Purification and characterization of recombinant *Xenopus* poly(A)⁺-binding protein expressed in a baculovirus system

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The poly(A)⁺-binding protein (PABP) is a highly conserved protein that binds to the poly(A)⁺ tail of mRNAs. PABP has been shown to regulate message stability and translational efficiency, yet the mechanisms remain unknown. To facilitate further dissection of the functions of this protein, we have expressed and purified *Xenopus* PABP using a baculovirus expression system. At 48 h after infection of insect *Spodoptera frugiperda* (Sf9) cells with recombinant virus, approx. 3% of cell protein was PABP. Purification of PABP was achieved by affinity chromatography on poly(A)⁺-Sepharese. The purified protein was indistinguishable from *Xenopus* PABP with respect to its immunoreactivity and electrophoretic mobility. Furthermore, the recombinant PABP was expressed and purified as a functional protein as indicated by its ability to bind to poly(A)⁺-Sepharese and its ability to enhance the translation of adenylated messages *in vitro*. By comparing protein extracts from various developmental stages of *Xenopus* embryos with known amounts of purified PABP, we determined the amount of PABP per embryo. This analysis suggested that there is less than one PABP molecule available per PABP-binding site at early stages of development, and only a slight excess of PABP at later stages.

INTRODUCTION

RNAs are normally found in cells as complexes with RNA-binding proteins. One of these highly conserved RNA-binding proteins is the poly(A)⁺-binding protein (PABP) that binds to the poly(A)⁺ tail of polyribosomal and non-polyribosomal mRNA. Studies *in vitro* (Jacobson & Favreau, 1983; Grossi de Sa *et al.*, 1988) and *in vivo* (Sachs & Davis, 1989) have implicated PABP in promoting the efficiency of the formation of 80 S initiation complexes, and thus facilitating mRNA translation. Interestingly, it has been shown by Gallie (1991) that the cap and the poly(A)⁺ tail work synergistically to regulate mRNA translational efficiency, possibly mediated by PABP. PABP cDNAs have been isolated from yeast (Sachs *et al.*, 1986), human (Grange *et al.*, 1987), *Xenopus* (Zelus *et al.*, 1989) and *Drosophila* (Lefrere *et al.*, 1990). Each of the proteins predicted by sequence analysis, which range in size from 69 to 78 kDa, contains four highly conserved RNA-binding domains, common to a family of other eukaryotic RNA-binding proteins (Bandzilius *et al.*, 1989).

Numerous studies have suggested that PABP concentrations are regulated. *In vitro*, serum-stimulated cells exhibit an increase in PABP synthesis without an increase in the rate of PABP transcription (Thomas & Thomas, 1986). Also, PABP mRNA contains an adenine-rich region in the 5' untranslated region (Sachs *et al.*, 1986; Grange *et al.*, 1987; Zelus *et al.*, 1989), and it has been proposed that PABP may regulate translation of its own message by binding to this region. Indeed, yeast PABP has the same affinity for this sequence *in vitro* as it does for poly(A)⁺ (Sachs *et al.*, 1986). An important outcome of studying the regulation of PABP concentrations may be the determination of which messages are translated more efficiently. It has been shown *in vitro* that some messages with longer poly(A)⁺ tails (and presumably more bound PABP) are translated more efficiently, though not all data support this correlation (reviewed by Munroe & Jacobson, 1990).

The PABP has also been shown to play a role in regulating message stability, as some adenylated messages are degraded faster in the absence of PABP *in vitro* (Bernstein *et al.*, 1989). It

has been proposed that a function of PABP is to maintain message stability by protecting the 3' region of the message from nucleolytic attack and hence from degradation. In contrast, results obtained *in vivo* show that depletion of PABP results in the lengthening of poly(A)⁺ tails thereby implying that PABP is necessary for poly(A)⁺ tail shortening (Sachs & Davis, 1989). Whatever the role of PABP may be in regulating message stability, these two lines of evidence suggest the potential importance of cellular regulation of PABP concentration. Dissection of the functions and mechanisms of action of vertebrate PABP would be facilitated by the availability of recombinant purified PABP, which has not heretofore been reported.

