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Cloning and Characterization of a *Xenopus* Poly(A) Polymerase

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During oocyte maturation and early embryogenesis in *Xenopus laevis*, the translation of several mRNAs is regulated by cytoplasmic poly(A) elongation, a reaction catalyzed by poly(A) polymerase (PAP). We have cloned, sequenced, and examined several biochemical properties of a *Xenopus* PAP. This protein is 87% identical to the amino-terminal portion of bovine PAP, which catalyzes the nuclear polyadenylation reaction, but lacks a large region of the corresponding carboxy terminus, which contains the nuclear localization signal. When injected into oocytes, the *Xenopus* PAP remains concentrated in the cytoplasm, suggesting that it is a specifically cytoplasmic enzyme. Oocytes contain several PAP mRNA-related transcripts, and the levels of at least the one encoding the putative cytoplasmic enzyme are relatively constant in oocytes and early embryos but decline after blastulation. When expressed in bacteria and purified by affinity and MonoQ-Sepharose chromatography, the protein has enzymatic activity and adds poly(A) to a model substrate. Importantly, affinity-purified antibodies directed against *Xenopus* PAP inhibit cytoplasmic polyadenylation in egg extracts. These data suggest that the PAP described here could participate in cytoplasmic polyadenylation during *Xenopus* oocyte maturation.

Early embryonic development in many animals is controlled by mRNAs and proteins inherited by the egg at the time of fertilization. In *Xenopus laevis*, for example, maternal mRNAs and proteins direct early development until the 4,000-cell midblastula stage, when they are generally degraded and replaced by zygotically derived gene products (19). Although maternal mRNA expression is regulated at multiple levels, it is clear that one major control mechanism is cytoplasmic poly(A) elongation (for reviews, see references 29, 47, and 48). Several studies have noted that mRNAs with short poly(A) tails sediment with nontranslating ribonucleoprotein particles; when the poly(A) tails of these mRNAs are elongated during oocyte maturation (i.e., the meiotic process by which an oocyte, arrested at prophase I, becomes an egg, arrested at metaphase II) or after fertilization, they sediment with polysomes (5, 16, 23, 24, 30). It was subsequently demonstrated that poly(A) elongation was causative for translational recruitment (12, 16, 24, 36, 42). Along the same line, mRNAs that lose their poly(A) tails during maturation are removed from polysomes (13, 41).

Two sequences in the 3′ untranslated region (UTR) of mRNAs are required for cytoplasmic polyadenylation during oocyte maturation and embryogenesis: the highly conserved polyadenylation hexanucleotide AAUAAA, also required for nuclear polyadenylation, and a less conserved cytoplasmic polyadenylation element (CPE), with the general structure $U_5A_{1\text{-}3}U$ (8, 16, 17, 24, 33) for maturation or $U_{12\text{-}18}$ for embryogenesis (35, 36). Proteins interacting with these elements have been reported for *Xenopus* mRNAs that are polyadenylated during oocyte maturation or early embryogenesis. An 82-kDa protein can be cross-linked to the CPE of G10 mRNA in egg extracts that efficiently polyadenylate this mRNA (17). The CPE of B4 mRNA is bound by a 62-kDa protein in both polyadenylation-deficient oocyte extracts and polyadenylation-proficient egg extracts. In egg extracts, this protein is phosphorylated via $p34^{cdc2}$, which is concomitant with the activation of B4 mRNA polyadenylation (11, 25). The CPE of c-*mos* mRNA is bound by an activity present in egg extracts, although in this case the AAUAAA hexanucleotide is also required (9). Finally, proteins of about 36 and 45 kDa that are present in egg extracts can be cross-linked to the CPEs of Cl1 and Cl2 mRNAs (35). All the factors noted above are considered to be "specificity" factors, in that they presumably distinguish those mRNAs that are to be adenylated from those that are not.

The enzyme catalyzing poly(A) elongation is the poly(A) polymerase (PAP). PAPs have been purified from prokaryotic and eukaryotic sources (for a review, see reference 6), and in some cases, multiple PAPs have been isolated from a single cell type (20, 26, 31, 38–40). Recently, a number of genes encoding PAPs have been cloned, including those of *Escherichia coli* (4), *Saccharomyces cerevisiae* (15), vaccinia virus (10), calves (27, 45), and HeLa cells (40). The yeast and mammalian enzymes show a high degree of homology in the amino-terminal region, where domains for RNA binding and polymerization have been mapped, indicating that the proteins are evolutionarily conserved. Cytoplasmic PAP activity has been detected in sea urchin eggs (7) and mammalian cells (31, 39, 40). Similarly, a cytoplasmic PAP activity must exist in *Xenopus* oocytes, because removal of the oocyte nucleus does not prevent cytoplasmic polyadenylation (8).

In this report, we describe the isolation of a cDNA encoding a *Xenopus* PAP. This protein, which is highly homologous to the amino-terminal region of calf PAP, lacks a consensus nuclear localization signal and appears to be a cytoplasmic enzyme. A fusion protein produced in bacteria catalyzes poly(A) addition in vitro, and antibodies generated against this protein inhibit cytoplasmic polyadenylation in egg extracts. These data suggest that the PAP described here may participate in cytoplasmic polyadenylation during *Xenopus* oocyte maturation.

MATERIALS AND METHODS

Amplification of *Xenopus* PAP cDNA fragments by PCR and sequencing. Calf PAP-specific oligonucleotides A, B, K, and H (see below) were used to amplify cDNA fragments from a *Xenopus* stage VI oocyte λgt10 cDNA library by PCR. An internal fragment was obtained with oligonucleotides A and B. The sequence upstream of this fragment was amplified from the library by nested PCR with oligonucleotides B and H, together with forward and reverse λgt10 primers. The sequence downstream from the internal fragment was also obtained from the library by nested PCR with oligonucleotides A and K, together with forward and

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reverse λgt10 primers. This sequence did not contain a stop codon. *Xenopus*-specific oligonucleotides C and D were therefore designed and used in a nested PCR with oligonucleotide *Not*I-T_{17} to amplify the 3′-end PAP sequence from ovary polyadenylated [poly(A)$^+$] RNA. Fragments of various sizes were obtained. The largest one, containing a stop codon, was selected to design oligonucleotide 3′PAP. This oligonucleotide, together with *Xenopus*-specific oligonucleotide 5′PAP, was annealed to ovary poly(A)$^+$ RNA in order to amplify the full-length PAP cDNA sequence. The sequences of the oligonucleotides and their positions on the corresponding PAP sequence (from the start codon) are as follows: A, TGGTGGGAAAATTTTTACATTTGGATCTTA (278 to 307); B, TTGATAACTGGTACAAATGCCTC (472 to 450); C, CCCGAGGATTTAG ACCTCC (525 to 543); D, GAATGGTTGTCGAGTGACT (593 to 611); H, CATGTCTTGGTGCAACACAC (369 to 350); K, GTGTGTTGCACCAAGA CATG (350 to 369); 5′PAP, GGAATTCCATATGCCGTTTCCTCTTGCAAG (*Nde*I, 1 to 20); 3′PAP, CCGCTCGAGTTAAGTATAACATCTCTTTTTA (*Xho*I, 1194 to 1173); forward λgt10, AGCAAGTTCAGCCTGGTTAAG; reverse λgt10, CTTATGAGTATTTCTTCCAGGGTA.

