

# Poly (A) polymerases in the nucleus and cytoplasm of frog oocytes: Dynamic changes during oocyte maturation and early development

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## ABSTRACT

Poly (A) can be added to mRNAs both in the nucleus and in the cytoplasm. During oocyte maturation and early embryonic development, cytoplasmic polyadenylation of preexisting mRNAs provides a common mechanism of translational control. In this report, to begin to understand the regulation of polyadenylation activities during early development, we analyze poly (A) polymerases (PAPs) in oocytes and early embryos of the frog, *Xenopus laevis*. We have cloned and sequenced a PAP cDNA that corresponds to a maternal mRNA present in frog oocytes. This PAP is similar in size and sequence to mammalian nuclear PAPs. By immunoblotting using monoclonal antibodies raised against human PAP, we demonstrate that oocytes contain multiple forms of PAP that display different electrophoretic mobilities. The oocyte nucleus contains primarily the slower migrating forms of PAP, whereas the cytoplasm contains primarily the faster migrating species. The nuclear forms of PAP are phosphorylated, accounting for their retarded mobility. During oocyte maturation and early postfertilization development, preexisting PAPs undergo regulated phosphorylation and dephosphorylation events. Using the cloned PAP cDNA, we demonstrate that the complex changes in PAP forms seen during oocyte maturation may be due to modifications of a single polypeptide. These results demonstrate that the oocyte contains a cytoplasmic polymerase closely related to the nuclear enzyme and suggest models for how its activity may be regulated during early development.

**Keywords:** 3' end formation; maternal mRNAs; oocytes; PAP; poly (A); translational control

## INTRODUCTION

The switch at fertilization from relative dormancy to mitotic cleavage divisions is one of the most striking aspects of early development. Because transcription typically is quiescent during this period, changes in protein synthesis require regulation of the stability or translational activity of maternal mRNAs. Translational stimulation of maternal mRNAs often is accompanied by the lengthening of their poly (A) tails (Paris & Philippe, 1990; Sheets et al., 1994). These polyadenylation reactions appear to be required to activate their translation (reviewed in Jackson & Standart, 1990; Bachvarova, 1992; Wickens, 1992). The number of mRNAs that receive poly (A) can be substantial; for ex-

ample, the number of mRNAs affected in sea urchin eggs is so large that the total mass of poly (A) in the egg doubles between fertilization and first cleavage (Wilt, 1973). The common correlation between translational activation and polyadenylation, as well as the large number of animal species in which that correlation exists, indicate that polyadenylation likely represents an important regulatory process during the first few hours of an animal's life.

Before fertilization, frog oocytes undergo maturation, in which they advance from first to second meiosis in response to progesterone. During this interval, specific mRNAs receive poly (A). This reaction is cytoplasmic and requires no nuclear components (Fox et al., 1989). Before meiotic maturation, poly (A) addition factors appear to be present in the cytoplasm but are quiescent. In contrast, in the nuclei of both frog and mouse oocytes, poly (A) addition factors are active and

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add long poly (A) tails to pre-mRNAs (Fox et al., 1989; Huarte et al., 1992). mRNAs carrying these long tails are then transported to the cytoplasm, where their poly (A) tails are shortened (Huarte et al., 1992). Later, at specific times during maturation or after fertilization, the poly (A) tails of specific mRNAs are lengthened or removed (Dworkin et al., 1985; Dworkin & Dworkin-Rastl, 1985; Paris et al., 1988; Fox et al., 1989; McGrew et al., 1989; Vassalli et al., 1989; Fox & Wickens, 1990; Paris & Richter, 1990; Varnum & Wormington, 1990; Huarte et al., 1992; Legagneux et al., 1992; Simon et al., 1992; Sheets et al., 1994; Stebbins-Boaz & Richter, 1994).

The mechanism of nuclear polyadenylation in vertebrates (reviewed in Wahle & Keller, 1992) provides a useful perspective on the regulation of polyadenylation in the cytoplasm. In the nucleus, a poly (A) polymerase (PAP) catalyzes the addition of a long (ca. 250 nt) poly (A) tail to most cellular mRNAs. The enzyme is highly conserved, and contains an RNA recognition motif (RRM), nuclear localization signals, polymerase modules characteristic of RNA-dependent polymerases, and a serine/threonine-rich carboxy-terminal domain (Raabe et al., 1991, 1994; Wahle et al., 1991; Thuresson et al., 1994). PAP interacts with cleavage and polyadenylation specificity factor (CPSF), a complex of three to four polypeptides that binds directly to the highly conserved sequence, AAUAAA (Bienroth et al., 1991; Murthy & Manley, 1992), as well as to the 3' end of the mRNA. The AAUAAA sequence, present near the 3' end of virtually all vertebrate mRNAs, is necessary and virtually sufficient for nuclear polyadenylation (Wilusz et al., 1989; Sheets et al., 1990; Wigley et al., 1990). The 3' end to which poly (A) is added (Moore et al., 1986; Sheets et al., 1987) is formed by an endonucleolytic cleavage reaction, to which polyadenylation is tightly coupled. However, polyadenylation can be experimentally uncoupled from cleavage by using synthetic substrates that end at the appropriate nucleotide (Sheets et al., 1987). In these "uncoupled" reactions, polyadenylation requires PAP, CPSF, and poly (A) binding protein II (Bienroth et al., 1993).