In the present study, we used the baculovirus expression system to produce recombinant *Xenopus* PABP. First, we have purified this protein and examined its binding to poly(A)⁺ and its ability to enhance translation of adenylated RNAs *in vitro*. We report that the recombinant PABP behaves similarly to PABPs from other species. The availability of this purified recombinant protein will facilitate future studies of the functions and mechanisms of action of PABP. Second, we have previously demonstrated a correlation between an increase in PABP during development and an increase in total poly(A)⁺ during *Xenopus* embryogenesis (Zelus *et al.*, 1989). In the present report, we have used the purified PABP to quantify the number of PABP molecules at different stages of *Xenopus* development, and compared this with the number of poly(A)⁺-binding sites in embryos.

MATERIALS AND METHODS

Materials

Molecular biology enzymes were obtained from New England Biolabs, Beverly, MA, U.S.A. Poly(A)⁺-Sepharese 4B was purchased from Pharmacia. The bicinchoninic acid (BCA) protein assay kit was supplied by Pierce Chemical Company. The ECL Western blotting detection kit was purchased from Amersham and the goat anti-(rabbit horseradish peroxidase) conjugate was from Bio-Rad.

Abbreviations used: PBS, phosphate-buffered saline; PABP, poly(A)⁺-binding protein; Sf9, *Spodoptera frugiperda*; AcNPV, *Autographa californica* nuclear polyhedrosis virus; AcNPV-BABP, PABP-expressing AcNPV.

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Cell culture and virus

Spodoptera frugiperda (Sf9) cells (Invitrogen, San Diego, CA, U.S.A.) were grown to 27 °C in TNM-FH medium.

Grace's insect cell culture medium (Gibco) supplemented with yeastolate (Difco), lactalbumin hydrosylate (Difco), 10% fetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.), 100 units of penicillin/ml and 100 µg of streptomycin/ml as described in Summers & Smith (1987). The cells were maintained as monolayer cultures. Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) was a gift from Dr. Max Summers (Texas A & M University, Texas Station, TX, U.S.A.).

Construction of transfer vector and production of recombinant baculovirus

The transfer vector pVL-DR, provided by Dr. David Russell (Sloan Kettering Institute), is a derivatized version of the original pVL941 vector (Luckow & Summers, 1989) with a polylinker cloned into the unique *Bam*HI site. Transfer vector pVL-PABP consisting of the full-length *Xenopus* PABP was constructed as follows. The 3' end of a 2.0 kb fragment encoding the *Xenopus* PABP was isolated from an expression vector using *Hind*III and the overhang was blunted with Klenow fragment. The expression vector was then digested with *Nco*I, and the PABP cDNA isolated and ligated into the unique *Nco*I and *Sma*I sites downstream of the polyhedrin promoter of the pVL-DR vector. Insertion and orientation were determined by *Hind*III and *Bam*HI digestions respectively. Correct insertion was verified by using oligonucleotide primers to pVL-DR and PABP cDNA to sequence across the fragment-vector junctions.

Recombinant baculovirus was produced by cotransfecting Sf9 cells (4×10^6 cells/60 mm culture dish) with total AcNPV DNA (4 µg) and pVL-PABP (2 µg) using standard calcium phosphate coprecipitation methods as described previously (Summers & Smith, 1987). The resulting culture supernatants were harvested 4–6 days later. Sf9 cells in a 96-well plate (2×10^5 cells/well) were infected with 10-fold serial dilutions of the culture supernatant. At 3 days after infection, cells were lysed with 0.2 ml of 0.5 M-NaOH, neutralized with 20 µl of 10 M-ammonium acetate, and dot-blotted on to a nitrocellulose filter. Filters were hybridized to a 32 P-labelled PABP cDNA probe, and four positive wells representing recombinant PABP baculovirus strains were selected for plaque purification (Summers & Smith, 1987). For the first round of plaque purification, 10-fold serial dilutions of recombinant virus stocks were used to infect Sf9 cells (2×10^6 cells/60 mm dish). These cells were overlaid with 1.5% low-melting-point agarose containing TNM-FH medium. At 4–6 days after infection, recombinant occlusion-negative plaques were identified by visual screening with a phase-contrast microscope. This process was repeated for the second round of visual screening. After a third round of purification, recombinant plaques were suspended in 0.5 ml of TNM-FH medium. This mixture was then analysed by dot-blot hybridization as described above with two different labelled probes: PABP cDNA to verify that these viral stocks contain the PABP insert, and an oligonucleotide specific to the wild-type polyhedrin gene to confirm that these viral stocks did not contain contaminating wild-type virus. Resulting recombinant PABP viral stocks were designated AcNPV-PABP.