To amplify the cDNA fragments from the library, 1 μl of library (10^4 PFU) was diluted in 50 μl of H_2O and incubated for 5 min at 70°C prior to PCR amplification. To obtain cDNA fragments from RNA, 1 μg of ovary poly(A)$^+$ RNA was used in the PCR.

The nucleotide sequence of the cDNA fragments was determined by the dideoxy method (34) and analyzed with GCG software (9a).

Expression of *Xenopus* PAP in *E. coli* and purification on MonoQ. *Xenopus* PAP was expressed in *E. coli* as a fusion protein with glutathione *S*-transferase (GST). A cDNA fragment encoding a PAP that lacked the 31 carboxy-terminal amino acids was obtained by PCR from a stage VI oocyte λgt10 library by using oligonucleotides forwardPAP-*Eco*RI (CGGAATTCATATGCCGTTTCCTCT TGCAAG) and reversePAP-*Eco*RI (CGGAATTCATAAAAGCAAAAGAAT TCCGGG). This fragment was digested with *Eco*RI and ligated to the *Eco*RI site of the pGEX-2T vector (Amrad). *E. coli* BL21 cells were subsequently transformed with this plasmid. Expression of the GST-PAP fusion protein was induced in a growing bacterial culture by adding isopropyl-β-D-thiogalactoside (IPTG) to 1 mM and incubating for 3 h at 37°C. The fusion protein was purified from the bacteria with a glutathione-agarose column as previously described (37) and dialyzed overnight against buffer D (25 mM Tris-HCl [pH 8], 10% glycerol, 0.5 mM dithiothreitol [DTT], 0.2 mM EDTA). The GST-PAP fusion protein preparation was adjusted to 50 mM NaCl and loaded on a MonoQ fast protein liquid chromatography (FPLC) column. The column was eluted with a 20-ml gradient from 50 to 500 mM NaCl followed by a 10-ml gradient from 0.5 to 1 M NaCl in buffer D. Fractions of 1 ml were collected and dialyzed against buffer D for further analysis. Protein concentrations were determined by the method of Bradford (3).

Expression of *Xenopus* PAP in reticulocyte lysates. The cDNA encoding the full-length *Xenopus* PAP, obtained by amplification of poly(A)$^+$ ovary RNA as mentioned above, was not properly expressed in bacteria and was therefore expressed in reticulocyte lysates. This cDNA was cloned into the *Nde*I and *Xho*I sites of the pET-14b vector (Novagen), downstream of a T7 promoter. PAP mRNA was synthesized in vitro by using T7 RNA polymerase as previously described (14) and translated in a reticulocyte lysate in the presence of [^{35}S]methionine by following the recommendations of the vendor (Promega).

In vitro polyadenylation assays. In vitro polyadenylation activity was determined by measuring the incorporation of either radiolabeled ATP or CTP into a poly(A) primer in the presence of $MnCl_2$ essentially as described by Raabe et al. (27). Typically, 40-μl reaction mixtures were set up in a buffer containing 0.5 mM $MnCl_2$, 0.3% polyvinyl alcohol, 86 mM Tris-HCl (pH 8.3), 860 μg of bovine serum albumin (BSA) per ml, 275 mM guanidine-HCl, 40 mM NaCl, 6% glycerol, 0.1 mM ATP, 1 μCi of [α-^{32}P]ATP, and 1.2 μg of poly(A) (average size, 400 nucleotides) and subsequently incubated for 2 h at room temperature (unless otherwise indicated). One microliter from each reaction mix was spotted onto Whatman 3MM paper and chromatographed (46). The paper was dried, squares containing the incorporated label were cut out, and the radioactivity was determined by Cerenkov counting. This chromatographic method to determine the incorporated radioactivity was equivalent to the classical trichloroacetic acid precipitation method. To measure the activity of the fractions from the MonoQ column, 10 μg of poly(A) and 5 μCi of [α-^{32}P]ATP were used in the reaction. PAP activity was similar whether poly(A) or oligo$(A)_{12}$ was used as the primer, provided that the same amounts (picomoles) of ends were present in the reaction. When labeled oligo$(A)_{12}$ was used, the reactions were performed in the same buffer conditions and the radioactive isotope was eliminated.

Affinity purification of α-PAP antibodies. Antibodies against *Xenopus* PAP were raised in rabbits and affinity purified as previously described (22). Briefly, GST-PAP fusion protein was digested with thrombin, and the fragments were resolved by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS). After electrotransfer to nitrocellulose paper, the protein fragments were visualized by Ponceau S staining. The band corresponding to PAP was cut out, blocked, and incubated for 4 h with a 1:10 dilution of anti-PAP (α-PAP) antibody. After washing for 30 min with TTBS (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.1% Tween 20), the PAP-specific antibodies were released from the paper by incubation with 100 mM triethylamine (pH 11.5) and immediately neutralized by adding 3 volumes of 1 M Tris-HCl, pH 6.8. Three successive incubations with triethylamine were performed, progressively releasing antibodies with increasing specificity and affinity for the PAP protein. The purified antibodies are referred to as Ab1, Ab2, and Ab3, respectively, and were concentrated by using Microcon 10 microconcentrators (Centricon). The final concentration was 10 ng/μl for Ab1 and Ab2. The concentration of Ab3 was below the detection limits of the Bradford assay and silver staining of proteins resolved by PAGE.

Extract preparation and inhibition of polyadenylation. Egg extracts were prepared as described by Murray and Kirschner (18). Ninety micrograms of extract was incubated in a final volume of 10 μl with either XB buffer (100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) or 20 ng of the following antibodies: α-PAP Ab1, α-PAP Ab2 (see above), α-E1A immunoglobulin G (IgG), or preimmune serum. α-PAP Ab3 (<20 ng; see above) was also used. After 15 min at room temperature, radiolabeled B4 mRNA 3′UTR (24) was added and further incubated for 1 h. The RNA was extracted with phenol-chloroform, precipitated, and resolved by PAGE in the presence of urea.

Oocyte injection. Manually defolliculated oocytes were injected with approximately 10 nl of radiolabeled PAP, E1A, or β-globin protein. To prevent the incorporation of free [^{35}S]methionine into protein, the oocytes were incubated in Barth's medium containing 10 μg of cycloheximide per ml.