The addition of poly (A) in the nucleus and cytoplasm differs in many respects. In the nucleus, virtually all mRNAs receive a 250–350-nt poly (A) tail; in the cytoplasm, during early development, only certain mRNAs receive poly (A), and do so at characteristic times, receiving very different lengths of poly (A) (Paris et al., 1988; Paris & Philippe, 1990; Simon et al., 1992; Sheets et al., 1994). Despite these differences, cytoplasmic and nuclear polyadenylation may be catalyzed by similar factors. The cytoplasmic polyadenylation reactions that occur during frog oocyte maturation can be reconstituted *in vitro* using fractions derived from a frog egg extract (Paris & Richter, 1990; Fox et al., 1992). One fraction contains an RNA binding activity, the other a PAP (Fox et al., 1992). The PAP fractions can

be replaced by recombinant or purified PAP, presumably nuclear in origin (Fox et al., 1992). Similarly, the RNA binding fraction can be replaced by the nuclear factor, CPSF (Bilger et al., 1994). The sequence specificity of polyadenylation *in vitro* by purified nuclear CPSF and PAP is as yet indistinguishable from that in the cytoplasm during oocyte maturation; in particular, efficient polyadenylation requires not only AAUAAA, but also a U-rich sequence nearby (Bilger et al., 1994).

Thus, nuclear and cytoplasmic polyadenylation may rely on the same core apparatus, including CPSF and PAP (Fox et al., 1992; Bilger et al., 1994). At least one CPSF subunit is present in the frog oocyte cytoplasm and is required for maturation-specific polyadenylation *in vitro* (A. Bilger, S. Ballantyne, D.L. Daniel, Jr., A. Jenny, & M. Wickens, submitted for publication). Proteins of 58 and 82 kDa have been identified that associate with RNAs containing AAUAAA and U-rich sequences (McGrew & Richter, 1990; Paris et al., 1991). These proteins are likely participants in cytoplasmic polyadenylation as well, either as constituents of the core apparatus, or as sequence-specific regulators of its activity (McGrew & Richter, 1990; Paris et al., 1991; Hake & Richter, 1994). The 58-kDa protein is a positive-acting polyadenylation factor *in vitro* (Hake & Richter, 1994). PAP activity has been detected in cytoplasmic extracts of sea urchin eggs (Wilt, 1973) and appears to become nuclear only after several cleavage divisions (Egrie & Wilt, 1979). Similarly, cytoplasmic extracts of somatic cells contain PAP activity (Ryner et al., 1989; reviewed in Edmonds, 1990) and proteins that are immunologically related to the nuclear enzyme (Thuresson et al., 1994).

In this report, to begin to understand the regulation of polyadenylation activities during early development, we analyze PAPs in frog oocytes and early embryos. We have cloned and sequenced a PAP cDNA that corresponds to a maternal mRNA present in frog oocytes. We demonstrate that multiple forms of PAP exist in frog oocytes, and analyze the difference between nuclear and cytoplasmic enzymes. PAP undergoes regulated phosphorylation and dephosphorylation events during oocyte maturation and early postfertilization development. These results demonstrate that the oocyte contains a cytoplasmic polymerase closely related to the nuclear enzyme and suggest models for how its activity may be regulated during early development.

