domain is sufficient to promote cell viability (40). However, illustrative of the essential nature of PABPs is the fact that deletion of the entire gene is lethal (40).

PABPs bind poly(A) approximately 100-fold more tightly than other polynucleotides, although in the absence of poly(A), PABPs bind poly(U), poly(G), and poly(C) (4, 5, 16, 52). Cytoplasmic PABPs from mammalian cells and yeasts bind poly(A) with a stoichiometry of 1 PABP per 25 to 57 adenosine residues in a "beads-on-a-string" type of configuration (4, 5, 40). Despite their high affinity for poly(A), PABPs can move from one poly(A) tract to another (41). Nuclear PABPs have been identified in yeast and mammalian cells and are derived from cytoplasmic PABP by cleavage of a portion of the carboxy terminus (39, 41). In contrast to the cytoplasmic form, nuclear PABPs do not confer the beads-on-a-string configuration on poly(A) tracts.

Because of their high affinity for poly(A) and their evolutionary conservation, it seems likely that PABPs play important roles in the metabolism or activity of poly(A)<sup>+</sup> mRNAs. Some hypotheses suggest that PABPs affect the translational activity of mRNAs. One proposal states that a certain PABP/adenosine residue ratio must exist on the poly(A) tail of a message for efficient translation to occur (20, 25, 47). This hypothesis predicts that poly(A)<sup>+</sup> messages compete for limited amounts of PABP to be efficiently translated. Sachs et al. (39) have proposed that PABP may regulate its own synthesis via a negative feedback pathway. Briefly, this hypothesis suggests that as PABP levels rise, PABPs bind 5' untranslated regions of their own messages, thereby preventing further PABP synthesis. More recently, it has been proposed that PABPs may also be involved in regulating the stability of poly(A)<sup>+</sup> mRNAs (7). This model proposes that PABP, when bound to the poly(A) tail, prevents degradation of mRNA. As the poly(A) tract is depleted of PABP, the

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message becomes more vulnerable to nuclease attack and therefore less stable. Although these hypotheses are compelling, there is not yet sufficient direct evidence to support them, and little is known about how the poly(A)-PABP complex functions in vivo.

Fertilization of sea urchin eggs results in a two- to three-fold increase in the total amount of detectable poly(A) in the cell (see reference 12 for a review). This increase is due to a cytoplasmic adenylation of stored maternal RNAs that results in the lengthening of existing poly(A) tracts as well as the 3' adenylation of previously poly(A)<sup>-</sup> RNAs (12, 48, 58). Fertilization of sea urchin eggs also results in the translational activation of stored maternal messages such that the rate of protein synthesis in two-cell embryos is 15- to 30-fold greater than that in unfertilized eggs (17, 23). Therefore, this system provides an opportunity to investigate PABPs under different conditions of translational activity and polyadenylation of native mRNPs.

In this study, we identified the PABPs in *Strongylocentrotus purpuratus* eggs. Sea urchin eggs have two PABPs with approximate molecular weights of 80,000 and 66,000 (the 80K and 66K PABPs). Unlike mammalian and yeast cells which partition the high-molecular-weight PABP to the cytoplasm and the low-molecular-weight PABP to the nucleus (39, 41), both sea urchin PABPs are found in a 1:1 ratio in both cytoplasmic and nuclear compartments of embryonic cells. Quantification of PABPs revealed that they are surprisingly abundant, composing approximately 0.6% of total cellular protein. We calculate that this is 50 times more PABP than required to bind all the poly(A) in unfertilized eggs and 15 times more PABP than required to bind all the poly(A) in two-cell embryos.

In our hands, sucrose or glycerol gradient centrifugation stripped PABP from poly(A)<sup>+</sup> RNA and, therefore, could not be used for reliable separation and quantification of free and complexed PABP. However, we found that large-pore gel filtration chromatography did not result in dissociation of PABP-poly(A) complexes. Analysis of PABP-poly(A) complexes from unfertilized eggs and two-cell embryos showed that fertilization results in a threefold increase in the amount of cellular poly(A) which is paralleled by an approximate fivefold increase in the amount of complexed PABP. Both translated and untranslated poly(A)<sup>+</sup> RNAs appeared to bind PABPs. However, greater than 95% of the PABP in sea urchins remained uncomplexed to poly(A).

## MATERIALS AND METHODS

**Embryo culture.** *S. purpuratus* adults were purchased from Marinas, Inc. Gametes were harvested by intracoelomic injection of 0.55 M KCl. Eggs were shed directly into Instant Ocean (Aquarium systems, Mentor, Ohio) artificial seawater at 15°C. Sperm were shed directly into a dry container and stored at 4°C until use. Eggs were dejellied before fertilization by settling through Instant Ocean at pH 4.5. Embryos at a concentration of 0.5 to 1.0% were cultured in Instant Ocean at 15°C with constant stirring.

**Preparation of homogenates and PMSs.** Eggs or embryos were concentrated by centrifugation in a tabletop or hand centrifuge, suspended, and washed twice in 20 volumes of ice-cold HB (250 mM NaCl, 25 mM EGTA, 10 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 6.8], 110 mM glycine, 250 mM glycerol) and once in ice-cold HB<sup>+</sup> (HB plus 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride). The volume of HB<sup>+</sup> to packed cells was adjusted to 1:1, and cells were homogenized with 3 to 10

strokes of a chilled, stainless-steel, Dounce-type homogenizer. Homogenization was monitored microscopically. Homogenates were centrifuged at 22,000 × *g* for 15 min at 4°C. The resulting supernatant is referred to as the postmitochondrial supernatant (PMS). Homogenates or PMSs were stored at -80°C until use.

**Preparation of nuclei.** Embryos were cultured as described above until the mesenchyme blastula stage. Nuclei were then prepared from these blastulae by the method of Hinegardner (22). Purified nuclei were treated with 10 μg of RNase A per ml and 30 μg of DNase I per ml for 20 min on ice. Nuclear proteins were precipitated with 2.5 volumes of ice-cold acetone, dried under vacuum, and dissolved in sodium dodecyl sulfate (SDS) sample buffer before SDS-polyacrylamide gel electrophoresis (PAGE) (3).

**Preparation of adult tissues.** Coelomic fluid (5 to 7 ml) was pipetted from the coelom of adult sea urchins. Coelomocytes were collected from the fluid by centrifugation for 5 min in a microcentrifuge. Pelleted material was examined microscopically to check for coelomocytes and to ensure that no germ cells contaminated the preparation. The pellet was then suspended and pelleted 2 times through ice-cold HB. The supernatant was discarded, and the pellet was sonicated in SDS sample buffer. Insoluble material was pelleted out of the sample buffer before SDS-PAGE. Tube feet were plucked from live adults and sonicated in SDS sample buffer.

**Purification of PABPs.** Sea urchin PABPs were purified from 60 ml of unfertilized egg homogenate essentially as described for rat and yeast PABPs (39, 41). Briefly, this procedure involves chromatography of PMS on Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.), washing the matrix with 4 M NaCl followed by a 0.5 M guanidine hydrochloride wash, and then eluting specifically bound proteins with 2 M guanidine hydrochloride. Poly(A) to a final concentration of 16 μg/ml and poly(C) to a final concentration of 1 mg/ml were added to the eluted proteins, followed by oligo(dT)-cellulose chromatography of the mixture. The oligo(dT)-cellulose was washed extensively with 0.23 M NaCl containing 1 mg of poly(C) per ml. Poly(A) and associated PABPs were eluted with 5 mM NaCl at 40°C. Poly(U)-Sepharose purification of PABPs is described in the next section.

**Polyclonal antibody preparation.** Egg PMS (60 ml) was passed over a Bio-Rad A1.5m gel filtration column equilibrated in RNase-free CB-I (250 mM NaCl, 10 mM EGTA, 5 mM MgCl<sub>2</sub>, 10 mM PIPES [pH 6.8], 360 mM glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The excluded peak was combined with 60 ml of poly(U)-Sepharose equilibrated with RNase-free CB-II (200 mM NaCl, 10 mM EDTA, 10 mM PIPES [pH 6.8]) and incubated at 4°C with gentle rocking for 2 h. The poly(U) matrix was settled by centrifugation, the supernatant was discarded, and the matrix was washed and settled three times through CB-II. The poly(U)-Sepharose was then poured into a jacketed column at 4°C and washed with CB-III (CB-II containing 0.5 M NaCl) until no UV-absorbing material could be detected. Poly(U)-binding proteins were eluted by raising the column temperature to 45°C and washing with 50% formamide in 15 mM KCl-5 mM MgCl<sub>2</sub>-5 mM PIPES [pH 6.8] at 45°C. The eluted proteins were dialyzed against CB-II and prepared for two-dimensional PAGE as described below. Antibodies to the PABPs were prepared by cutting the three most abundant isoelectric species of the 66K PABP from Coomassie-stained gels, emulsifying the acrylamide in Freund complete adjuvant, and injecting the preparation into a New Zealand White rabbit. Subsequent boosts with Freund incomplete

adjuvant were made 4 weeks after the initial injection and every 2 weeks thereafter. Antiserum was harvested by standard techniques.