RNA extraction. Oocytes were released from an ovary by treatment with 1.5 mg of collagenase per ml, which digested the follicle cells. "Ovary" was obtained by taking oocytes from all stages, whereas stage VI oocytes were obtained by selecting fully grown oocytes through repeated washes of the collagenase-treated ovary with 0.5 M sucrose in Barth's solution. Ovulated eggs were fertilized and collected at stages 4.5, 8.5, 9, 13.5, 20.5, and 30 (21). Total RNA was extracted by using the RNA STAT-60 reagent (Tel-Test B, Inc.) following the recommendations of the vendor. Poly(A)$^+$ RNA was selected on oligo(dT) columns as previously described (1).

RNase protection assays. For the RNase protection assay shown in Fig. 4, poly(A)$^+$ RNA was annealed to a probe complementary to the 3′ end of *Xenopus* PAP mRNA, starting at position 921 from the initiation codon. This probe also contained a poly(U) tract of 15 residues. For the experiment shown in Fig. 9, 50 μg of total RNA from oocytes, eggs, and embryos was annealed to an antisense RNA probe complementary to positions 300 to 441 from the start codon of *Xenopus* PAP cDNA. This probe was used instead of that in Fig. 4 because the latter showed a high background when annealed to total RNA. To ensure that the quantity and quality of the RNAs from all stages were similar, we measured the optical density at 260 and 280 nm, examined an ethidium bromide-stained agarose gel, and performed a dot blot hybridization of serial dilutions of the RNAs to a gel-purified U_{73} probe.

RNA was mixed with the probe in 20 μl of hybridization buffer (80% formamide, 0.4 M NaCl, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4], 5 mM EDTA). The RNA was denatured by incubation at 85°C for 5 min and transferred to 45°C for overnight annealing. The samples were then digested for 1 h at 30°C with 10 μg of RNase A per ml in a buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 300 mM NaCl. Samples were subsequently adjusted to 0.6% SDS and digested with 0.17 mg of proteinase K per ml for 30 min at 37°C. The protected products were analyzed by PAGE and autoradiography. The gels were also exposed in a PhosphorImager for quantification.

RESULTS

Cloning *Xenopus* PAP cDNA. The sequences in the N-terminal half of the yeast and calf PAPs are 47% identical at the amino acid level and 44% identical at the nucleotide level (45). Using this conserved region, we designed oligonucleotides to amplify sequences from a *Xenopus* λgt10 stage VI oocyte cDNA library (provided by D. Melton) or ovary poly(A)$^+$ RNA. We obtained a *Xenopus* PAP cDNA in five steps by PCR amplification (Fig. 1). An internal fragment was amplified with oligonucleotides A and B (step 1). Oligonucleotide H and an oligonucleotide derived from the λgt10 vector were used to amplify 5′ sequences (step 2). Two 5′ fragments were obtained, both containing a putative initiation codon but different lengths of 5′UTR sequence. For step 3, another oligonucleotide (K), together with a vector-derived oligonucleotide, was used to amplify 3′ sequences. Two fragments were obtained, neither of which contained a stop codon. To determine the sequences at the 3′ end of PAP mRNA, ovary poly(A)$^+$ RNA was annealed with *Not*I-T_{17} oligonucleotide and reverse transcribed. cDNA amplification (step 4) was then accomplished with *Not*I-T_{17} and a *Xenopus*-specific oligonucleotide (D) whose sequence was derived from the fragments obtained in step 3. Several fragments were obtained. The largest one, containing a termination codon, was selected. Fragments corresponding to a different PAP, more similar to the calf protein,

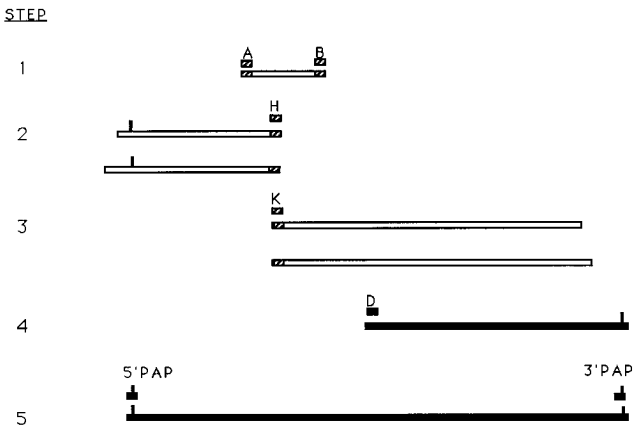

FIG. 1. Strategy for the isolation of a *Xenopus* PAP cDNA. Several clones were obtained by PCR with ovary poly(A)$^+$ RNA or an oocyte stage VI λgt10 cDNA library as the template material. Open rectangles represent cDNA fragments obtained from the oocyte library. Solid rectangles represent fragments obtained from ovary poly(A)$^+$ RNA. The oligonucleotides used for the amplification reactions are calf-specific oligonucleotides A, B, K, and H (represented as hatched small rectangles) and *Xenopus*-specific oligonucleotides D, 5′PAP, and 3′PAP (represented as solid small rectangles). The vertical bars represent the initiation and termination codons. For details on the oligonucleotide sequences, see Materials and Methods.

were obtained in steps 1 and 4, the latter by using oligonucleotide K instead of D. Oligonucleotides spanning the initiation and termination codons were used in a final PCR amplification from ovary poly(A)$^+$ RNA to obtain the full-length cDNA of *Xenopus* PAP (step 5).

The sequence contained an open reading frame encoding a 394-amino-acid protein (Fig. 2). Interestingly, the sequence did not contain any 3′UTR. Two copies of the polyadenylation hexanucleotide were included in the open reading frame, and the termination codon coincided with the beginning of the poly(A) tail (Fig. 2). These features were consistently present in PCR fragments amplified in independent PCRs.

The predicted protein sequence of *Xenopus* PAP contains a ribonucleoprotein-type RNA binding domain at the N terminus (Fig. 3) and a sequence that has been proposed to represent a polymerase module (27, 28) but lacks the nuclear localization signals that have been described for calf PAP (27, 28, 45). In this regard, the protein described here is similar to apparently truncated forms of PAP encoded by cDNAs isolated from HeLa (45) and calf (28) cell libraries.

Although *Xenopus* PAP shows 87% overall identity with calf PAP, there are clusters of amino acid differences, especially at the amino terminus (including a deletion of three amino acids) and the 26 carboxy-terminal amino acids, which are totally unrelated to calf PAP (Fig. 3). No differences exist in the highly conserved sequence elements of the RNA-binding domain and polymerase module domain described for calf PAP (28).