Time course of PABP expression

Sf9 cells (7×10^6 cells/100 mm dish) were infected with AcNPV-PABP for 1 h, after which time the inoculum was replaced with fresh medium. Sf9 cells were harvested at specific time-intervals after the infection, and washed in ice-cold PBS (140 mM-NaCl, 2.7 mM-KCl, 8.13 mM- Na_2HPO_4 , and 1.47 mM-

KH_2PO_4) before being lysed in electrophoresis sample buffer. The samples were screened for PABP by Western blotting with 1:1000-fold dilution of anti-PABP serum (Zelus *et al.*, 1989) followed by 1:3000-fold dilution of horseradish peroxidase coupled to goat anti-rabbit IgG. Immunoreactive proteins were visualized with the ECL Western-blotting detection system.

Purification of PABP

Sf9 cells (1.2×10^6 cells/ml) were infected with AcNPV-PABP with a multiplicity of infection of eight plaque-forming units per cell. At 1 h after infection, the medium was replaced with fresh TNM-FH medium, and the cells were harvested 48 h after infection. Cell pellets were washed once with ice-cold hypotonic homogenization buffer (10 mM-Tris/HCl, pH 7.5, 10 mM-NaCl, 1.5 mM-MgCl₂, 5 µg of leupeptin/ml, 200 mM-phenylmethanesulphonyl fluoride, and 1.2 µg of pepstatin/ml), resuspended (6×10^6 cells/ml) in the same buffer, and incubated on ice for 40 min. Cell contents were released by 30 strokes with a Dounce homogenizer, adjusted to 5 mM-EDTA/100 mM-NaCl/15 mM-2-mercaptoethanol. After 10 min on ice, the mixture was centrifuged at 7200 g for 5 min. All subsequent work was conducted at 4 °C. Purification of PABP was essentially as described (Fukami & Itano, 1976; Sachs & Kornberg, 1990) with the following modifications. Poly(A)⁺-Sephacrose was activated according to the manufacturer's protocol, and equilibrated in buffer A (60 mM-KCl, 40 mM-NaCl, 0.1 mM-spermidine, 15 mM-2-mercaptoethanol, 15 mM-Tris/HCl, pH 7.5, 10 mM-EDTA and 200 µM-phenylmethanesulphonyl fluoride). The supernatant fraction was batch-bound to equilibrated poly(A)⁺-Sephacrose by gently rocking for 2 h at 4 °C. The resin was poured into a plastic column, and was washed successively with 12 ml of buffer A, 12 ml of buffer A containing 2.5 µg of poly(C)⁺ RNA/ml and 50 µg of tRNA/ml (added to block non-specific polynucleotide-binding proteins), 12 ml of buffer A containing 1 M-NaCl, and finally 4 ml of buffer A containing 1 M-guanidinium chloride. Bound PABP was eluted with 4 ml of buffer A containing 1.5 M-guanidinium chloride, then renatured and concentrated by 14 h of dialysis in a Prodon membrane (Bio-Molecular Dynamics, Beaverton, OR, U.S.A.) against buffer B (100 mM-KCl, 10 mM-Hepes, 10 mM-NaCl, 1 mM-MgCl₂ and 0.1 mM-EDTA, pH 7.2), and PABP was stored at -80 °C.

PABP polynucleotide-binding specificity

Purified PABP (5 µg) was incubated at 4 °C with 25 µl of packed poly(A)⁺-Sephacrose in the presence of 10 µg of each competitor polynucleotide in 200 µl (total) of buffer A described above. After 1 h, the poly(A)⁺-Sephacrose was washed with 2×200 µl of buffer A, then boiled in 50 µl of SDS/PAGE buffer. The entire mixture was analysed by SDS/10%-PAGE (Zelus *et al.*, 1990) and proteins were either stained with Coomassie Brilliant Blue or transferred to nitrocellulose and probed with anti-PABP serum.