## RESULTS

### PAP cDNAs from frog oocytes

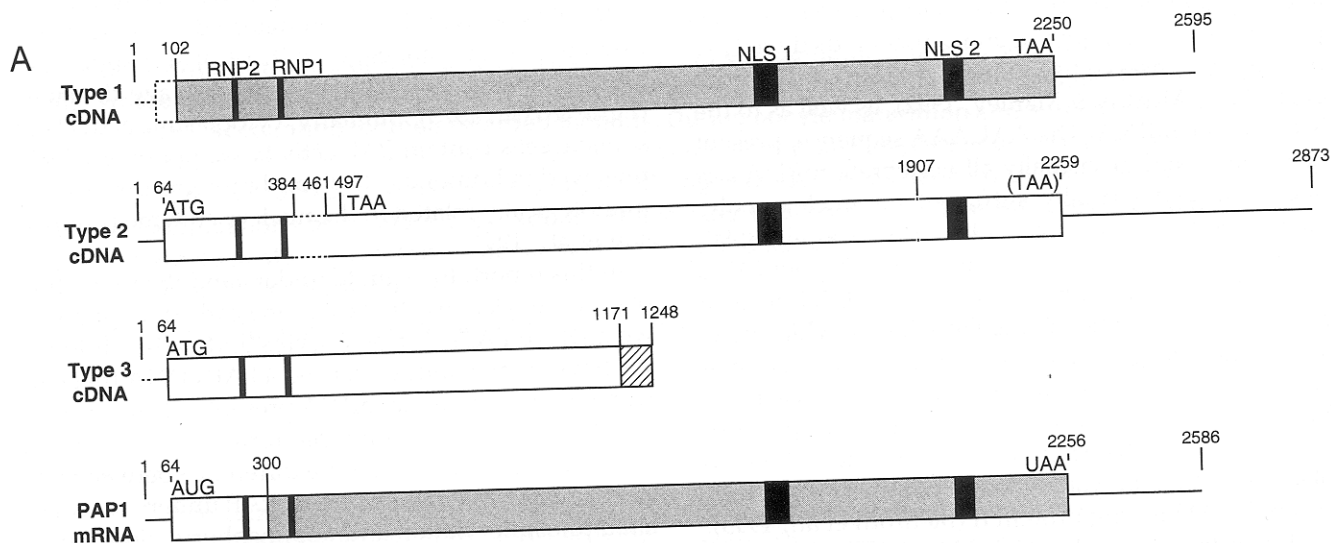
To identify and characterize PAPs in frog oocytes, we first isolated cDNAs encoding PAP-related sequences from a *Xenopus laevis* ovarian cDNA library. To obtain a hybridization probe, we used degenerate oligonucleotides derived from conserved portions of PAP in yeast and calf as primers for PCR, using egg cDNA as tem-

plate (Lingner et al., 1991; Wahle et al., 1991; see Materials and methods for details). Using this probe, we screened approximately  $10^5$  plaques from a frog ovary cDNA library (Rebagliati et al., 1985) and identified 15 plaques that hybridized.

Based on their nucleotide sequences, the positive cDNAs fell into at least three classes, as diagrammed in Figure 1A. Type 1 cDNA contains an open reading frame of 716 amino acids that is 83% identical in sequence to the calf enzyme. Like the mammalian enzyme, the protein encoded by frog Type 1 cDNA contains a putative RRM domain (characterized by RNP1 and RNP2 sequences), a putative polymerase module (Raabe et al., 1991, 1994), two putative nuclear localization signals (Robbins et al., 1991), and a serine/threonine-rich carboxy-terminal domain. Type 2 cDNA contains a 76-nt deletion removing nucleotides 384–461, and a single nucleotide deletion at position 1907; thereafter, allowing for the frameshift, the open reading frame continues. In Type 3 cDNA, the open reading frame at amino acid 395 directly abuts poly (A), without a termination codon. Unusual PAP cDNA se-

quences analogous to Type 3 appear to be characteristic of the PAP gene because they also have been isolated from mouse (W. Zhao & J. Manley, pers. comm.) and human (L. Thuresson & A. Virtanen, pers. comm.) libraries.

In this report, we focus on Type 1 cDNA and the protein it encodes. It corresponds to a maternal mRNA and encodes a protein similar to the most prominent endogenous enzyme (see below). mRNAs derived from Type 1 cDNA were used in the injection experiments described in subsequent sections. Because our Type 1 cDNAs lacked a 5' terminal sequence including the AUG initiation codon, we fused the long Type 1 open reading frame with the N-terminal region of the Type 2 sequence. The resulting mRNA, designated PAP1 mRNA, is diagrammed in Figure 1A and its sequence presented in Figure 1B. The protein it encodes, derived by conceptual translation, is aligned with the sequences of calf, human, and yeast PAPs in Figure 1C. The protein encoded by PAP1 mRNA is 83% identical to the calf nuclear enzyme and diverges most from the calf enzyme sequence in the C-terminal region.