Because of the unusual sequence features of the 3′ end of *Xenopus* PAP mRNA, we wanted to confirm that it indeed was present in oocytes by using an RNase protection assay that can discriminate between two sequences containing a two-base mismatch. For this purpose, we used a probe complementary to the 3′ end of *Xenopus* PAP mRNA starting at nucleotide 921 (from the initiation codon), which was annealed to poly(A)$^+$ RNA from stage VI oocytes or ovary (i.e., oocytes of all stages). As a positive control, we included the full-length nonadenylated PAP RNA cloned in step 5 of Fig. 1. The results shown in Fig. 4 indicate that a fragment of the same size as the one protected in the positive control (lane 1) was protected when RNA from oocytes or ovary was used (lanes 2 and 3, respectively). This fragment (fragment B) corresponds to the nonadenylated form of the PAP mRNA. Two other fragments were also protected, corresponding to the polyadenylated form of PAP mRNA (fragment A) and to a different mRNA with sequences in common with the PAP mRNA described here (fragment C). The ratio of fragments B and C was about 1 to 3, as measured in the phosphorImager, and was similar in the oocyte and ovary lanes. The size of fragment C corresponds to the size of the probe that is complementary to conserved sequences between *Xenopus* and mammalian PAPs (Fig. 4), suggesting that fragment C represents another PAP sequence. From these results, we conclude that several PAP mRNAs exist in *Xenopus* oocytes and that the one we have cloned does indeed have the unusual features at the 3′ end mentioned previously.

The cDNA encodes a functional PAP. We expressed *Xenopus* PAP in *E. coli* as a fusion protein with GST. This protein lacked the 31 carboxy-terminal residues, because full-length PAP fused to GST was not properly expressed. The protein was purified in a glutathione column and used in an in vitro polyadenylation assay in the presence of Mn^{2+}, which decreases the specificity of PAPs for RNA primers. Reactions were performed at room temperature under the buffer conditions previously described for calf PAP (27). The activity was measured as the incorporation of radioactive ATP or CTP into a poly(A) primer. Figure 5A shows that the fusion protein was able to catalyze poly(A) addition but not poly(C) addition to the primer. The GST protein alone did not show any activity in parallel reactions. Shown another way, Fig. 5B demonstrates that *Xenopus* PAP incorporated ATP to the 3′ end of a labeled oligo$(A)_{12}$ primer. In addition, GST-PAP activity was temperature dependent, being maximum at 18 to 20°C and decreasing at higher temperatures (Fig. 5C).

To further demonstrate that the polyadenylation activity was due to the expressed *Xenopus* PAP and not to a bacterial contaminant, we fractionated the GST-PAP preparation on a MonoQ column with a salt gradient (Fig. 6). Two major protein peaks eluting between 0.2 and 0.25 M NaCl (fractions 8 and 9) contained the PAP activity (Fig. 6A). An analysis of the proteins in the various fractions (Fig. 6B) and a Western blot (immunoblot) performed in parallel with an affinity-purified antibody against *Xenopus* PAP (Fig. 6C) indicated that both peaks also contained the GST-PAP protein. As with the unfractionated GST-PAP, the activity was maximum at 18°C (data not shown). These results were consistently reproduced when different GST-PAP preparations were used. The small activity peak present in fraction 6 was, however, not reproducible. Thus, the cDNA that we have cloned indeed encodes a *Xenopus* PAP.

Two other proteins of a size similar to the GST-PAP fusion protein were fractionated on the MonoQ column (Fig. 6B, lanes 15 to 20). These proteins do not show PAP activity and do not represent *Xenopus* PAP because they are not recognized by the α-PAP antibody. One of these is probably DnaK, a 70-kDa protein involved in the degradation of "abnormal" proteins in *E. coli* that is often found as a contaminant in GST fusion protein preparations (26a).

Inhibition of cytoplasmic polyadenylation by α-PAP antibodies. Antibodies directed against the GST-PAP fusion protein were generated in rabbits and affinity purified. Antibodies Ab1, Ab2, and Ab3 were obtained, and their specificity was analyzed by Western blot of a *Xenopus* PAP that was released from its GST counterpart by digestion with thrombin. Figure 7A shows that 300 ng (lanes 1, 3, and 5) or 100 ng (lanes 2, 4, and 6) of thrombin-digested GST-PAP protein was well rec-