Transcription and translation of messages *in vitro*

PABP RNA consisted of the PABP coding region, flanked by the 5' and 3' β -globin untranslated region, and a 30-nucleotide poly(A)⁺ tail. Vimentin RNA (Christian *et al.*, 1990) included the full vimentin coding and non-coding region with a 60-nucleotide poly(A)⁺ tail. mRNA was synthesized from cDNA constructs by transcription *in vitro* (Krieg & Melton, 1984). Equivalent amounts of mRNA in 1 µl were prebound for 15 min to 145 pg of purified PABP in 1 µl, or mixed with 1 µl of buffer B, followed by addition of 18 µl of rabbit reticulocyte lysate. Synthetic mRNA was then translated in a message-dependent rabbit reticulocyte lysate in the presence of [35 S]methionine for 20 min at 30 °C under conditions where mRNA was limiting and

translation rates were linear with respect to time. Products of the translation *in vitro* were analysed by SDS/PAGE followed by enhancement in the fluorographic reagent Amplify (Amersham) and autoradiography.

Quantification of PABP in *Xenopus* embryos

Non-yolk proteins of *Xenopus* embryos were isolated by homogenization of tissue in 20 μ l per embryo of 15 mM-Tris/HCl (pH 7.5)/1 mM-phenylmethanesulphonyl fluoride. An equal volume of 1,1,2-trichlorotrifluoroethane was added, and after being vortexed, the suspension was centrifuged at 12000 *g* for 10 min. Gel electrophoresis buffer was added to the supernatant, followed by boiling for 5 min. Two-embryo, three-embryo and four-embryo equivalents of protein extracts from various stages of *Xenopus* development were separated by SDS/10%-PAGE and analysed by Western-blot analysis as described above. Between 20 and 500 pg of purified recombinant PABP were co-electrophoresed with the embryo samples. To ensure accuracy of quantification, standard curves were derived from two separate preparations of recombinant PABP, yielding equivalent curves. Moreover, the data in Table 1 reflect the averages of five separate sets of protein from *Xenopus* embryos with negligible differences being noted between each set. Protein bands from various developmental stages were quantified by laser densitometry scanning. The values that are obtained from this type of analysis are expressed as areas under the curve for each signal which is directly proportional to the density of the signal. These values were plotted against the known amounts of purified protein, and an equation describing the linear relationship between the two ordinates was calculated. The amounts of *Xenopus* PABP at each stage of development were then calculated by solving for the *Y* value, using the area under the curve from each signal on the immunoblot for the *X* value. It is important to note that the standard curves of purified PABP yielded a linear standard curve, and that all samples of embryos analysed yielded signals within the linear range of the standard curves.

RESULTS

Construction of recombinant baculovirus

Transfer vector pVL-PABP (Fig. 1) was constructed by inserting a 2.0 kb cDNA fragment encoding the full-length *Xenopus* PABP (Zelus *et al.*, 1989) into the *Nco*I and *Sma*I sites of the vector pVL-DR as described in the Materials and methods section. Sf9 cells were co-transfected with wild-type AcNPV DNA and pVL-PABP, and the supernatant was harvested after 4 days. The recombinant virus was purified by three rounds of plaque purification (Summers & Smith, 1987). Amplification of purified AcNPV-PABP stocks yielded titres between 1×10^8 and 3×10^8 plaque-forming units/ml as determined by two separate measures: end-point dilution and plaque assay with the aid of Neutral Red dye to visualize plaques (Summers & Smith, 1987). The identity of the viral stocks was confirmed by dot-blot hybridization with a ³²P-labelled PABP cDNA probe (results not shown). No occlusion bodies characteristic of wild-type plaques were observed by light microscopy, and analysis of viral DNA by dot-blot hybridization did not result in a hybridization signal when stocks were probed with an oligonucleotide specific to the polyhedrin gene (results not shown). Together these data demonstrate that the purified AcNPV-PABP recombinants contain the *Xenopus* PABP cDNA insert and are free from wild-type AcNPV baculovirus.