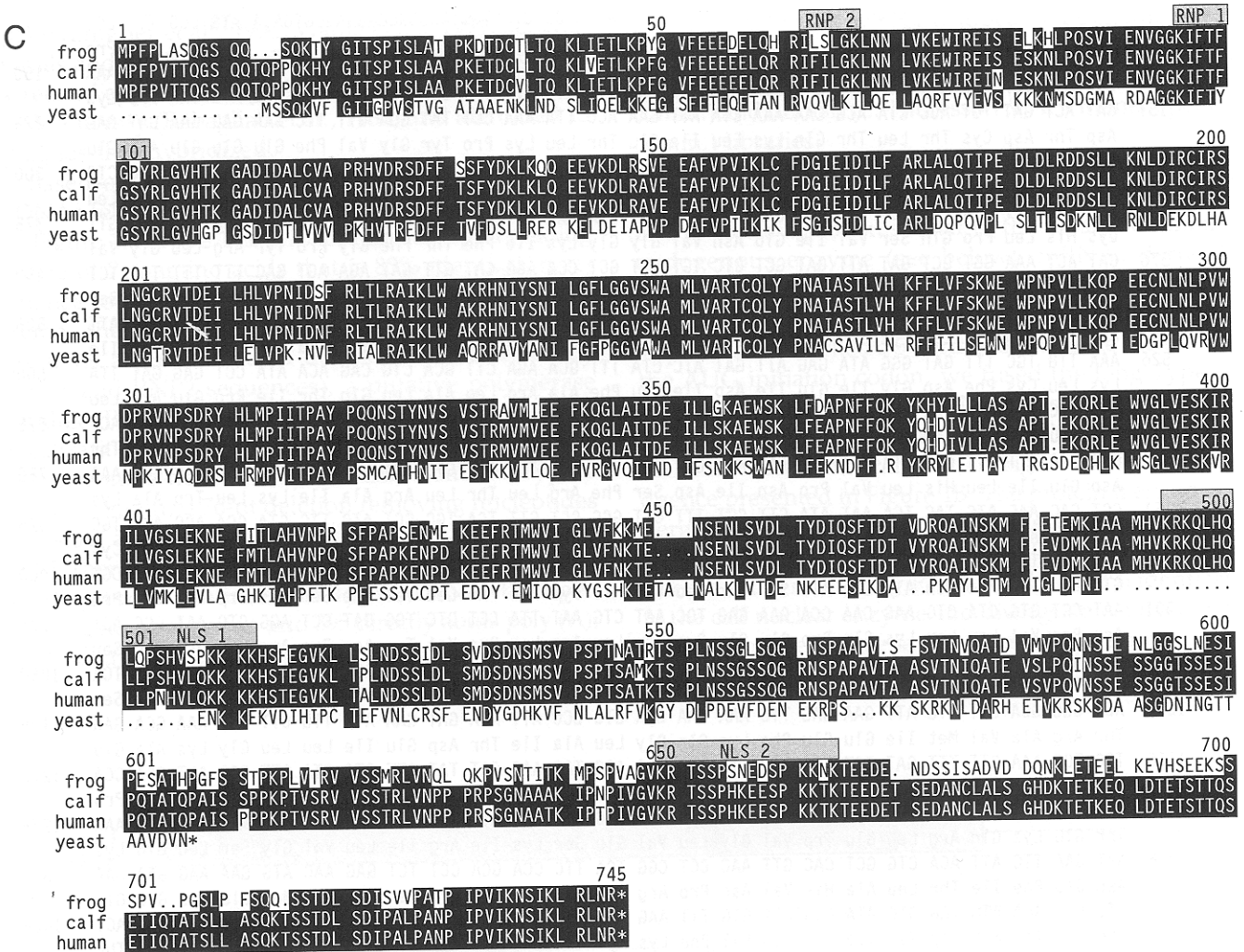


**FIGURE 1.** Frog poly(A) polymerase cDNAs. PAP-related cDNAs were isolated from a frog ovarian cDNA library. Three distinct classes of cDNA (Types 1, 2, and 3) were obtained. **A:** Schematic of the relationship between the three types of cDNAs isolated and the PAP1 mRNA. Boxes correspond to the predicted ORFs and the shading indicates regions of identical nucleotide sequence. Gray- and white-shaded regions are very similar to one another; Type 1 (gray box) and Type 2 (white box) sequences are 93% identical at both the nucleotide and amino acid levels. The location of putative RNA recognition motifs (RRM) and nuclear localization signals (NLS) are indicated. Positions of putative initiation (ATG, AUG) and termination (TAA, UAA) codons are indicated. Dashed lines correspond to nucleotide sequence not present in a cDNA but present in another. Lines indicate 5' and 3' UTRs. Nucleotides are numbered, with 1 designating the first nucleotide of Type 2 cDNA, extrapolated to Types 1 and 3 by alignment of identical regions. Type 1 cDNA lacks the 5'-most sequence present in Type 2, as indicated by dots at the end of the Type 1 structure. The missing sequence includes the first 13 amino acids of the Type 2 protein. In Type 2 cDNA, a deletion of 76 nt (indicated by dots between nt 384 and 461) generates a frameshift that would cause termination at nt 497, at the TAA indicated. The open reading downstream of that termination codon continues to be similar to Type 1 sequence throughout its length. (The TAA at the end of that open reading frame is indicated by a TAA in parentheses.) The open reading frame of Type 3 cDNA is identical to Type 2 up to nt 1171, except that Type 3 lacks the 76-nt deletion present in Type 2 and instead contains sequences similar to Type 1 (and other PAPs) in that region. At position 1171 of Type 3, the similarity between Type 3 and Types 1 and 2 ends. Thereafter, the Type 3 open reading frame diverges entirely (indicated by a cross-hatched box) and abuts the poly (A) tract directly, without an intervening termination codon. Types 1, 2, and 3 sequences are available through GenBank using accession numbers U19973 (Type 1), U19974 (Type 2), and U19975 (Type 3). (Panels B and C appear on the next two pages.)