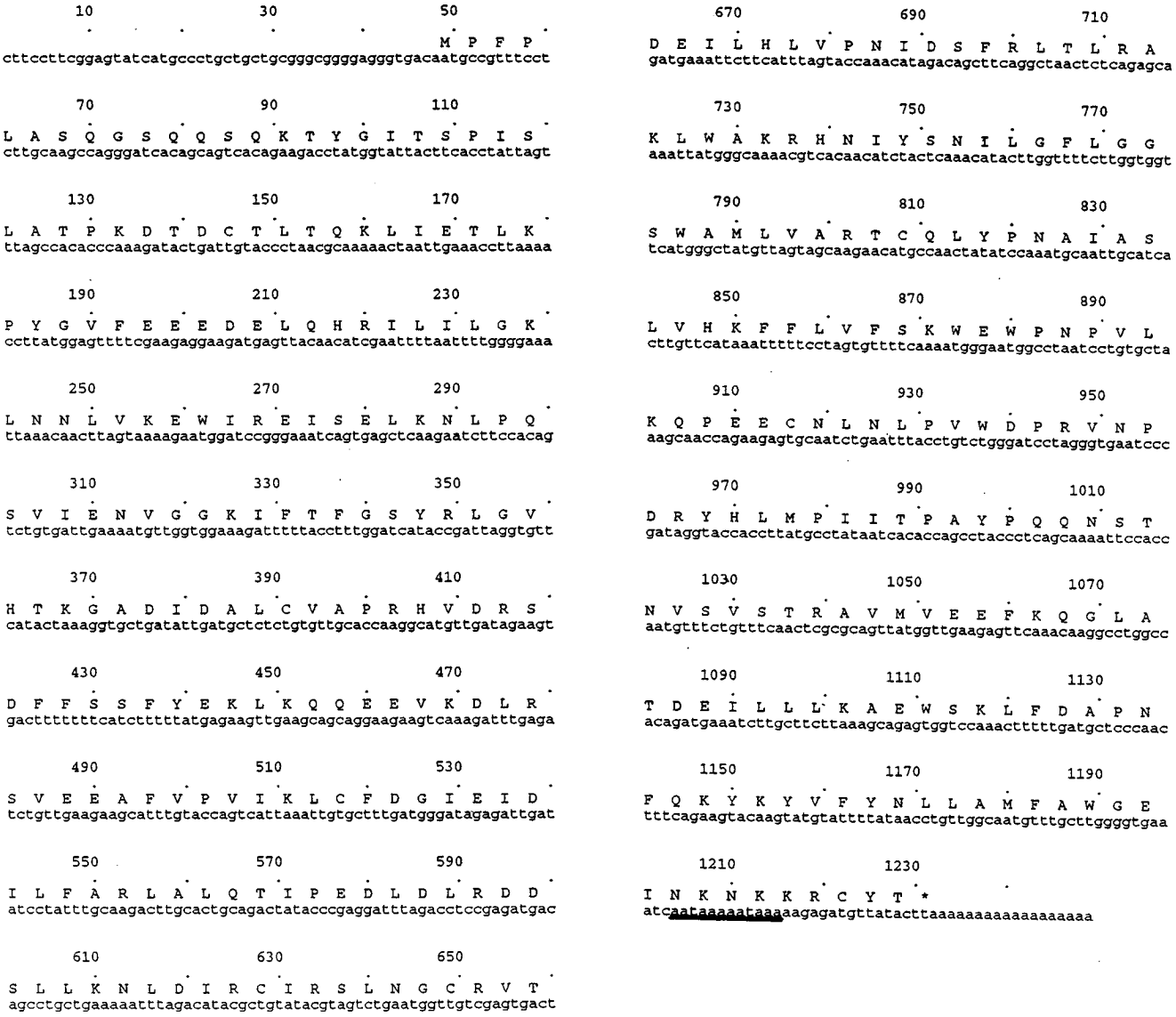

FIG. 2. Nucleotide sequence and predicted amino acid sequence of a cDNA encoding *Xenopus* PAP. Two copies of the hexanucleotide sequence AAUAAA are underlined.

ognized by the antibodies. In addition to PAP, Ab1 could detect GST and DnaK (see above) with similar efficiencies, indicating that it was somewhat impure. However, this was not the case with the purer Ab2 and Ab3 antibodies. These antibodies were added to egg extracts to determine whether they could inhibit the polyadenylation of B4 mRNA 3′UTR (Fig. 7B). Under conditions supporting CPE-dependent cytoplasmic polyadenylation in vitro (17, 24), the egg extract efficiently polyadenylated B4 mRNA (Fig. 7B, compare lane 1 and lane 2). However, preincubation of the extract with increasingly purified α-PAP antibodies progressively inhibited polyadenylation (Fig. 7B, lanes 3 to 5). Polyadenylation was not inhibited when preimmune serum or an unrelated IgG was used in the assay (Fig. 7B, lanes 6 and 7). Thus, these results demonstrate that antibodies directed against *Xenopus* PAP inhibit cytoplasmic polyadenylation in vitro.

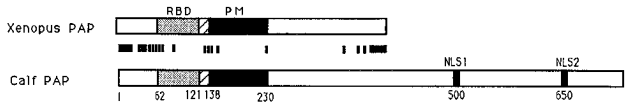

FIG. 3. Schematic representation of the sequence features of *Xenopus* and calf PAPs. The numbers indicate amino acid positions according to Raabe et al. (29, 30). Note that these numbers should be shifted three positions for *Xenopus* PAP because of a three-amino-acid amino-terminal deletion with respect to calf PAP. The RNA-binding domain (RBD, amino acids 62 to 138) is represented as a stippled rectangle; the polymerase module (PM, amino acids 121 to 230) and nuclear localization signals NLS1 (amino acids 489 to 507) and NLS2 (amino acids 644 to 659) are shown as solid rectangles. RBD and PM partially overlap (hatched rectangle). The vertical bars between the two PAP schemes indicate amino acid differences between *Xenopus* and calf PAPs.

PAP is located in the oocyte cytoplasm. To determine the localization of the PAP in oocytes, we attempted both Western blotting with a variety of sensitive detection methods and immunoselection of metabolically labeled protein from nuclear and cytoplasmic compartments. In all cases, the amounts of PAP were below our limits of detection. As an alternative

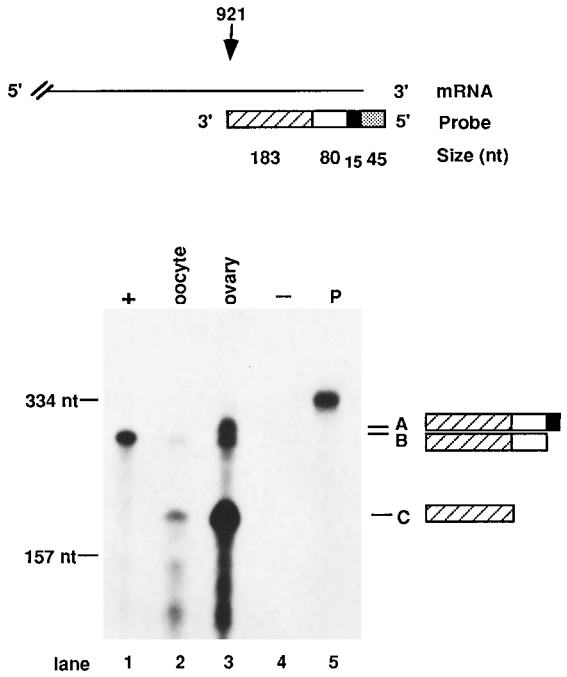

FIG. 4. RNase protection of the 3′ end of *Xenopus* PAP mRNA. A probe complementary to the 3′ end of *Xenopus* PAP mRNA was annealed to 50 pg of in vitro-synthesized PAP mRNA (lane 1), 3 μg of poly(A)$^+$ stage VI oocyte RNA (lane 2), 7 μg of poly(A)$^+$ ovary RNA (lane 3), or no RNA (lane 4). The probe was 334 nucleotides (nt) in length, containing 263 nt complementary to PAP sequences (hatched and open boxes), 15 uridylate residues (black box), and 45 nt of polylinker (stippled box). Of the 263 nt complementary to PAP sequences, 183 nt were 84% conserved between calf and *Xenopus* PAPs, and 80 nt were divergent. The protected products were analyzed by PAGE and autoradiography. The probe (lane 5) was included as a marker.

strategy, we cloned the full-length PAP cDNA downstream of the T7 promoter of the pET-14b vector. mRNA encoding the full-length PAP was synthesized in vitro and translated in a reticulocyte lysate in the presence of [^{35}S]methionine. The radiolabeled protein was injected into oocytes, and at different times, oocyte cytoplasms and nuclei were separated manually. PAP was then resolved by SDS-PAGE and autoradiography. As controls, we performed identical experiments with the adenovirus E1A protein, which is nuclear, and β-globin, which is cytoplasmic. The results (Fig. 8) show that the PAP protein, as well as globin, remained in the cytoplasm after 5 h, whereas E1A was translocated to the nucleus. From this, we surmise that the endogenous PAP is also likely to be a cytoplasmic protein in *Xenopus* oocytes.