An antibody was also prepared from the poly(U)-Sephrose eluate without subjecting it to two-dimensional PAGE. Injection of the proteins, boosting, and antiserum harvesting were performed as described above. This antiserum, termed anti-PU, contained antibodies for both PABPs as well as other poly(U)-binding proteins. The titer of the antibodies to the PABPs in this preparation was at least fivefold greater than that of the anti-PABP serum, although precise characterization was not done.

Anti-ribosome antiserum was prepared from highly purified ribosomes from unfertilized sea urchin eggs. PMS (30 to 40 units of optical density at 260 nm [ $OD_{260}$ ]) was layered onto a 36 ml 15 to 40% linear sucrose gradient made up in CB-I. The gradient was centrifuged at 22,000 rpm in a Beckman SW27 rotor at 4°C for 8 h. The 80S peak collected from this gradient was brought to 10 mM EDTA and recentrifuged on a similar gradient made up in CB-II. The 40S peak was collected from this gradient and recentrifuged, and a conservative cut of the 40S peak was used as the antigen.

**Fractionation of PMSs on gel filtration columns.** PMS (1,000  $OD_{260}$  units) from eggs or embryos was fractionated by gel filtration on Bio-Rad A1.5m columns (2.5 by 50 cm) (9). In some experiments, 200  $OD_{260}$  units of PMS were fractionated on A1.5m or A150m columns (1.5 by 50 cm). The excluded fractions from the A1.5m columns were termed peak 1 fractions. In experiments in which peak 1 was rechromatographed, 30 to 50  $OD_{260}$  units were loaded onto A1.5m columns (1.5 by 50 cm). Columns were equilibrated with either LS-CB (CB-I containing 100 mM NaCl) or HS-CB (CB-I containing 500 mM NaCl) before use. A1.5m columns (2.5 by 50 cm) were run at 70 ml/h, A1.5m columns (1.5 by 50 cm) were run at 36 ml/h, and A150m columns (1.5 by 50 cm) were run at 6 ml/h. All gel filtration chromatography was done at 4°C. In all cases, 20 equal fractions were collected per column run and prepared for protein, RNA, poly(A), and Western blot (immunoblot) analysis as described below.

**Glycerol and sucrose gradient centrifugation.** Peak 1 or PMS (30  $OD_{260}$  units) was layered onto linear 26-ml 15 to 50% (wt/vol) glycerol gradients or 15 to 40% (wt/vol) sucrose gradients made up in LS-CB. Each gradient had a 7-ml pad of 80% (wt/vol) glycerol or 60% (wt/vol) sucrose made up in LS-EB (100 mM NaCl, 10 mM EDTA, 10 mM PIPES [pH 6.8], 1 mM dithiothreitol) (28). Control gradients made up in LS-EB throughout were also prepared. Samples to be centrifuged on the LS-EB gradients were brought to a final concentration of 10 mM EDTA before centrifugation. The gradients were centrifuged in a Beckman SW28 rotor at 28,000 rpm for 5.5 h at 4°C. Gradients were collected by upward displacement with heavy sucrose. Ten equal fractions were collected per gradient and prepared for Western blot and poly(A) analysis as described below.

**Protein, RNA, and poly(A) analysis.** The protein concentration in various samples was determined by the method of Schaffner and Weissman (42). Samples to be used for RNA isolation were subjected to proteinase K digestion in the presence of SDS, followed by phenol-chloroform extraction and ethanol precipitation (57). Precipitates were dissolved in RNase-free distilled water. Total RNA concentration was determined from  $OD_{260}$  readings, and poly(A) concentration was determined by hybridization with [ $^3H$ ]poly(U).

**PAGE, Western blot analysis, and autoradiography.** Samples to be electrophoresed on one-dimensional SDS gels

were either precipitated with 4.5 volumes of ice-cold ethanol overnight at  $-20^{\circ}C$ , pelleted by centrifugation, washed with ice-cold acetone, dried under vacuum, and dissolved in SDS sample buffer or diluted 1:1 in  $2\times$  SDS sample buffer (3). Two-dimensional gel samples were incubated with 10  $\mu$ g of RNase A per ml and 30  $\mu$ g of DNase I per ml for 10 min on ice, precipitated as above, and dissolved in urea sample buffer (34). Samples were run on either 12.5% polyacrylamide-SDS gels or two-dimensional gels with a 12.5% polyacrylamide second dimension.

Proteins were transferred to nitrocellulose by electrophoresis in 25 mM Tris (pH 8.3)–192 mM glycine–10% methanol–0.01% SDS for 900 mA-h (53). The nitrocellulose blots were stained with 0.1% amido black to check for efficient transfer, rinsed for 5 min in distilled water, and then blocked for 30 min in BLOTTO (27). The blot was incubated with primary antibody at an appropriate dilution (1:100 for anti-PABP and 1:250 for anti-PU and antiribosome) in BLOTTO overnight at room temperature. Blots were rinsed four times in BLOTTO for 15 min each, incubated for 4 h to overnight with  $10^5$  cpm of  $^{125}I$ -protein A per ml in BLOTTO, rinsed as before, air dried, and exposed to X-ray film.

Autoradiography was done on preflashed XAR double-sided or SB5 single-sided Kodak X-ray film. Exposure times were adjusted so that films to be analyzed densitometrically were within the linear response range of the film. Densitometric analysis was performed with a Hoefer Scanning Densitometer.

**Quantification of PABP and poly(A).** The fraction of poly(A) [percent poly(A)] is expressed as the ratio of poly(A) [as determined by hybridization to [ $^3H$ ]poly(U)] to total RNA (as determined by  $OD_{260}$  readings). For these experiments, it was determined that 0.022% RNA in the egg and 0.069% of the total mass of two-cell embryos is poly(A). There are 2.8 ng of RNA per egg or embryo (17), and the molecular weight of AMP is 347.5; therefore, there are  $1.07 \times 10^9$  adenosine residues per egg and  $3.35 \times 10^9$  adenosine residues per two-cell embryo.

PABPs were purified by the Blue Sepharose–oligo (dT)-cellulose method (41) or by poly(U)-Sephrose chromatography. The purified proteins, unfertilized egg, and embryo homogenate for which total protein had been determined and bovine serum albumin (BSA) standards (0.05 to 1  $\mu$ g) were run on a one-dimensional SDS-polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose blot was stained with Schaffner-Weissman stain (42). The stained blot was scanned with a Hoefer Scanning Densitometer, and the amount of protein in the purified 66K and 80K PABPs was determined by comparison with the BSA standards. The blot was then probed with anti-PABP serum and  $^{125}I$ -protein A and autoradiographed as described above. The amount of protein per unit of OD in the purified 66K and 80K samples was calculated. The OD of the 66K and 80K PABPs in unfertilized egg and two-cell embryo homogenates was converted to mass by comparison with the densitometric profile of the purified sample. The 80K PABP gives a slightly lower OD per unit protein than the 66K PABP, indicating that anti-PABP has a higher affinity for the 66K PABP. The fraction of PABP in homogenates was calculated by dividing the amount of 66K and 80K PABPs by the total amount of protein in the sample. We calculated that the 66K and 80K PABPs each represent approximately 0.3% of total protein in eggs and embryos. *S. purpuratus* eggs and embryos contain 40 ng of protein (21); therefore, there are  $1.01 \times 10^9$  66K PABPs per egg or embryo and  $9.6 \times 10^8$  80K PABPs per egg or embryo.

## RESULTS

**Identification and purification of sea urchin PABPs.** Poly(U)-Sepharose chromatography of cytoplasmic proteins of sea urchin eggs resulted in the purification of two major proteins with molecular weights of 66,000 and 80,000. The molecular weights and poly(U)-binding properties of these two proteins suggested that they were the sea urchin PABPs. Therefore, to identify specific PABPs (as opposed to non-specific polyribonucleotide-binding proteins) of sea urchin embryos, we performed the PABP purification procedure of Sachs and Kornberg (41). Briefly, this method entails isolating PABPs from cell homogenates by Blue Sepharose chromatography, eluting bound proteins with 0.5 M guanidine hydrochloride, adding poly(A) and excess poly(C) to the eluted proteins, followed by oligo(dT)-cellulose chromatography of the mixture. Nonspecific RNA-binding proteins are separated from true PABPs by extensive washing of the oligo(dT)-cellulose column with poly(C). Figure 1 shows two-dimensional gels of the proteins purified by both poly(U)-Sepharose chromatography and the Sachs and Kornberg procedure (41). While the poly(U)-Sepharose method purifies a number of less abundant proteins in addition to the major 66K and 80K proteins, the Sachs and Kornberg method (41) purifies only the 66K and 80K PABPs. The acidic 38K protein seen in the gel in Fig. 1A is probably the single-stranded nucleic acid-binding protein previously reported by others to copurify with PABPs by this procedure (39).