PABP expression in Sf9 cells

To determine whether infection of Sf9 cells with this purified recombinant baculovirus resulted in the expression of *Xenopus*

PABP, Sf9 cells were infected with either wild-type AcNPV (Fig. 2a) or AcNPV-PABP virus (Fig. 2b), and cell extracts were prepared at 12, 24, 36, 48, 60 and 72 h after infection. These cell extracts were analysed by Western-blot analysis with anti-PABP serum. Expression of PABP (Fig. 2b) was apparent at 24 h after infection with levels of expression increasing up to 72 h after infection. This immunoreactive protein is not present in cell extracts from wild-type AcNPV-infected cells (Fig. 2a). The identification of *Xenopus* PABP in these experiments was confirmed by co-electrophoresis of *Xenopus* samples. Two other proteins of unknown identity cross-react with the anti-PABP serum in the non-infected, wild-type AcNPV-infected and AcNPV-PABP-infected Sf9 cells. These data show that the production of *Xenopus* PABP in infected Sf9 cells is dependent on infection with recombinant virus.

Purification of PABP-AcNPV

PABP-infected Sf9 cells were homogenized and centrifuged, and PABP in the supernatant was purified to homogeneity by poly(A)⁺-Sephacrose affinity chromatography (see the Materials and methods section). Results of a typical purification are shown in Fig. 3. Coomassie Brilliant Blue staining (Fig. 3a) and Western-

Fig. 1. Transfer vector pVL-PABP

The cDNA of *Xenopus* PABP (hatched) was inserted into the baculovirus vector pVL-DR at the *Nco*I and *Pst*I sites following the polyhedrin promoter. This vector [redrawn from Vissavajhala & Ross (1990)] uses the start site of the inserted gene, resulting in the expression of an unfused protein.

Fig. 2. Time course of PABP expression

Extracts from cells (about 3×10^5 /lane) infected with AcNPV (a) or AcNPV-PABP (b) were analysed by Western-blot analysis (SDS/10% polyacrylamide gel) with anti-PABP serum. The arrow in (b) denotes specific detection of *Xenopus* PABP. NI, non-infected.

Fig. 3. Purification of PABP to homogeneity by poly(A)⁺-Sepharose affinity chromatography

To isolate PABP, Sf9 cells were infected with AcNPV-PABP with a multiplicity of infection of eight and the cells were harvested 48 h after infection. (a) Coomassie Brilliant Blue-stained SDS/10%-polyacrylamide gel of the purified protein; (b) immunoreactivity of the PABP via Western-blot analysis of these same samples. Lane 1, starting material (8 μ g of protein) loaded on to the poly(A)⁺-Sepharose column; lane 2, after elution with 1 M-guanidinium chloride (7 μ g of protein); lane 3, after elution with 1.5 M-guanidinium chloride (3 μ g of protein).

Fig. 4. Analysis of polynucleotide-binding specificity of purified PABP

Coomassie Brilliant Blue-stained SDS/10%-polyacrylamide gel of: lane 1, 5 μ g of purified PABP bound to poly(A)⁺-Sepharose, lane 2, purified PABP bound to poly(A)⁺-Sepharose in the presence of 10 μ g of poly(A)⁺, and lane 3, purified PABP bound to poly(A)⁺-Sepharose in the presence of 10 μ g of poly(C)⁺.

blot analysis (Fig. 3b) of the eluate of the poly(A)⁺-Sepharose column clearly illustrate the enrichment of the approx. 70 kDa PABP. A protein of approximate molecular mass 50 kDa is eluted from the column with 1 M-guanidinium chloride in addition to the PABP (Fig. 3a, lane 2). This lower band is not immunoreactive with PABP antiserum as demonstrated by Western-blot analysis (Fig. 3b, lane 2). Subsequent elution of the column with 1.5 M-guanidinium chloride yields PABP with negligible contamination with the 50 kDa protein or other proteins, and is therefore considered to be purified PABP (Fig. 3a, lane 3, and silver-stained gels, not shown). This purified PABP is strongly immunoreactive with anti-PABP serum (Fig. 3b, lane 3). Significantly, the recombinant PABP from the 1.5 M-guanidinium chloride elution migrated with the same mobility as the 70 kDa *Xenopus* PABP detected in *Xenopus* embryos (results not shown), confirming that the complete coding sequence for *Xenopus* PABP was contained within the recombinant baculovirus. Together, these data show that the expressed PABP has been purified from infected Sf9 cells by poly(A)⁺-Sepharose affinity chromatography.