PAP mRNA is developmentally regulated. We used RNase protection assays to determine the relative amount of PAP mRNA in early development. A *Xenopus*-specific probe complementary to the 5′ end of the PAP cDNA (nucleotides 300 to 441 from the start codon) was annealed to 50 μg of total RNA from different developmental stages. The results in Fig. 9 show that PAP mRNA levels are relatively constant in oocytes and embryos up to the blastula stage (stage 9) but drop thereafter. From the amount of in vitro-synthesized mRNA used as a positive control in the assay, we calculate that the proportion of PAP mRNA in an oocyte is about 0.001% of the poly(A)$^+$ RNA. As previously mentioned, the probe only protects sequences that are virtually 100% complementary under our RNase protection assay conditions. We cannot exclude, however, the possibility that other PAP mRNAs identical in the region complementary to the probe are also detected.

DISCUSSION

In this paper, we describe the isolation of a cDNA encoding a *Xenopus* PAP. The protein expressed from this cDNA in bacteria specifically adds adenylate residues to the 3′ end of an

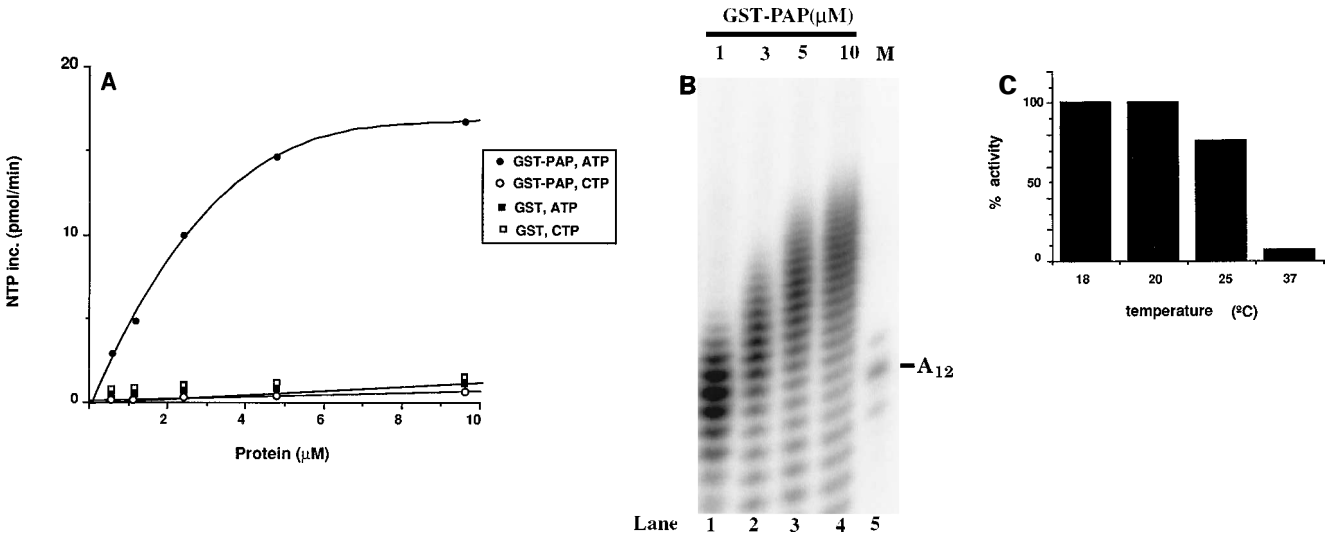

FIG. 5. Activity of *Xenopus* PAP. (A) Activity profile of *Xenopus* PAP in the presence of either ATP or CTP. The final concentration of the GST-PAP fusion protein or the GST control used in the assay is indicated on the abscissa. The activity was measured as the incorporation (inc.) of radiolabeled ATP or CTP on a poly(A) primer and was expressed as picomoles of nucleoside triphosphate (NTP) incorporated per minute (ordinate). For details on the reaction conditions, see Materials and Methods. (B) In vitro polyadenylation of labeled A_{12} oligonucleotide by *Xenopus* PAP. GST-PAP was used at a final concentration of 1, 3, 5, or 10 μM (lanes 1 to 4, respectively) in a standard polyadenylation reaction. Lane 5 shows the labeled oligonucleotide used as a marker. The polyadenylated products were separated in a 20% acrylamide gel in the presence of urea and visualized in a PhosphorImager. (C) Effect of temperature on the activity of *Xenopus* PAP. GST-PAP fusion protein was used in an in vitro polyadenylation assay in the presence of Mn^{2+}. The reaction conditions were similar to those used in the experiment shown in panel A except that the temperature was changed from 18 to 37°C (abscissa). GST-PAP was used at either 1 or 5 μM with similar results. The activity is represented on the ordinate as a percentage of maximum activity.