Anti-PABP, a polyclonal antibody raised against poly(U)-Sepharose-purified 66K protein (Fig. 1C; see Fig. 2 for characterization of all antibodies used in these experiments), recognized the 66K and 80K Blue Sepharose-purified PABPs as well as both 66K and 80K poly(U)-Sepharose-purified proteins (data not shown). Thus, the 66K and 80K proteins isolated by poly(U)-Sepharose chromatography are the same antigens as the 66K and 80K sea urchin PABPs.

**The 66K and 80K PABPs are closely related proteins.** Anti-PABP was made by separating poly(U)-Sepharose-purified protein on two-dimensional polyacrylamide gels and excising the three most basic spots of the 66K PABP from the gels and injecting them into rabbits (Fig. 1B and 2). This antibody is specific for all isoelectric species of both molecular weight forms of PABP, indicating that all isoforms of both PABPs have common antigenic properties (Fig. 1C).

The relatedness of the 66K and 80K PABPs was further investigated by peptide mapping (11). Partial proteolysis of the two molecular weight forms of PABP with V8 protease (Fig. 3) revealed that these proteins generate essentially identical peptides, again indicating that they are closely related proteins.

**Sea urchin PABPs are related to yeast PABPs.** One reason for the general lack of knowledge about PABP function stems from the lack of consistency in the methods used to isolate and identify these proteins. It is frequently unclear whether or not the PABPs identified in one system are the bona fide homologs of the PABPs from another (14, 44; discussed in reference 39). Because of this, we felt it was necessary to compare the proteins identified as the sea urchin PABPs with well-characterized PABPs from another system.

Purified cytoplasmic (termed p68) and nuclear (termed p55) PABPs from the yeast *S. cerevisiae* were kindly provided by Alan Sachs, Stanford University (see reference 40 for the purification procedure). The two yeast PABPs and poly(U)-Sepharose-purified sea urchin PABPs were sub-

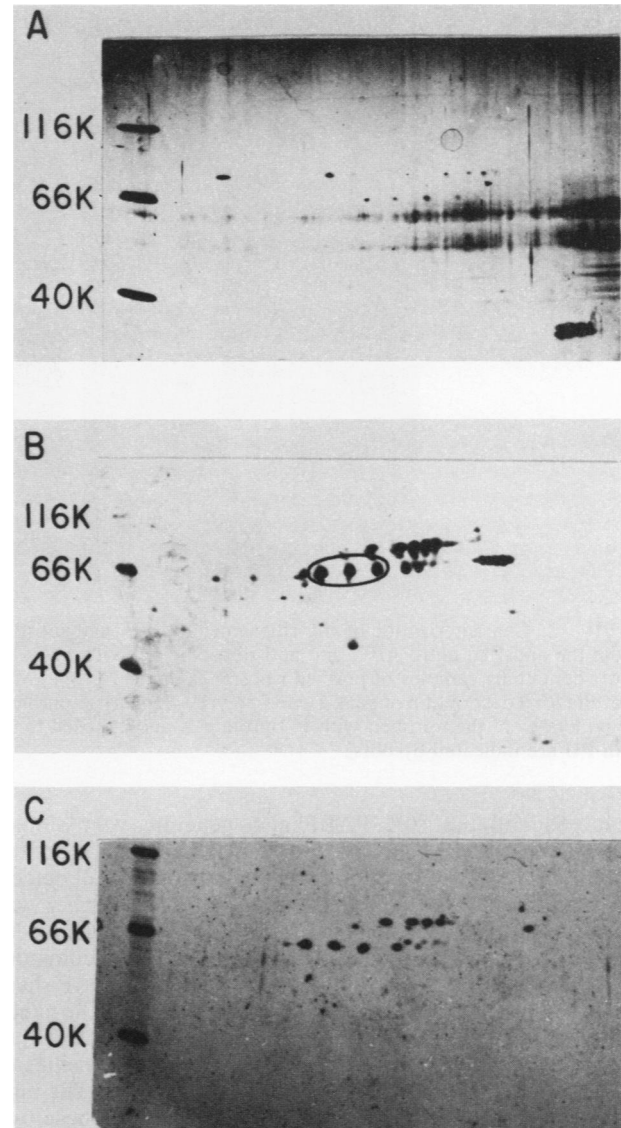


FIG. 1. Multiple isoforms of the 66K and 80K PABPs are antigenically related. (A) Two-dimensional polyacrylamide gel analysis of the proteins purified by Blue Sepharose chromatography. (B) Two-dimensional PAGE was performed on poly(U)-Sepharose-binding proteins. The circled proteins in panel B were excised from the gel and used to produce anti-PABP. Proteins were visualized by silver staining in both panels A and B. (C) PMS from sea urchin eggs was subjected to two-dimensional PAGE, Western blotted, and probed with anti-PABP (see also Fig. 2) and  $^{125}\text{I}$ -protein A.

jected to two-dimensional PAGE and visualized by either silver staining or Coomassie blue staining. PABPs from both organisms resolved into similar patterns of spots on these gels (Fig. 4). Furthermore, determination of the isoelectric points of individual spots revealed that the isoelectric variants of yeast p68 and sea urchin 66K PABP are identical.

Yeast and sea urchin PABPs were also compared by peptide analysis (Fig. 3). As mentioned previously, the two sea urchin PABPs generate essentially identical peptides upon partial proteolysis. The two yeast PABPs also generate almost identical patterns; yeast p68 yields two large peptides not shared with p55 and the p55 digest yields one small peptide not found in p68. Careful scrutiny of Fig. 3 reveals

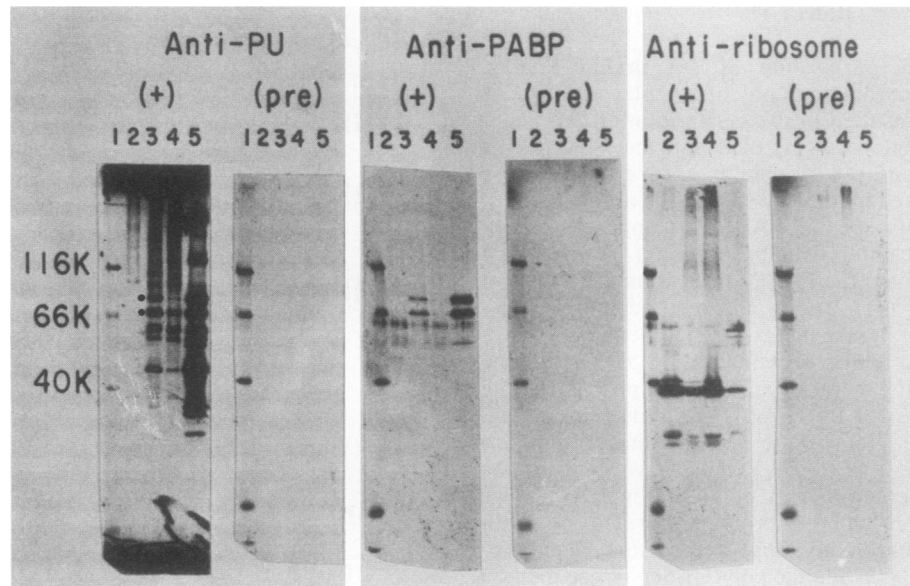


FIG. 2. Characterization of anti-ribosome, anti-PU, and anti-PABP polyclonal antisera. Preimmune (pre) and polyclonal immune (+) sera from the anti-PU, anti-PABP, and anti-ribosome antibody preparations (described in Materials and Methods) were used to probe Western blots containing samples of purified ribosomes (lanes 2), egg PMS (lanes 3), peak 1 fraction from unfertilized eggs (lanes 4; see Fig. 8 and Results for a description of peak 1), and poly(U)-Sepharose-purified proteins (lanes 5). Lanes 1 contain  $^{14}\text{C}$ -labeled molecular weight markers. The blots were then probed with  $^{125}\text{I}$ -protein A and exposed to X-ray film for visualization of antigenic proteins. The PABPs detected by anti-PU are indicated by dots.

that p68 and the 80K PABP also generate very similar digestion patterns. To derive a simple numerical index of the similarity of these two peptide maps, we counted all detectable peptides in the p68 and 80K lanes. This number was divided into the total number of peptides (based on molecular weight) that the 80K PABP and p68 have in common. This comparison revealed that p68 and the 80K PABP share 75% of detectable peptides. Further comparison of the bands shared by p68 and the 80K PABP revealed that not only are the molecular weights the same, but the band intensities of any given shared pair of peptides are comparable. The high degree of similarity between the peptide maps of these two PABPs provides further evidence that the proteins we identified as sea urchin PABPs are the homologs of yeast PABPs.