<b>B</b>	1	CCCTCTTCTGCCGCTTCCCTTTGGAGTATCATGCCCTGCTGCTGCGGGCGGGGAGGGTGACA <b>ATG</b> CCG TTT CCT	75
		Met Pro Phe Pro	
76	CTT GCA AGC CAG GGA TCA CAG CAG TCA CAG AAG ACC TAT GGT ATT ACT TCA CCT ATT AGT TTA	GCC ACA CCC AAA	150
	Leu Ala Ser Gln Gly Ser Gln Gln Ser Gln Lys Thr Tyr Gly Ile Thr Ser Pro Ile Ser Leu Ala Thr Pro Lys		
151	GAT ACT GAT TGT ACC CTA ACG CAA AAA CTA ATT GAA ACC TTA AAA CCT TAT GGA GTT TTC GAA GAG GAA GAT GAG		225
	Asp Thr Asp Cys Thr Leu Thr Gln Lys Leu Ile Glu Thr Leu Lys Pro Tyr Gly Val Phe Glu Glu Glu Asp Glu		
226	TTA CAA CAT CGA ATT TTA AGT TTG GGG AAA TTA AAC AAC TTA GTA AAA GAA TGG ATC CGG GAA ATC AGT <u>GAG CTC</u>		300
	Leu Gln His Arg Ile Leu Ser Leu Gly Lys Leu Asn Asn Leu Val Lys Glu Trp Ile Arg Glu Ile Ser Glu Leu		
301	AAG CAT CTT CCA CAG TCT GTC ATT GAA AAT GTA GGT GGA AAG ATT TTT ACT TTT GGA CCA TAC CGA TTA GGT GTT		375
	Lys His Leu Pro Gln Ser Val Ile Glu Asn Val Gly Gly Lys Ile Phe Thr Phe Gly Pro Tyr Arg Leu Gly Val		
376	CAT ACT AAA GGT GCT GAT ATT GAT GCT CTC TGT GTT GCT CCA AGG CAT GTT GAT AGA AGT GAC TTT TTT TCA TCT		450
	His Thr Lys Gly Ala Asp Ile Asp Ala Leu Cys Val Ala Pro Arg His Val Asp Arg Ser Asp Phe Phe Ser Ser		
451	TTT TAT GAC AAG TTG AAG CAG CAG GAA GAA GTC AAA GAT TTG AGA TCT GTT GAA GAA GCA TTT GTA CCA GTG ATT		525
	Phe Tyr Asp Lys Leu Lys Gln Gln Glu Glu Val Lys Asp Leu Arg Ser Val Glu Glu Ala Phe Val Pro Val Ile		
526	AAA TTG TGC TTT GAT GGG ATA GAG ATT GAT ATC TTA TTT GCA AGA CTT GCA CTG CAG ACA ATA CCT GAG GAT TTA		600
	Lys Leu Cys Phe Asp Gly Ile Glu Ile Asp Ile Leu Phe Ala Leu Glu Thr Ile Pro Glu Asp Leu		
601	GAC CTC CGA GAT GAC AGC CTG CTG AAA AAT TTA GAC ATA CGA TGT ATA CGT AGT CTG AAT GGT TGC CGA GTG ACT		675
	Asp Leu Arg Asp Asp Ser Leu Leu Lys Asn Leu Asp Ile Arg Cys Ile Arg Ser Leu Asn Gly Cys Arg Val Thr		
676	GAT GAA ATT CTT CAT TTA GTA CCA AAC ATA GAC AGC TTC AGG TTA ACT CTC AGA GCA ATC AAA TTA TGG GCC AAA		750
	Asp Glu Ile Leu His Leu Val Pro Asn Ile Asp Ser Phe Arg Leu Thr Leu Arg Ala Ile Lys Leu Trp Ala Lys		
751	CGT CAC AAC ATC TAC TCA AAT ATA CTT GGT TTT CTT GGC GGT GTT TCA TGG GCT ATG TTG GTA GCA AGG ACA TGC		825
	Arg His Asn Ile Tyr Ser Asn Ile Leu Gly Phe Leu Gly Val Ser Trp Ala Met Leu Val Ala Arg Thr Cys		