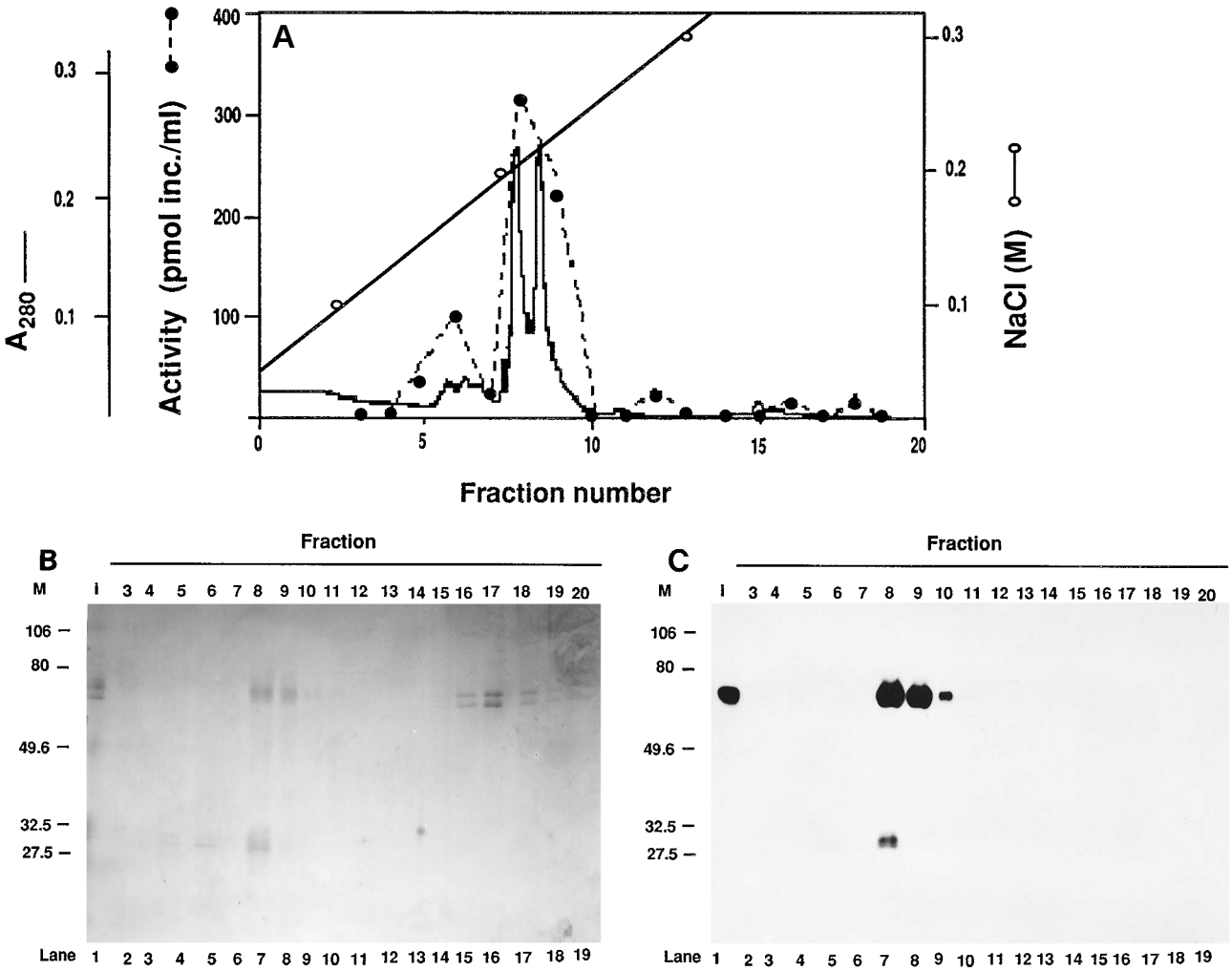

FIG. 6. Chromatography of PAP on MonoQ. (A) Profile of the MonoQ column showing A_{280}, NaCl gradient, and PAP activity measured in the presence of Mn^{2+}. inc., incorporated. (B) SDS-PAGE of fractions from the MonoQ column. The positions of molecular size markers are shown to the left (in kilodaltons). Lane 1, 100 ng of input GST-PAP; lanes 2 to 19, 2 μl of each fraction. Proteins were visualized by silver staining. (C) Western blot of fractions from the MonoQ column. A duplicate of the gel shown in panel B was transferred to nitrocellulose and incubated with an affinity-purified antibody against *Xenopus* PAP (Ab3 of Fig. 7A). Goat α-rabbit IgG labeled with horseradish peroxidase was used as the secondary antibody. Immunocomplexes were visualized by fluorography with the enhanced chemiluminescence system (NEN-Dupont).

RNA primer. Affinity-purified antibodies generated against this protein inhibit cytoplasmic polyadenylation in egg extracts. Furthermore, the *Xenopus* PAP described here appears to be located in the oocyte cytoplasm. Finally, the mRNA encoding *Xenopus* PAP is developmentally regulated, in that it is most abundant in oocytes and early embryos up to the blastula stage.

The sequence of *Xenopus* PAP cDNA contains some unusual features. It does not include a 3′UTR, and a sequence that is normally present in the 3′UTR (i.e., the polyadenylation hexanucleotide) is contained within the coding region. In addition, the first two adenylate residues of the poly(A) tail are part of the stop codon. These features may be the consequence of alternative splicing or alternative polyadenylation, as has been suggested previously to explain the existence of several calf PAP mRNAs (31, 45). Indeed, there also appear to be multiple *Xenopus* PAP-related mRNAs, because fragments corresponding to different mRNAs are detected by RNase protection (Fig. 4, fragment C) or PCR (not shown). One can speculate that any of these PAP-related mRNAs could encode the nuclear enzyme.

A fragment containing the 363 amino-terminal amino acids of *Xenopus* PAP specifically catalyzes poly(A) elongation (Fig. 5 and 6). Interestingly, a fragment containing the 371 amino-terminal amino acids of mammalian PAP, which is 87% homologous to *Xenopus* PAP, does not have activity in vitro (28, 45), underscoring the unique nature of the *Xenopus* enzyme. Consistent with the frog physiological temperature, *Xenopus* PAP is more active at 18°C than at 37°C. The specific activity of the enzyme, however, is 100-fold lower than that of HeLa cell PAP under roughly the same conditions (40). This could be due to several reasons: (i) the GST-PAP protein used to measure activity is a bacterially expressed fusion protein that lacks the 31 carboxy-terminal amino acids, which could be important for activity; (ii) only a small proportion of the bacterially expressed protein could be folded in an active form; or (iii) the activity of the *Xenopus* PAP that we have cloned could be intrinsically lower than that of the mammalian PAPs and may require additional factors.

One line of evidence supporting the notion that the PAP reported here may function in cytoplasmic polyadenylation is

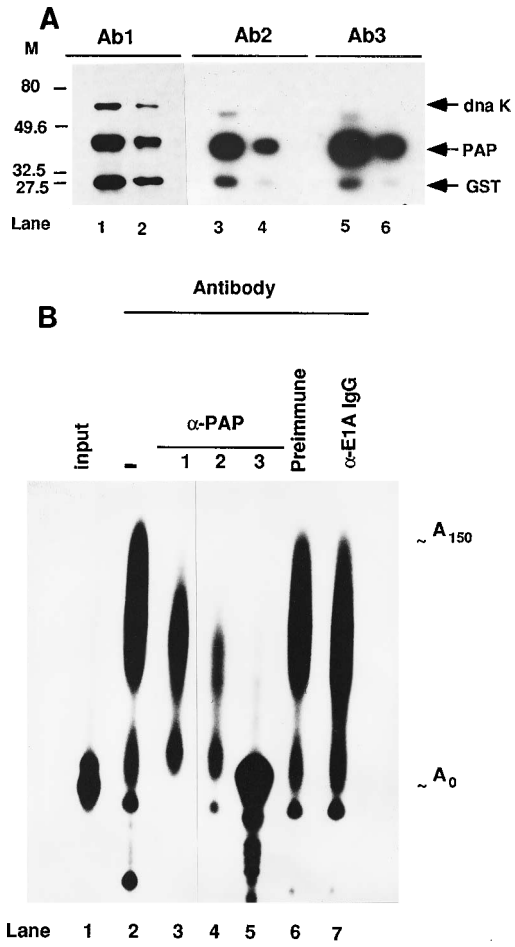

FIG. 7. Inhibition of polyadenylation by α-PAP antibodies. (A) Purification of α-PAP antibodies measured by Western blot. Affinity-purified antibodies against *Xenopus* PAP were obtained and named Ab1, Ab2, and Ab3. Either 300 ng (lanes 1, 3, and 5) or 100 ng (lanes 2, 4, and 6) of thrombin-digested GST-PAP protein was run on SDS-PAGE, transferred to nitrocellulose, and incubated with either Ab1, Ab2, or Ab3. The filters were subsequently incubated with horseradish peroxidase-labeled goat α-rabbit IgG, and bands were visualized by fluorography. The bands corresponding to DnaK (see text), PAP, and GST are indicated by arrows. The positions of molecular size markers are shown at the left (in kilodaltons). (B) Inhibition of polyadenylation in *Xenopus* egg extracts by α-PAP antibodies. Egg extracts were preincubated with buffer (lane 2), 20 ng of α-PAP Ab1 and Ab2 (lanes 3 and 4), less than 20 ng of α-PAP Ab3 (lane 5), 20 ng of preimmune serum (lane 6), or 20 ng of an unrelated antibody (α-E1A IgG, lane 7). After 15 min at room temperature, the extracts were mixed with radiolabeled B4 mRNA 3′UTR (lane 1), incubated for 1 h, and processed as described in Materials and Methods. The RNA was analyzed on a 5% denaturing polyacrylamide gel, followed by autoradiography.