**Both 66K and 80K PABPs are present in cytoplasmic and nuclear fractions of embryonic cells.** Yeast and mammalian cells contain two PABPs: a cytoplasmic protein of approximately 70,000 to 80,000 molecular weight and a nuclear protein of about 50,000 to 60,000 molecular weight (41). Although the function of these two proteins has not yet been elucidated, they display different poly(A) binding characteristics, suggesting that they perform different functions in the cell (4, 5). Sea urchins are unusual in that both molecular weight PABPs can be isolated from cytoplasmic preparations (i.e., PMSs) of sea urchin eggs and embryos (Fig. 5). Since eggs contain stores of material to be used by the developing embryo, we hypothesized that the presence of the 66K PABP in cytoplasmic fractions was due to stockpiling of this protein for use later in development. However, densitometric analysis of the lanes designated p in Fig. 5 revealed that the ratio of 80K to 66K PABP remains 1:1 in PMSs at least until the late gastrula stage. This is despite the fact that early development is characterized by rapid nuclear replication during which maternal stores of nuclear proteins would, presumably, be depleted. During early development, nuclei are also becoming smaller and less fragile and there-

fore less likely to contaminate PMS preparations. Further analysis of the blot in Fig. 5 also revealed that crude homogenates and PMSs contain the same amount of PABP, indicating that detectable amounts of PABPs are not lost in mitochondrial pellets. The high-molecular-weight band detected by anti-PABP in the lane labeled unf h in Fig. 5 was not seen on subsequent blots of the same sample and probably represents incompletely solubilized protein.

To further address the issue of the relationship between the two sea urchin PABPs and the nuclear and cytoplasmic forms of PABP from mammalian and yeast cells, we isolated and analyzed the nuclear proteins of mesenchyme blastulae. Highly purified nuclei were separated from the cytoplasmic fraction by the method of Hinegardner (22). Microscopic examination of purified nuclei showed no cytoplasmic contamination in these preparations. Proteins from nuclear and cytoplasmic preparations were separated by SDS-PAGE, Western blotted, and probed with anti-PABP and  $^{125}\text{I}$ -protein A. The Western blot in Fig. 6 shows that although both forms are present, the nuclear fraction is enriched for neither the 80K nor the 66K PABP. In the absence of additional experiments to determine whether the presence of PABP in the nuclear fraction is due to cytoplasmic contamination, we cannot be entirely convinced that both forms are found in the nuclei of intact cells. However, the small volume of the nucleus compared with the much greater cytoplasmic volume leads us to predict that, if one form of PABP was exclusively nuclear, it would represent a smaller proportion of the protein of the cell than the cytoplasmic form. Since we found both sea urchin PABPs in equal amounts in crude homogenates as well as PMSs, we interpret this to mean that sea urchin cells are unusual because they contain two cytoplasmic forms of PABP.

**The 66K and 80K PABPs are both constituents of adult tissues.** To test the possibility that embryonic forms of sea urchin PABP are distinct from those of differentiated adult

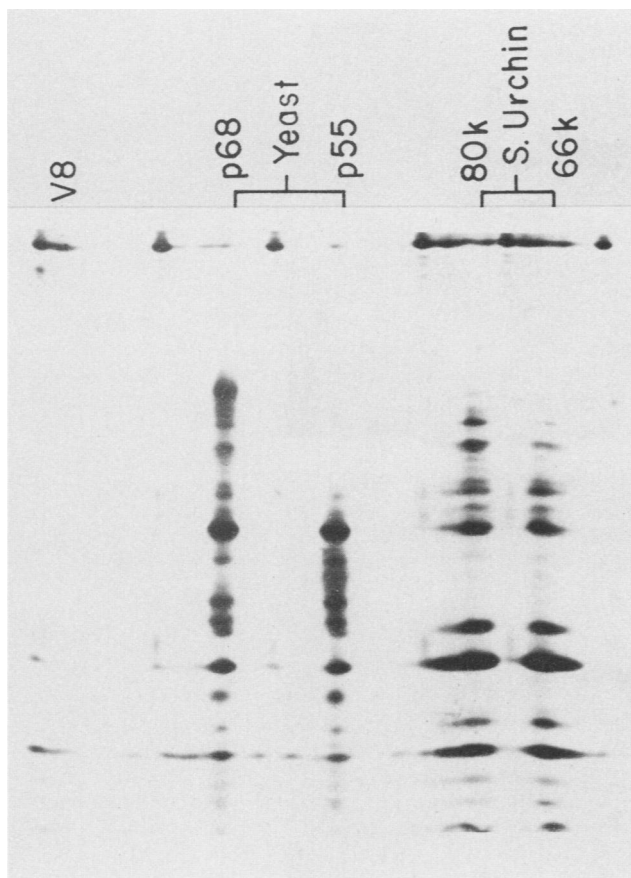


FIG. 3. Yeast and sea urchin PABPs generate similar peptides upon partial proteolysis with V8 protease. Yeast p68 and p55 and sea urchin 80K and 66K PABPs were partially digested with V8 protease by the method of Cleveland et al. (11). The resulting peptides were separated on a 20% SDS-polyacrylamide gel and visualized by silver staining. V8 protease generates the background peptides shown in the first lane of the gel.

tissues, we analyzed the proteins of tube feet and coelomocytes for the presence of the embryonic 66K and 80K PABPs. These tissues were sonicated in SDS sample buffer and run on an SDS-polyacrylamide gel along with homogenate from two-cell embryos. The Western blot of this gel was analyzed for PABP as previously described. Densitometric analysis of the autoradiograph in Fig. 7 showed that both adult and embryonic tissues contain the 66K and 80K PABPs in a 1:1 ratio. An additional 50K protein recognized by anti-PABP in coelomocytes has not yet been characterized.

**PABPs are abundant proteins.** We used purified PABP as a standard to measure the absolute amount of PABP in sea urchin eggs and embryos. The binding of anti-PABP to known amounts of purified PABP and unknown amounts of PABP in homogenates was compared (see Materials and Methods for details). In both eggs and embryos, we found that the 66K PABP is 0.3% of total protein and the 80K PABP is 0.3% of total protein. Therefore, 0.6% of the protein of the cell is PABP.

The ratio of PABP to adenosine residues (see Materials and Methods for calculations) (Table 1) is 1:0.54 in eggs and 1:1.68 in two-cell embryos. Nuclease protection assays have shown that 1 cytoplasmic PABP binds approximately 27 adenosine residues (4, 5). Assuming sea urchin PABPs bind similar numbers of adenosines, then unfertilized eggs have

approximately 50 times more PABP and two-cell embryos have 16 times more PABP than required to bind all the poly(A) in the cell.

**Poly(A)-PABP complexes are stable during gel filtration chromatography but not during density gradient centrifugation.** A 1.5m gel filtration chromatography of PMSs from eggs and embryos (18) separates PABP into two fractions. A small amount of PABP coisolates with poly(A) in the excluded fractions from these columns (peak 1; see below for a description). However, in agreement with our calculations suggesting that large excesses of PABP exist in cells, most of the PABP in eggs and embryos fractionates as free protein (Fig. 8). Since fractionation of PMSs on these columns does not separate poly(A) into nonpolysomal and polysomal fractions, density gradient centrifugation was used to investigate and compare free, nonpolysomal, and polysomal PABP.

We have recently developed a density gradient technique in which samples are centrifuged through linear 15 to 40% sucrose or 15 to 50% glycerol gradients onto EDTA containing cushions underlying the gradients (28). As polysomes migrate into the cushion, the ribosomes dissociate into 40S and 60S ribosomal subunits, greatly reducing further sedimentation of polysomal material. Thus, all the polysomal poly(A)<sup>+</sup> mRNA fractionates as a single peak at the cushion-gradient interface, whereas untranslated poly(A)<sup>+</sup> RNA migrates as a broader peak in the middle of the gradient, and free protein and other small molecules remain in top fractions.