826	CAA CTA TAT CCA AAT GCA ATT GCA TCG ACT CTT GTT CAT AAA TTT TTC CTA GTG TTT TCA AAA TGG GAA TGG CCT		900
	Gln Leu Tyr Pro Asn Ala Ile Ala Ser Thr Leu Val His Lys Phe Phe Leu Val Phe Ser Lys Trp Glu Trp Pro		
901	AAT CCT GTG CTA CTG AAG CAA CCA GAA GAG TGC AAT CTG AAT TTA CCT GTC TGG GAT CCT AGG GTG AAT CCC AGT		975
	Asn Pro Val Leu Leu Lys Gln Pro Glu Glu Cys Asn Leu Asn Leu Pro Val Trp Asp Pro Arg Val Asn Pro Ser		
976	GAT AGG TAC CAC CTT ATG CCT ATA ATC ACA CCA GCC TAC CCT CAG CAA AAT TCT ACA TAC AAT GTT TCG GTG TCA		1050
	Asp Arg Tyr His Leu Met Pro Ile Ile Thr Pro Ala Tyr Pro Gln Gln Asn Ser Thr Tyr Asn Val Ser Val Ser		
1051	ACT CGC GCA GTT ATG ATT GAA GAG TTC AAA CAA GGA CTG GCC ATT ACA GAT GAA ATC TTG CTT GGT AAA GCA GAG		1125
	Thr Arg Ala Val Met Ile Glu Glu Phe Lys Gln Gly Leu Ala Ile Thr Asp Gly Ile Leu Leu Gly Lys Ala Glu		
1126	TGG TCC AAA CTT TTT GAT GCT CCC AAC TTC TTT CAA AAG TAC AAG CAT TAT ATT CTC CTA CTT GCA AGT GCA CCA		1200
	Trp Ser Lys Leu Phe Asp Ala Pro Asn Phe Phe Gln Lys Tyr Lys His Tyr Ile Leu Leu Leu Ala Ser Ala Pro		
1201	ACG GAA AAA CAG CGG CTG GAA TGG GTA GGC TTG GTA GAG TCA AAG ATT CGC ATT CTT GTT GGA AGT TTA GAG AAA		1275
	Thr Glu Lys Gln Arg Leu Glu Trp Val Gly Leu Val Glu Ser Lys Ile Arg Ile Leu Val Gly Ser Leu Glu Lys		
1276	AAT GAA TTC ATT ACA CTG GCT CAC GTT AAC CCT CGG TCA TTT CCA GCA CCT TCT GAG AAC ATG GAA AAG GAA GAA		1350
	Asn Glu Phe Ile Thr Leu Ala His Val Asn Pro Arg Ser Phe Pro Ala Pro Ser Glu Asn Met Glu Lys Glu Glu		
1351	TTC CGT ACA ATG TGG GTC ATA GGG CTA GTG TTT AAG AAA ATG GAA AAT TCC GAA AAT TTG AGT GTT GAT CTA ACT		1425
	Phe Arg Thr Met Trp Val Ile Gly Leu Val Phe Lys Lys Met Glu Asn Ser Glu Asn Leu Ser Val Asp Leu Thr		
1426	TAT GAC ATT CAG TCT TTC ACT GAC ACA GTG GAC CGG CAG GCC ATT AAC AGC AAA ATG TTT GAA ACT GAA ATG AAA		1500
	Tyr Asp Ile Gln Ser Phe Thr Asp Thr Val Asp Arg Gln Ala Ile Asn Ser Lys Met Phe Glu Thr Glu Met Lys		
1501	ATT GCT GCA ATG CAC GTC AAG AGG AAG CAA CTC CAT CAA CTG CAA CCT AGT CAT GTG TCT CCC AAA AAG AAA AAG		1575
	Ile Ala Ala Met His Val Lys Arg Lys Gln Leu His Gln Leu Gln Pro Ser His Val Ser Pro Lys Lys Lys Lys		
1576	CAT TCA TTT GAA GGT GTA AAA CTG TTA TCT CTG AAT GAC AGC AGC ATT GAT TTA TCA GTG GAC AGT GAC AAC AGC		1650