To determine the yields for three of the purified PABP preparations, the amount of PABP at various stages of the purification was determined. Briefly, known amounts of purified recombinant PABP were analysed by Western-blot analysis and probed with anti-PABP serum to generate a standard curve which was linear in the range of 20–400 pg of PABP. The amount of PABP at the various stages of purification was then determined

by comparison with the standard curve. Between 2 and 4% of total protein of the infected Sf9 cells is recombinant PABP. After Dounce homogenization and low-speed centrifugation, approx. 60% of the PABP was detected in the supernatant fraction and approx. 30% of the PABP was detected in the pellet. Between 50 and 60% of the PABP in the supernatant can be recovered from a poly(A)⁺-Sepharose column. We found that more than 3.5 mg of infected Sf9 cell supernatant material (equivalent to approx. 60 μ g of PABP) will saturate 1 ml of poly(A)⁺-Sepharose. These data indicate that PABP can be purified to homogeneity by the described methods.

Polynucleotide-binding specificity of purified PABP

To determine the specificity of purified PABP for binding poly(A)⁺ or poly(C)⁺, we incubated purified PABP with poly(A)⁺-Sepharose in the presence of either competitor polynucleotide. PABP that bound to the poly(A)⁺-Sepharose was analysed by SDS/PAGE and Coomassie Brilliant Blue staining. The PABP bound to poly(A)⁺-Sepharose in the absence of any competitor polynucleotide is shown in Fig. 4, lane 1. Addition of competitor poly(A)⁺ resulted in significantly less PABP-binding to the poly(A)⁺-Sepharose (Fig. 4, lane 2). However, the addition of competitor poly(C)⁺ did not appreciably decrease the amount of PABP bound to poly(A)⁺-Sepharose (Fig. 4, lane 3). When the relative amount of purified PABP bound to poly(A)⁺-Sepharose in the presence or absence of the competitor polynucleotides was analysed by Western-blot analysis with anti-PABP serum and the signals were compared by laser densitometry, between 40 and 50% of the original signal was competed for by free poly(A)⁺, whereas free poly(C)⁺ decreased the binding of PABP to poly(A)⁺-Sepharose by only 10% (multiple experiments, quantification not shown). Together these data demonstrate that purified PABP binds to poly(A)⁺ RNA more effectively than to poly(C)⁺ RNA. These data are consistent with previous studies on the specificity of PABP (Nietfeld *et al.*, 1990; Burd *et al.*, 1991).

Enhancement of the translation *in vitro* of adenylated RNAs by PABP

To characterize the activity of this purified PABP further, the effects of purified PABP were determined on the translation *in vitro* of two adenylated RNAs. PABP and vimentin synthetic mRNAs were synthesized by transcription *in vitro*. mRNAs without PABP or mRNAs prebound with PABP for 15 min were translated *in vitro* in a message-dependent rabbit reticulocyte lysate, and separated by SDS/PAGE. The ³⁵S-labelled translation products were visualized by autoradiography and quantified by densitometry. In several independent experiments with different preparations of purified recombinant PABP, the translation of the PABP mRNA was enhanced 1.5-fold (Fig. 5, lane 1 compared with lane 3) and translation of vimentin mRNA was enhanced 3-fold (Fig. 5, lane 2 compared with lane 4) in the presence of purified PABP. To test whether the RNAs were degraded during the translation or binding to PABP, RNAs extracted at the end of the translation were analysed by Northern-blot analysis, and were found to be intact and of comparable abundance between samples (results not shown). These data show that purified PABP enhances the translation of two adenylated messages *in vitro*.