PMSs from two-cell embryos were centrifuged as described above. To ensure that material fractionating in the polysomal peaks was truly polysomal, we also ran a control containing 10 mM EDTA throughout the gradient. Only material released from the polysome peak in the EDTA control is considered polysomal. Nine or 10 fractions were collected per gradient, and proteins from each fraction were Western blotted and probed simultaneously with anti-PU and anti-ribosome antisera (see Fig. 2 for characterization of anti-PU and anti-ribosome antisera). Although anti-PU has a high nonspecific background and reacts with several unidentified poly(U)-binding proteins as well as the PABPs, it was used in this experiment because it contains a higher titer of antibodies to PABPs than anti-PABP does (Fig. 2). The anti-ribosome serum provided an internal control in which the presence of ribosomal material in polysome peaks could be monitored. Figure 9 shows the results of this experiment. Most of the PABP detected by anti-PU cofractionated with free protein in the top three fractions of the gradients. A very small amount of PABP was detected in the polysomal fraction, but since PABPs were also present in the "polysomal" fraction of the EDTA control, these must represent nonspecific aggregates formed during centrifugation. Analysis of both sucrose and glycerol gradients yielded identical results.

We considered two possible interpretations of these results. The first is that the proteins we identified as the sea urchin PABPs are not bound to poly(A) *in vivo*. The second is that density gradient centrifugation strips PABP from poly(A). The second possibility seemed more reasonable because, during gel filtration chromatography of PMSs from eggs and embryos, a small amount of PABP fractionates with poly(A). Furthermore, the observed increase in poly(A) occurring after fertilization correlates with an increased amount of PABP in poly(A)-containing fractions (Fig. 8). This indicated that cofractionation of PABP with poly(A) during column chromatography was not due to nonspecific interactions.

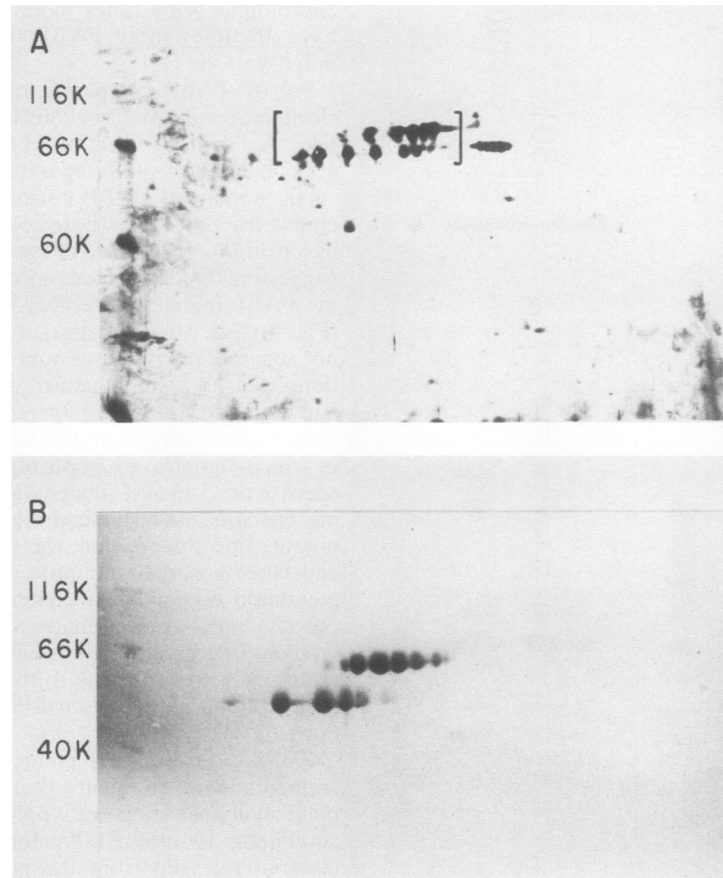


FIG. 4. Yeast and sea urchin PABPs generate similar patterns of spots on two-dimensional polyacrylamide gels. Purified yeast and poly(U)-Sepharose-purified sea urchin PABPs were subjected to two-dimensional PAGE as described in Materials and Methods. Sea urchin PABPs (A; in brackets) were visualized by silver staining; yeast PABPs (B) were visualized by staining in 0.25% Coomassie blue. Yeast p68 and sea urchin 80K PABPs are superimposable in the isoelectric focusing dimension.

To ensure that the PABP cofractionating with poly(A)<sup>+</sup> RNA during gel filtration chromatography was specifically bound to poly(A), we performed several control experiments. A1.5m gel filtration columns fractionate sea urchin PMSs into three UV-absorbing peaks. The excluded fraction, peak 1, contains ribosomes, polysomes, and free mRNPs, while peaks 2 and 3 contain free protein (including RNases and free PABP), tRNAs, and free nucleotides (18). In the first control experiments, peak 1 material from two-cell PMS was rechromatographed on A1.5m columns with or without prior treatment with RNases A and T<sub>1</sub>. RNases A and T<sub>1</sub> hydrolyze all ribonucleotide bonds except for poly(A) tracts. Fractions were then analyzed for RNA, poly(A), and PABP (Fig. 10). Without RNase treatment, both PABP and poly(A) refractionated as peak 1 material (Fig. 10A). In the RNase-treated sample, hydrolysis of peak 1 RNA was incomplete, probably due to protection of the RNA by mRNP proteins. In this experiment, 18% of the 260-nm-light-absorbing material was still detectable after RNase treatment. However, 75% of the poly(A) present before RNase treatment was recovered from column fractions after rechromatography. Sixty-seven percent of the poly(A) detected remained in peak 1, while 33% shifted to included fractions (Fig. 10B, top). Western blot analysis of the PABP in fractions from the same column showed that 65% of the

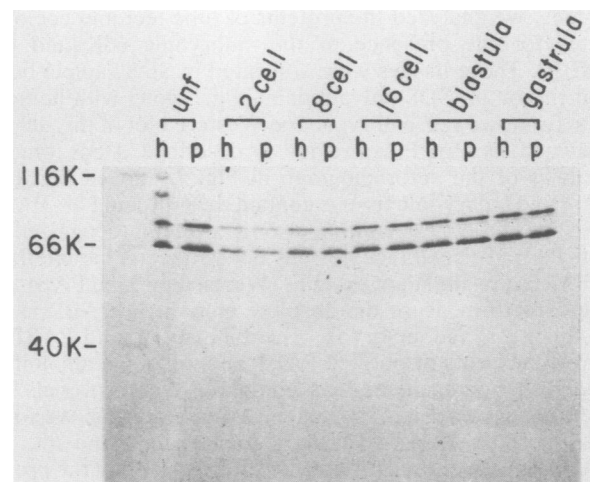


FIG. 5. Ratios of 66K to 80K forms of PABP remain 1:1 throughout early development. Crude homogenates (h) and an equal amount in cell equivalents of their resulting PMSs (p) from eggs (unf) and embryos at various stages of early development were subjected to SDS-PAGE and Western blotting. The blot was probed with anti-PABP and <sup>125</sup>I-protein A. 66K and 80K PABPs were visualized by autoradiography.

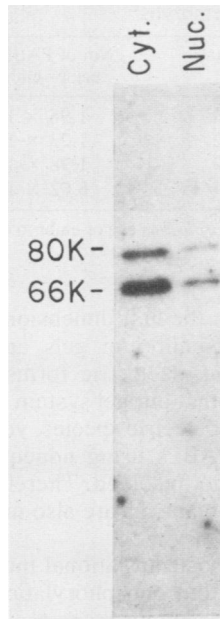


FIG. 6. Both molecular weight forms of PABP are found in the nuclear (Nuc.) and cytoplasmic (Cyt.) fractions isolated from mesenchyme blastulae. Nuclear and cytoplasmic fractions were prepared from mesenchyme blastulae by the method of Hinegardner (22). Equal amounts of protein (as determined by the Schaffner-Weissman assay [42]) from each fraction were separated by SDS-PAGE, blotted to nitrocellulose, and probed with anti-PABP and  $^{125}\text{I}$ -protein A.

PABP remained with the peak 1 poly(A), while 35% shifted to included fractions containing poly(A) (Fig. 10B, bottom). Thus, the fractionation of PABP correlates well with the fractionation of poly(A), indicating that the PABP in peak 1 is specifically complexed to poly(A) and does not associate with other ribonucleotide sequences to any significant degree.

Peak 1 poly(A)-PABP complexes were also shown to be resistant to high salt treatment. Equal amounts of PMS from two-cell embryos were fractionated on A1.5m gel filtration columns in the presence of either 100 or 500 mM NaCl. Densitometric analysis of Western blots probed with anti-PABP showed that high salt has no effect on the fractionation of PABP (data not shown).

These control experiments clearly indicated that peak 1 is tightly and specifically associated with poly(A). Therefore, to test the hypothesis that PABP is removed from poly(A) upon gradient centrifugation, we centrifuged peak 1 fractions from two-cell embryos on gradients identical to those described for Fig. 9. Nine equal fractions were collected per gradient and analyzed for poly(A) and PABP. After centrifugation, peak 1 PABPs fractionated with free protein, unassociated with either untranslated or translated poly(A)<sup>+</sup> RNA (Fig. 11). Identical results were obtained with both sucrose and glycerol gradients. These results demonstrate unequivocally that PABPs are removed from their association with poly(A) by centrifugation and that density gradient centrifugation cannot be used to quantify complexed and uncomplexed PABP.