	His Ser Phe Glu Gly Val Lys Leu Leu Ser Leu Asn Asp Ser Ser Ile Asp Leu Ser Val Asp Ser Asn Ser Ser		
1651	ATG TCT GTG CCT TCT CCT ACC AAT GCC ACA AGA ACC AGT CCA CTA AAT TCT TCT GGA TTG TCT CAG GGA AAC AGC		1725
	Met Ser Val Pro Ser Pro Thr Asn Ala Thr Arg Thr Ser Pro Leu Asn Ser Ser Gly Leu Ser Gln Gly Asn Ser		
1726	CCA GCA GCA CCA GTA TCC TTT TCT GTG ACC AAC GTC CAG GCT ACT GAT GTG ATG GTG CCA CAG AAT AAT TCC ACT		1800
	Pro Ala Ala Pro Val Ser Phe Ser Val Thr Asn Val Gln Ala Thr Asp Val Met Val Pro Gln Asn Asn Ser Thr		
1801	GAA AAT TTA GGG GGT AGT TTG AAT GAA AGC ATT CCT GAA TCT GCC ACA CAC CCA GGT TTT TCT TCA ACG CCA AAA		1875
	Glu Asn Leu Gly Gly Ser Leu Asn Glu Ser Ile Pro Glu Ser Ala Thr His Pro Gly Phe Ser Thr Pro Lys		
1876	CCT TTG GTG ACC CGA GTT GTT TCT TCC ATG CGT TTG GTT AAC CAA CTT CAA AAG CCT GTC TCA AAC ACA ATT ACA		1950
	Pro Leu Val Thr Arg Val Val Ser Ser Met Arg Leu Val Asn Gln Leu Gln Lys Pro Val Ser Asn Thr Ile Thr		
1951	AAA ATG CCC AGT CCA GTG GCA GGT GTG AAA AGG ACA TCT TCT CCT TCG AAT GAA GAC AGC CCC AAA AAA AAC AAA		2025
	Lys Met Pro Ser Pro Val Ala Gly Val Lys Arg Thr Ser Ser Pro Ser Asn Glu Asp Ser Pro Lys Lys Asn Lys		
2026	ACA GAG GAG GAT GAA AAT GAT AGC AGT ATT TCC GCA GAC GTG GAT GAT CAA AAC AAA TTA GAA ACT GAA GAA CTT		2100
	Thr Glu Glu Asp Glu Asn Asp Ser Ser Ile Ser Ala Asp Val Asp Asp Gln Asn Lys Leu Glu Thr Glu Glu Leu		
2101	AAG GAA GTC CAT AGC GAA GAA AAA TCC TCA TCC CCT GTG CCA GGT TCA CTG CCA TTC TCT CAG CAA TCA AGT ACA		2175
	Lys Glu Val His Ser Glu Glu Lys Ser Ser Ser Pro Val Pro Gly Ser Leu Pro Phe Ser Gln Gln Ser Ser Thr		
2176	GAT CTG TCT GAT ATT TCT GTG GTC CCT GCT ACT CCT ATT CCT GTT ATC AAG AAT TCA ATA AAA CTA AGA CTG AAC		2250
	Asp Leu Ser Asp Ile Ser Val Val Pro Ala Thr Pro Ile Pro Val Ile Lys Asn Ser Ile Lys Leu Arg Leu Asn		
2251	CGA <b>TAA</b> ACACAATTTCCCTAGGGTCCATAAACATGTCACCAGTTTCAGCTTTTTTCTGCTGCAAGGCCAAACTGCGGAGGTTAGCAGGGCACA		
	Arg End		
	GCCGCTGACACTTTAGACTTTTATTCTGAAGGTGTCCTGGGCTACAGGCAGCCTTTCTGGAAGTCCAGGTTAGTATGACGAGAATAAAGTGCTATTAAT		
	GTGTTGACAGCATAAAGGACTCTATTCCCTTTCAAGAGAGATGAAAAAATATTACTGCATATCTGTCTGACTTAACATTCTGACTGAAATAAT		
	GGTTGATGTTTATTGCAAAATACAACCTGGCCTAAAACTTAAAAAATAAAAAA		