Quantification of PABP in *Xenopus* developmental stages

To quantify the concentration of PABP during *Xenopus* development, we analysed known amounts of purified recombinant PABP by Western-blot analysis with anti-PABP serum. The standard curves generated from these data were then used to determine the amount of PABP in protein preparations

Fig. 5. Enhancement of the translation *in vitro* of two adenylated RNAs by PABP

Fluorogram of [³⁵S]methionine-labelled translation products analysed by SDS/PAGE. Synthetic adenylated PABP message (lanes 1 and 3) and synthetic adenylated vimentin message (lanes 2 and 4) were translated in a message-dependent rabbit reticulocyte lysate in the presence (+) or absence (–) of 145 pg of purified recombinant PABP.

Fig. 6. Quantification of PABP in *Xenopus* embryos

(a) Representative Western blot of two-embryo equivalents of total protein from *Xenopus* developmental stages probed with anti-PABP serum: lane 1, cleavage; lane 2, blastula; lane 3, gastrula; lane 4, neurula; lane 5, early tadpole. (b) Representative Western blot of increasing amounts of purified PABP probed with anti-PABP serum: lane 1, 50 pg; lane 2, 100 pg; lane 3, 150 pg; lane 4, 200 pg; lane 5, 250 pg; lane 6, 300 pg.

Table 1. Quantification of PABP at *Xenopus* developmental stages

Amounts of PABP at various *Xenopus* embryo stages were determined as described in the Materials and methods section. The number of PABP molecules/embryo was determined using 68.5 kDa as the molecular mass of PABP. The number of PABP binding sites/embryo was estimated on the basis of the quantity of poly(A)⁺ at each stage of development (Sagata *et al.*, 1980) and 25 adenine residues/PABP binding site (Sachs *et al.*, 1987). ND, Not detectable, below limit of sensitivity of 5 pg.

Embryo stage	PABP (pg/embryo)	10 ^{–10} × PABP molecules/embryo	10 ^{–10} × PABP binding sites/embryo	PABP molecules/binding site
Cleavage	ND	–	9	–
Blastula	7	6	16	< 1
Gastrula	35	31	13	2
Neurula	70	61	16	4
Early tadpole	156	140	25	6
Late tadpole	227	200	54	4

from embryos at different stages of *Xenopus* development. A representative example of a Western blot used for laser densitometry scanning is shown in Fig. 6. Immunoreactive bands of known amounts of purified PABP ranging from 50 to 300 pg (Fig. 6b) and of unknown amounts of *Xenopus* PABP in two-embryo equivalents from various stages of *Xenopus* development (Fig. 6a) were scanned using laser densitometry. In Fig. 6, *Xenopus* PABP was not detected at the cleavage (Fig. 6a, lane 1) or blastula (Fig. 6a, lane 2) stages of development. However, in other experiments with three-embryo and four-embryo equivalents of protein, an immunoreactive band was evident at the

blastula stage, and the amount was determined as described above.

We found that PABP increases during development from less than 5 pg/embryo (Table 1, column 2) at the cleavage stages to more than 200 pg/embryo at the late tadpole stage of development. These data confirm an earlier report that PABP concentrations increase during development (Zelus *et al.*, 1989). Together with determined values for the steady-state concentrations of poly(A)⁺ present at different stages of *Xenopus* development [Sagata *et al.* (1980) recalculated for Table 1, column 4] and the estimation of one PABP molecule binding to every 25 adenine residues (Sachs & Kornberg, 1986), the number of PABP molecules per PABP binding site were estimated (Table 1, column 5).

DISCUSSION

Previous studies have strongly suggested an important role for PABP in regulating mRNA stability (Bernstein *et al.*, 1989) and the initiation of translation (Grossi de Sa *et al.*, 1988; Sachs & Davis, 1989). However, the mechanisms by which PABPs influence these processes have not yet been elucidated. As a prelude to studying the potential involvement and mechanisms of the PABP in modulating translational efficiency and mRNA stability in developing *Xenopus* embryos, we have expressed and purified recombinant *Xenopus* PABP using the eukaryotic baculovirus expression system. After characterization of the purified PABP, it was employed as a standard to quantify the levels of expression of PABP during development of *Xenopus*.