**PABP binds untranslated and translated poly(A)<sup>+</sup> RNA, although most cellular PABP is free protein.** Since we found that density gradient centrifugation strips PABP from RNA, we used gel filtration chromatography to separate intact poly(A)-PABP complexes from free PABP. Fractions from

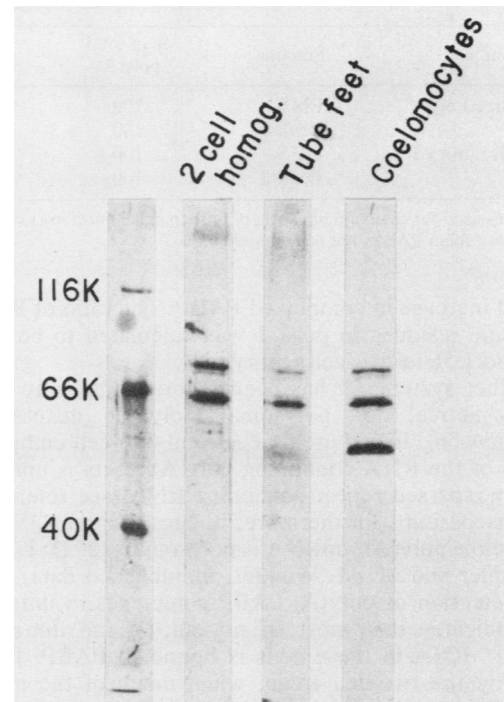


FIG. 7. 66K and 80K forms of PABP are both constituents of adult tissues. Proteins from tube feet and coelomocytes from adult sea urchins and crude homogenate from two-cell embryos were subjected to SDS-PAGE, Western blotted, and probed with anti-PABP and  $^{125}\text{I}$ -protein A. The resulting autoradiograph, shown here, revealed that both molecular weight forms of sea urchin PABP are present in these tissues. A 50K protein in coelomocytes was also detected by anti-PABP.

A1.5m gel filtration columns were analyzed for poly(A) by hybridization to [ $^3\text{H}$ ]poly(U) and for PABP by densitometric analysis of Western blots (Fig. 8; data summarized in Table 1). A total of 100% of the PABP was recovered from these columns. Recovery of poly(A) was 85% for unfertilized egg PMS and 100% for two-cell PMS. The autoradiographs shown in Fig. 8 were overexposed so that the PABP bands in peak 1 would be distinct enough for clear photographic reproduction. Upon overexposure, several low-molecular-weight bands cofractionating with PABP could be detected (some can also be seen in Fig. 2). These have not been identified but are not likely to be degradation products of PABP because protease inhibitors were used during all procedures. In addition, we have never observed these low-molecular-weight bands when alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies are used to detect anti-PABP on Western blots.

Greater than 95% of cellular PABP in both eggs and embryos fractionates as free PABP, indicative of a large excess of uncomplexed cellular PABP (Fig. 8B and C). [ $^3\text{H}$ ]poly(U) hybridization analysis showed that poly(A) is restricted to peak 1 (Fig. 8A). In this experiment, the amount of poly(A) rose from 0.022% of the mass of RNA in eggs to 0.069% in embryos, an increase of approximately threefold. Densitometric analysis of the blots in Fig. 8B and C demonstrated that there is a concurrent increase in the amount of PABP in peak 1. In eggs, 0.62% of total PABP fractionates with poly(A) in peak 1, while 3.04% of the PABP in two-cell embryos fractionates in peak 1. Thus, the threefold increase in poly(A) seen at fertilization occurs concurrently with a



TABLE 1. Quantification of poly(A) and PABP in eggs and embryos<sup>a</sup>

Source	Fraction	% of total poly(A)	No. of A residues (10 <sup>9</sup> ) per egg or embryo	% of total PABP	No. of PABPs per egg or embryo	Ratio of PABP:A
Unfertilized egg	PMS	100	1.07	100.00	1.98 × 10 <sup>9</sup>	1:0.54
	Peak 1	100	1.07	0.62	1.23 × 10 <sup>7</sup>	1:86
Two-cell embryo	PMS	100	3.35	100.00	1.98 × 10 <sup>9</sup>	1:1.69
	Peak 1	100	3.35	3.04	6.02 × 10 <sup>7</sup>	1:56

<sup>a</sup> Values used for calculations: 40 ng of protein per *S. purpuratus* egg or embryo (21); 2.8 ng of RNA per *S. purpuratus* egg or embryo (17);  $M_r$  of AMP is 347.2;  $M_r$ s of sea urchin PABPs are 66,000 and 80,000.

fivefold increase in complexed PABP. The ratio of PABP to adenosine residues in peak 1 was calculated to be 1:86 in eggs and 1:56 in two-cell embryos.

In other systems, it has been reported that only translationally active, i.e., polysomal, poly(A)<sup>+</sup> mRNA binds PABP (54–56). In sea urchin eggs and two-cell embryos, 50 to 65% of the RNA containing poly(A) tracts is untranslatable interspersed repeat-containing RNA (see reference 12 for a discussion). Furthermore, in eggs less than 1% of the translatable poly(A)<sup>+</sup> mRNA is polysomal (12, 23; L. Kelso-Winemiller and M. M. Winkler, unpublished data). Therefore, detection of poly(A)-PABP complexes in unfertilized eggs indicates that most, if not all, of the untranslated poly(A)<sup>+</sup> RNA in these cells is bound to PABP. Furthermore, by the two-cell stage, when much of the maternal poly(A)<sup>+</sup> message has moved into polysomes, a fivefold increase in the amount of PABP complexed to poly(A) is observed. In contrast, there has been an approximate 20-fold increase in the amount of poly(A)<sup>+</sup> mRNA being translated (12, 23). Thus, the increase in bound PABP correlates with the increase in total cellular poly(A) rather than the increase in translationally active poly(A)<sup>+</sup> message. This suggests that all poly(A) tracts, regardless of their translational status, bind PABP. To test this more rigorously, we fractionated unfertilized egg and two-cell embryo PMSs on A150m gel filtration columns (data not shown). The excluded fractions from these columns contain only polysomal material. Monosomes, ribosomal subunits, and free mRNPs are included in the columns. Analysis of these columns shows that PABP fractionates with polysomal material, nonpolysomal material, and free protein. Therefore, it appears that all poly(A)<sup>+</sup> RNA in sea urchins can bind PABP.

## DISCUSSION

The 66K and 80K PABPs of the sea urchin were identified as the homologs of the previously characterized mammalian, avian, and yeast PABPs by (i) their specific interaction with poly(A) in the presence of other polyribonucleotides (39, 41); (ii) their molecular weights (e.g., 1, 5, 8, 16, 19, 24, 26, 30, 46, 56); and (iii) their similarity to yeast PABPs based on two-dimensional PAGE analysis and peptide mapping.

Two-dimensional PAGE resolves both yeast and sea urchin PABPs into several posttranslationally modified forms. Previously published two-dimensional gels of PABPs from yeast, frog, and avian cells have not revealed the presence of posttranslational modifications (1, 56, 59), although there is a report stating that posttranslational modifications were observed in yeast PABPs (39). The difference between our results and those of other workers may lie in the fact that different methods were used to separate PABPs in the first dimension. All previously published two-dimensional analyses of PABPs have employed nonequilibrium pH electrophoresis for separation of proteins in the first dimension (1, 56, 59). On the other hand, we (and in reference 39) employ

isoelectric focusing in the first dimension. Further evidence suggesting that nonequilibrium gels are not optimal for resolving the different isoelectric forms of PABPs comes from the observation that our gel system resolves both yeast PABPs into several isoelectric species, yet when Adam et al. (1) analyzed yeast PABPs using nonequilibrium focusing, multiple forms were not resolved. Therefore, it is likely that the PABPs of other organisms are also multiply posttranslationally modified.

The nature of the posttranslational modifications has not yet been determined, but phosphorylation is a likely candidate. The literature documents many cases of mRNP protein phosphorylation (6, 10, 13, 15, 29, 38, 49). This group includes HD40 from *Artemia salina*, a helix-destabilizing protein with poly(A)-binding activity (49). For the most part, the functional significance of mRNP protein phosphorylation is not known. However, it has recently been shown that a 60K mRNP protein from *Xenopus* oocytes inhibits translation in its phosphorylated state (29). Future work in our laboratory will be directed toward determining the nature of the PABP posttranslational modifications as well as their possible functional roles.