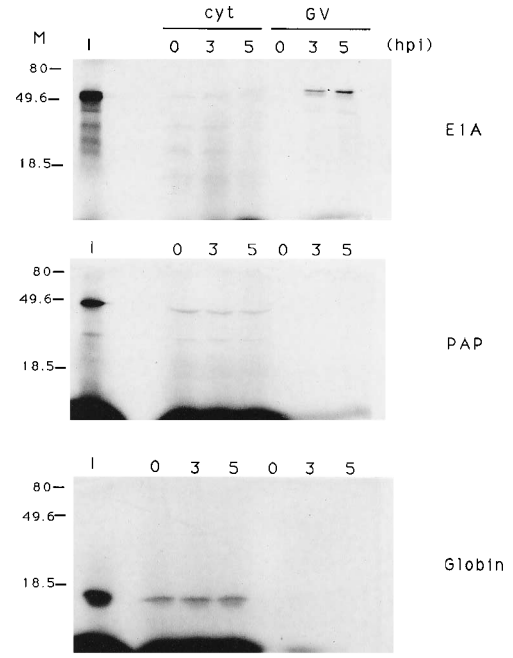

FIG. 8. Intracellular localization of *Xenopus* PAP in stage VI oocytes. Oocytes were injected with in vitro-synthesized radiolabeled E1A, PAP, or globin. Groups of 10 oocytes were taken at 0, 3, and 5 h postinjection (hpi), the cytoplasms (cyt) were separated manually from the nuclei (GV, germinal vesicle), and the proteins were analyzed by SDS-PAGE. Lane I, uninjected in vitro-synthesized protein used as a marker. The positions of size markers are shown to the left (in kilodaltons).

that purified antibodies generated against this protein inhibit CPE-mediated polyadenylation in egg extracts. We cannot exclude, however, that other PAPs containing similar epitopes are being recognized and inhibited by the antibody. The fact that very small amounts of antibody inhibit polyadenylation suggests that the protein is not abundant, which probably explains our failure to detect this protein by Western blotting or immunoprecipitation. This correlates with the small amount of PAP mRNA that is detected in oocytes by the RNase protection assay (0.001% of the mRNA). Another line of evidence suggesting that this PAP may be specific for cytoplasmic polyadenylation is that it contains no discernible nuclear localization signal and it remains localized to the cytoplasm following oocyte injection. Finally, the decline of PAP mRNA levels after the blastula stage correlates with the completion of Cl2 and Cl1 mRNA polyadenylation (35, 36), which are the latest cytoplasmic polyadenylation events described to date.

Do the same components catalyze cytoplasmic and nuclear polyadenylation? Although cytoplasmic and nuclear polyadenylation are in some respects similar, in other ways they are quite different (for reviews, see references 32, 43, 44, 47, and 48). For example, while both reactions are dependent upon the AAUAAA hexanucleotide, only the cytoplasmic process re-

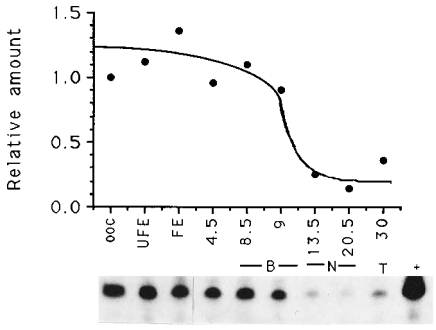

FIG. 9. RNase protection of *Xenopus* PAP mRNA during development. Fifty micrograms of total RNA from stage VI oocyte (ooc), unfertilized egg (UFE), fertilized egg (FE), early cleavage (stage 4.5), blastula (B, stages 8.5 and 9), early neurula (N, stage 13.5), late neurula (N, stage 20.5), and tadpole (T, stage 30) embryos was annealed with radiolabeled antisense RNA complementary to nucleotides 300 to 441 of PAP mRNA and digested with RNase A. Note that this probe is different from that used in Fig. 4. The protected products were analyzed by PAGE and autoradiography and quantified in a phosphorImager. The graphic shows the relative amount of RNA in each stage, arbitrarily setting the amount present in oocytes to 1. A total of 200 pg of in vitro-synthesized *Xenopus* PAP mRNA was used as a positive control (+).

quires a CPE. As a consequence, nuclear polyadenylation is common to most pre-mRNAs, but the cytoplasmic process is selective for those mRNAs that contain a CPE. Moreover, the nuclear reaction becomes independent of the AAUAAA sequence after 10 adenylates have been added to the growing poly(A) tail; this is not the case for the cytoplasmic reaction. Do these differences imply that separate sets of factors catalyze nuclear and cytoplasmic polyadenylation? Fox et al. (9) have reported that a fraction from *Xenopus* egg extracts contains an RNA-binding activity able to recognize the CPE and the sequence AAUAAA. When this fraction is mixed with purified calf PAP, cytoplasmic polyadenylation is reconstituted. In addition, Bilger et al. (2) have shown that CPE-containing RNAs are preferentially polyadenylated by purified calf PAP and CPSF, a protein complex that recognizes the hexanucleotide AAUAAA in the nucleus. Furthermore, polyadenylation is stimulated in oocyte extracts by the addition of cleavage and polyadenylation specificity factor (CPSF) (2). These observations led those investigators to propose that nuclear polyadenylation and cytoplasmic polyadenylation are catalyzed by similar or identical components. However, two additional observations suggest that cytoplasmic polyadenylation is catalyzed by at least some unique components. First, a CPE-binding protein (CPEB) identified by Paris et al. (25), which is now known to be required for B4 mRNA polyadenylation, is a primarily cytoplasmic protein (11). Second, the PAP identified in this report could correspond to an active cytoplasmic factor involved in CPE-dependent polyadenylation. Of course, there may be additional factors, e.g., the CPSF-like factor proposed by Bilger et al. (2), that are common to both nuclear and cytoplasmic polyadenylation. Using bacterially expressed CPEB and PAP in affinity chromatography-type experiments, we hope to isolate and identify such factors.

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