Xenopus PABP was purified from baculovirus-infected cells by poly(A)⁺-Sephacryl affinity chromatography. The levels of expression of PABP in these cells was between 2 and 4 % of total protein, significantly higher than any available tissue source and similar to the expression of other baculovirus-expressed proteins (Luckow & Summers, 1989). Several lines of evidence support the idea that this purified PABP is full-length and functional. The use of poly(A)⁺-Sephacryl affinity chromatography as the final step in purification ensures the isolation of only proteins that bind poly(A)⁺. The co-migration by SDS/PAGE of the purified PABP with endogenous PABP from *Xenopus* embryos, and their detection by anti-PABP serum, suggests that the expression system faithfully synthesized the full-length PABP, and that it was not detectably degraded during purification. The demonstration that purified PABP preferentially binds poly(A)⁺ compared with poly(C)⁺ is in agreement with similar binding studies conducted with yeast PABP (Burd *et al.*, 1991) and with a *Xenopus* PABP-β-galactosidase fusion protein isolated from *Escherichia coli* (Nietfeld *et al.*, 1990).

Although the above analysis demonstrated that the purified PABP preferentially binds poly(A)⁺ *in vitro*, we were interested in employing an independent functional assay based on translation *in vitro* to test the activity of the purified recombinant PABP. Previous experiments have suggested that the addition of PABP purified from tissue sources reverses the inhibitory effect of poly(A)⁺ on protein synthesis *in vitro* (Jacobson & Favreau, 1983; Grossi de Sa *et al.*, 1988). Our observation that the purified PABP enhances the translation of its own adenylated message [containing 3' but not 5' poly(A)⁺] and that of the adenylated test message vimentin *in vitro* provides additional evidence that the purified PABP is functional, and that PABP enhances the translation of adenylated RNAs.

Before initiating experiments to test directly the involvement of PABP in modulating translation and mRNA stability in *Xenopus* embryos, it is essential to establish the level of expression of PABP. By quantifying the number of PABP molecules per embryo, and by estimating the number of poly(A)⁺-binding sites

per embryo, we predict that at no time during early embryonic development is PABP in vast excess relative to the availability of poly(A)⁺-binding sites. To further this prediction it would be interesting to investigate the amount of free PABP relative to poly(A)⁺-associated PABP present at various stages of development. However, three factors complicate the design and interpretation of these experiments. First, PABP can be stripped from poly(A)⁺ during analysis (Drawbridge *et al.*, 1990), hence any free PABP may previously have been associated with poly(A)⁺ before disruption of the cells. Second, because we detect negligible PABP in the oocyte or egg, these experiments would need to be conducted with embryos. However, in embryos the level of expression of PABP varies greatly between tissues (Zelus *et al.*, 1989), which invalidates a biochemical analysis based on disruption of the embryo. Third, there are additional PABPs in *Xenopus* which are distinct from the one studied here, as evidenced by u.v. cross-linking of additional proteins to poly(A)⁺ (Swiderski & Richter, 1988). Therefore any quantification of PABPs relative to potential binding sites needs to monitor all PABPs.

The steady-state levels of expression of PABP may differ in various developmental systems. Using a sensitive chemiluminescence detection system for Western-blot analysis, we established that PABP is expressed by the blastula stage of development, and that steady-state concentrations subsequently increase. These data contrast with the expression of PABP in the sea-urchin egg, where PABP is not only detectable, but may also be in 50-fold excess over the poly(A)⁺-binding sites in the egg (Drawbridge *et al.*, 1990). Also differing from the expression of PABP in *Xenopus*, concentrations of PABP remain constant during sea-urchin development. One possible explanation for the differences between the expression of PABP in sea-urchins and *Xenopus* is that our analysis of PABP in *Xenopus* was based solely on the concentrations of the 68.5 kDa PABP. Other PABPs have been detected in *Xenopus* oocytes (Richter & Smith, 1983; Lorenz & Richter, 1985; Swiderski & Richter, 1988), but have not been characterized with respect to abundance during development. Therefore it is possible that additional PABPs are present in *Xenopus* embryos and that they change in abundance relative to the PABP analysed in the present study.

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