Like the sea urchin, yeast and mammalian cells have two molecular weight forms of PABP. However, yeasts and mammals compartmentalize the larger PABP to the cytoplasm and the smaller PABP to the nucleus (39, 41), while in the sea urchin system, both proteins are located in the cytoplasm and possibly in the nucleus. Since no enrichment of either sea urchin PABP was observed in either the nuclear or cytoplasmic compartment, the relationship of the two sea urchin PABPs to the cytoplasmic and nuclear forms of other organisms remains unclear.

Density gradient centrifugation was shown to strip PABP from poly(A)-PABP complexes. This is a surprising result because gradient centrifugation has been a widely used method for the isolation of poly(A)-PABP complexes (e.g., see references 8, 31, 32, 35, 45, and 54–56). However, it is important to mention that no quantitative studies have been done employing this methodology. There are several possible explanations for the discrepancy in results. (i) Sea urchin PABP binds poly(A) differently from other species. Since actual binding studies have only been performed on rat, mouse, and yeast PABPs (4, 5, 40), this issue cannot be addressed until similar experiments are performed with the PABPs of sea urchins and other species. However, most investigators believe that the ubiquitous occurrence of poly(A) tails and PABPs indicates an evolutionarily conserved function which is, presumably, related to the poly(A)-binding properties of PABP (e.g., see references 1, 5, 7, 8, 19, 32, 39, 40, 54, 56, and 59). (ii) Different centrifugation conditions may allow some poly(A)-PABP complexes to remain intact. Some workers have reported isolating poly(A)-PABP complexes by centrifugation through 5 to 20% or 10 to 30% sucrose (e.g., see references 1, 4, 5, 8, 35,

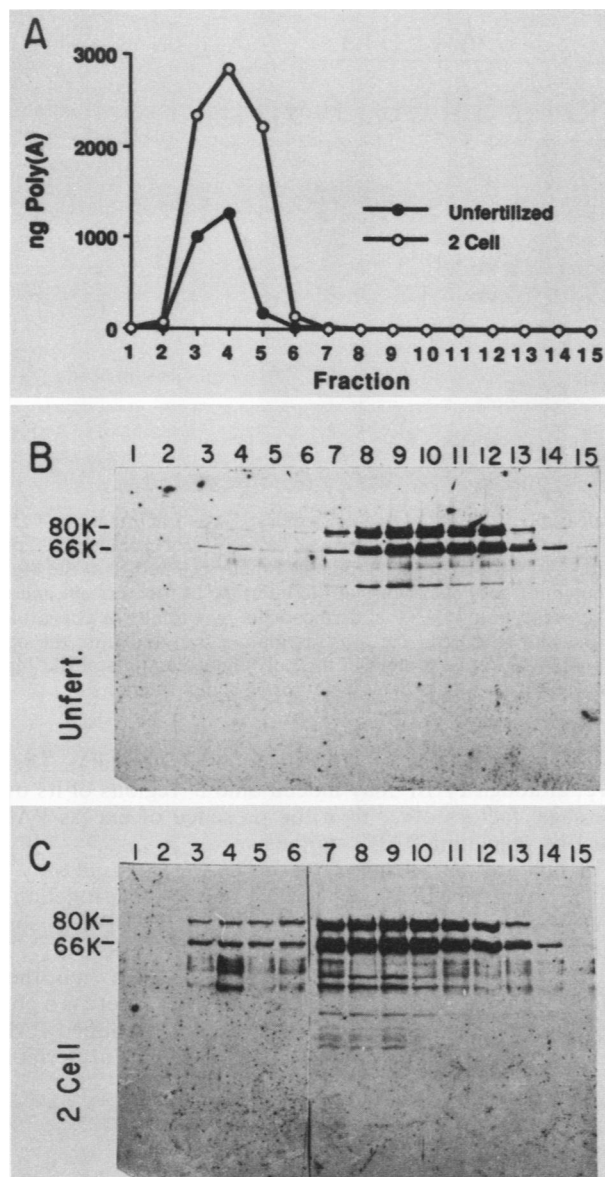


FIG. 8. Increase in poly(A) triggered by fertilization is coupled to an increase in complexed PABP. Equal amounts of protein (as determined by the Schaffner-Weissman assay [42]) from unfertilized egg and two-cell-embryo PMS were fractionated on A1.5m gel filtration columns. RNA was extracted from 1.5-ml samples of each fraction, and the total amount of poly(A) in those fractions was determined by hybridization to [ $^3$ H]poly(U) (A). The fractionation of PABP on the same columns was determined by Western blot analysis. A 10- $\mu$ l sample of each fraction was dissolved in an equal volume of 2 $\times$  SDS sample buffer, boiled for 5 min, and then subjected to SDS-PAGE. The gels were blotted to nitrocellulose, and the blots were probed with anti-PABP and [ $^{125}$ I]-protein A followed by autoradiography. (B) Fractionation of PABPs from unfertilized eggs; (C) fractionation of PABPs from two-cell embryos. The autoradiographs shown in panels B and C were overexposed for better photographic reproduction of the PABP in fractions 3 to 5 (collectively termed peak 1).

45, 54, and 56). Poly(A)-PABP complexes are then isolated from a broad peak near the top of these gradients. It is possible that some poly(A)-PABP complexes remain intact under these conditions. However, since these studies are not

quantitative, it cannot be ascertained whether or not all poly(A)-PABP complexes remain intact during these procedures. (iii) PABP cofractionate with poly(A) on gradients as a nonspecific aggregate. We showed that PABP can be isolated from polysomal fractions of gradients. However, since this material was not released from polysomes by EDTA, it cannot be associated with polysomal poly(A)<sup>+</sup> mRNA. This is a critical observation because, on the basis of gradient experiments, it has previously been reported that PABP associates only with polysomal poly(A)<sup>+</sup> mRNA (54-56). Furthermore, EDTA controls were not performed in these studies, and polysomal PABP was isolated from a polysomal pellet. In light of our findings, data such as these may need to be reevaluated.

It is becoming increasingly clear that gel filtration chromatography is superior to density gradient centrifugation for isolation of intact macromolecular complexes. Eucaryotic initiation factor 2-GDP complexes remain intact during gel filtration chromatography, whereas gradient centrifugation causes disruption of this complex (36). Previous work in our laboratory has demonstrated that intact translationally repressed mRNPs can also be isolated from sea urchin eggs by this method (18). Additionally, the results presented here demonstrate that although PABP is stripped from poly(A) quite readily during gradient centrifugation, poly(A)-PABP complexes are stable during gel filtration chromatography even in the presence of high salt.

Data from *in vitro* experiments have led to the formulation of several hypotheses concerning the possible functions of PABPs. The observation that exogenous poly(A) is a potent inhibitor of translation in reticulocyte lysates led to the idea that poly(A)<sup>+</sup> mRNAs might compete for PABP (25). mRNAs successful at binding PABPs would have a translational advantage over messages depleted in PABP. Evidence supporting this idea comes from experiments in cell-free pea and reticulocyte lysate systems. Exogenous PABP added to PABP-depleted cell extracts was shown to increase the translational efficiency of poly(A)<sup>+</sup> message (47), and reticulocyte lysate "poisoned" with added poly(A) can be rescued by addition of purified PABP (20). Other *in vitro* experiments have shown that added PABP protects poly(A)<sup>+</sup> mRNA from degradation in PABP-depleted extracts (7), suggesting that poly(A)<sup>+</sup> mRNAs compete for PABP to prevent nuclease attack. Sequence analysis of the yeast PABP gene has revealed that PABP mRNA has a 5' untranslated region with a high affinity for PABP (39, 40), suggesting that PABP regulates its own synthesis via a negative feedback loop. This hypothesis meshes nicely with those proposing that poly(A) competes for limited amounts of PABP because it suggests how tightly controlled levels of PABP could be maintained in cells.

One prediction of these various hypotheses is that there should be less PABP in cells than required to bind all the poly(A) present. We showed in sea urchin eggs and embryos that there is many times more PABP than required to bind all the poly(A) in the cell and that levels of PABP remain constant throughout development. Furthermore, it is not likely that this situation is unique to embryonic systems because careful analysis of the literature suggests that the slime mold *Dictyostelium discoideum* and mammalian cells also contain large amounts of PABP, which probably exists free in the cytoplasm (16, 31, 32, 46).

These models also predict that translationally active mRNA should bind more PABP than untranslated poly(A)<sup>+</sup> mRNA. In the past, findings based on gradient data have concurred with that prediction (54, 56). However, it has

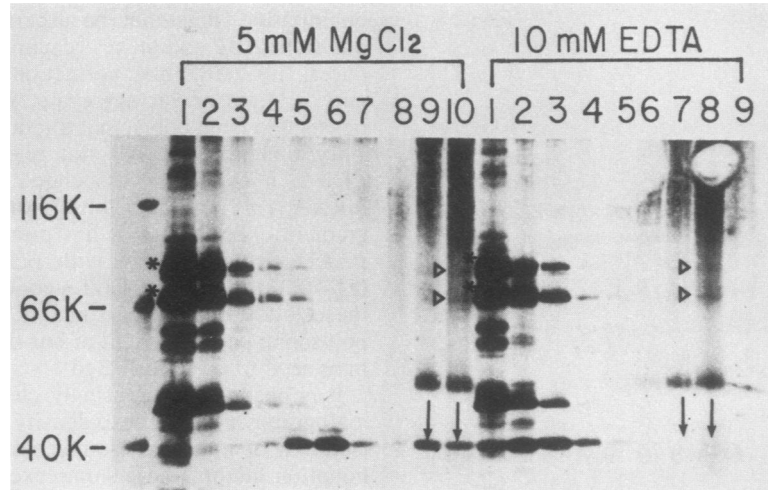


FIG. 9. PABP found in polysomal fractions of gradients is not EDTA releasable. PMS from two-cell embryos was fractionated on 15 to 50% glycerol gradients containing either 5 mM  $Mg^{2+}$  or 10 mM EDTA. Both gradients were underlaid with an 80% glycerol cushion containing 10 mM EDTA. The gradients were fractionated into 9 or 10 fractions. Fraction 1 is at the top of the gradient. Fractions 9 and 10 of the  $Mg^{2+}$ -containing gradient contain the polysome peak. Fraction 8 of the EDTA-containing gradient occurs at the interface of the gradient and the 80% glycerol cushion. The proteins in each fraction were analyzed for the presence of PABP and ribosomes by probing Western blots simultaneously with anti-PU and anti-ribosome antibodies. The asterisks indicate PABPs in the top fractions of the gradients; the open triangles indicate PABPs in the polysomal fractions. The arrows indicate a 40K ribosomal protein in the polysomal fractions of the  $Mg^{2+}$  gradient. In the EDTA control gradient, the arrows indicate that the 40K protein is no longer detectable in polysomal fractions.

already been mentioned that these results may be artifactual. Our data suggest that both untranslated and translated poly(A)<sup>+</sup> message binds PABP. Until experiments in other systems are repeated with appropriate controls and/or more appropriate methods, we conclude that all poly(A) tracts can bind PABP.

The idea that PABP synthesis is regulated autogenously predicts that (i) as PABP levels rise, PABP synthesis slows, and that (ii) excess PABP should never appear in normal cells. Not only have we shown that PABP is in vast excess in sea urchins, but we also find that PABP is synthesized in the egg and throughout early development (M. T. Peeler, J.

Drawbridge, and M. M. Winkler, unpublished data). Therefore, although PABP may indeed bind 5' regions of its own message, factors other than the presence of excess PABP must be regulating PABP synthesis.

In light of our results, we believe it is necessary to reinterpret various data concerning the possible function of PABPs. In vitro experiments show that PABP has some effect on translation and stability of poly(A)<sup>+</sup> mRNAs (7, 20, 25, 47). However, our data demonstrate that in vivo, these effects would be mitigated due to the fact that there is a great excess of PABP in cells. We have recently identified PABP as a component of sea urchin cytoskeletons. Furthermore,

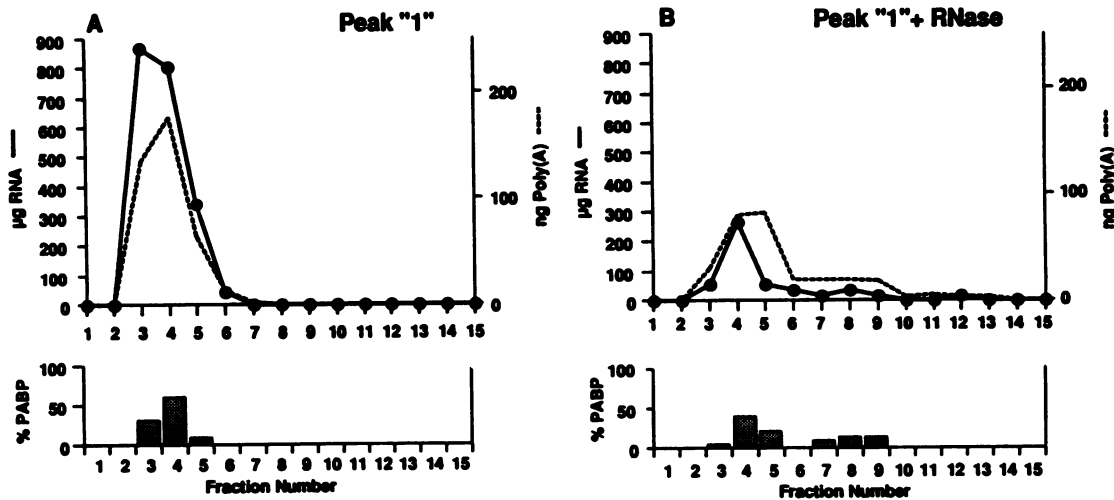


FIG. 10. Poly(A)-PABP complexes are stable during gel filtration chromatography. The association of peak 1 PABP with poly(A) was investigated by rechromatography of peak 1 from two-cell embryos with and without prior treatment with 10  $\mu$ g of RNase A per ml and 1  $\mu$ g of RNase T<sub>1</sub> per ml. Percentage of total PABP in each fraction was determined by densitometric analysis of Western blots probed with anti-PABP (bar graphs). RNA was extracted from each fraction, RNA content was determined by OD<sub>260</sub> readings (solid lines), and poly(A) content was determined by hybridization to [<sup>3</sup>H]poly(U) (dashed lines). (A) Rechromatography of untreated peak 1; (B) rechromatography of peak 1 treated with RNases A and T<sub>1</sub>.

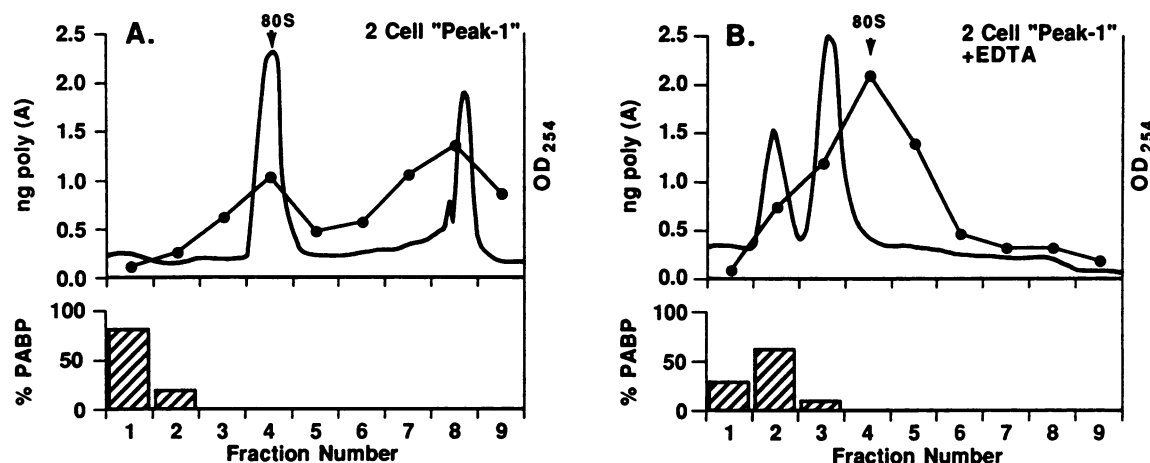


FIG. 11. Poly(A)-PABP complexes are disrupted by density gradient centrifugation. Peak 1 fractions from two-cell embryos were fractionated on  $Mg^{2+}$ -containing(A) and EDTA-containing (B) gradients identical to those described in the legend to Fig. 9. Nine equal fractions were collected per gradient and analyzed for poly(A) by hybridization to [ $^3H$ ]poly(U) and for PABP by densitometric analysis of Western blots probed with anti-PABP. The upper panel of each graph shows the OD<sub>254</sub> tracing of the gradient and the poly(A) content of each fraction. The lower panels show the fraction of total detectable PABP (expressed as percent) in each fraction of the gradient. The direction of sedimentation is from left to right.

fertilization of the egg causes a mobilization of poly(A)<sup>+</sup> RNA as well as PABP to this structure (Drawbridge and Winkler, unpublished data). Work is now in progress to determine how the function of PABP might be involved in the localization of poly(A)<sup>+</sup> RNAs to the cytoskeleton.

#### ACKNOWLEDGMENTS

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