2601

**FIGURE 1.** Continued. **B:** Nucleotide sequence and conceptual translation of the PAP1 mRNA. The *Sst* I site used to join the 5' Type 2 sequence and 3' Type 1 sequence is underlined. Numbering as in panel A.



**FIGURE 1.** Continued. **C:** Alignment of the predicted frog PAP1 protein with homologs from calf, human, and yeast (*Saccharomyces cerevisiae*) cells. Boxed regions correspond to amino acids conserved between two or more sequences. Amino acids are numbered with 1 designating the initiating methionine of mammalian PAPs. Positions of putative RNP and NLS domains are indicated.

### Maternal mRNA encoding PAP

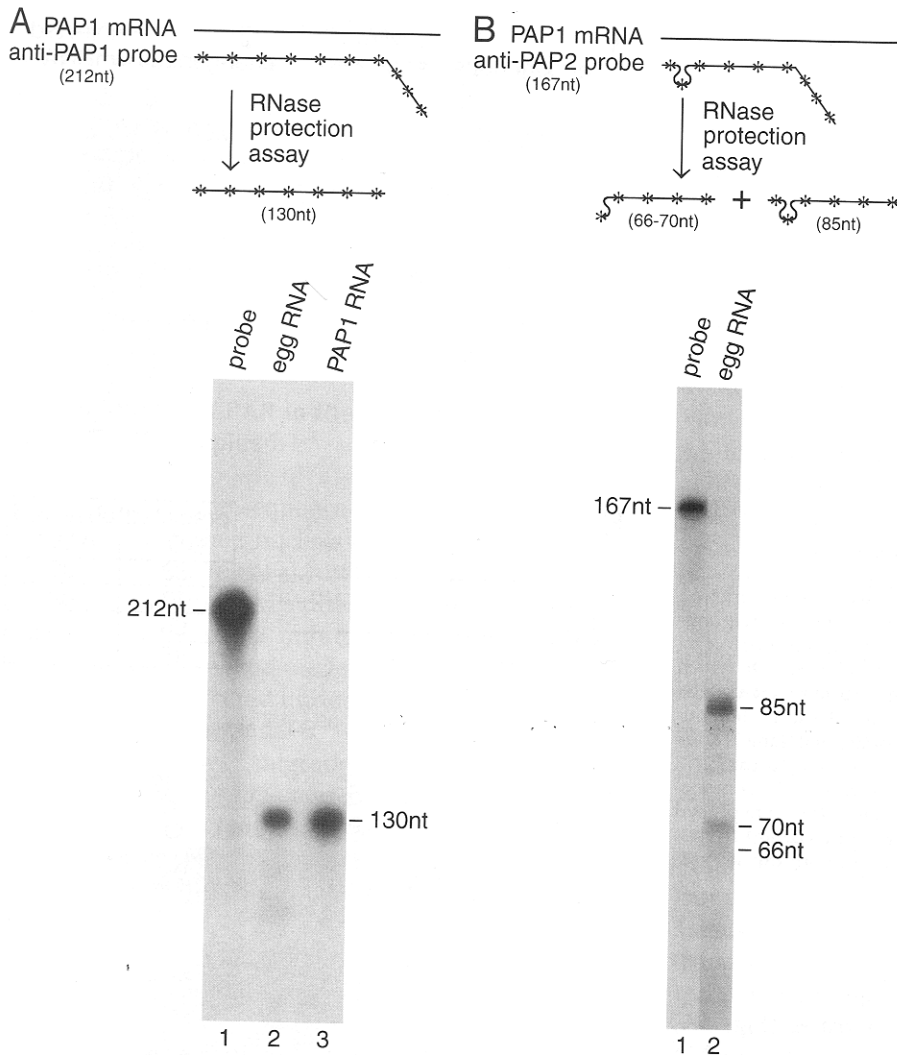
To determine whether PAP mRNA was a maternal mRNA, we performed RNase mapping experiments using RNA from frog eggs and a labeled RNA probe derived from the Type 1 sequence (Fig. 2A). The probe was hybridized either to total egg RNA (Fig. 2A, lane 2) or to synthetic RNA prepared by transcription of Type 1 cDNA in vitro (Fig. 2A, lane 3). The hybridization mixture was treated with single-strand-specific RNases, and the products were analyzed by electrophoresis and autoradiography. Egg RNA protects 130 nt of the probe, corresponding to the entire region of PAP sequence (Fig. 2A, lane 2); the protected species comigrates with the band protected by the synthetic Type 1 transcript (Fig. 2A, lane 3). We conclude that frog eggs contain a maternal mRNA encoding a PAP.

To confirm the presence of Type 1 mRNA, and to distinguish it from Types 2 and 3, RNase mapping experiments were repeated using a probe derived from

Type 2 sequence. A labeled RNA probe was prepared corresponding to a portion of the Type 2 cDNA sequence (Fig. 2B, lane 1). Type 1 mRNA lacks three nucleotides that are present in the Type 2 and 3 sequence in this region. As a result, Type 1 mRNAs will protect bands of between 66 and 70 nt, whereas Type 2 and 3 mRNAs will protect 85 nt (see diagram in Fig. 2B). Egg RNA yields two bands of 66 and 70 nt (Fig. 2B, lane 2), which comigrate with the marker prepared using synthetic Type 1 mRNA (data not shown). An 85-nt band also is detected; this may correspond to either Type 2 or 3 mRNAs or to incomplete digestion of hybrids formed with Type 1 mRNA. We conclude that Type 1 mRNA is present as a maternal mRNA in frog eggs.

### PAP proteins in frog oocytes

To detect endogenous PAP(s) in oocytes, we performed immunoblotting experiments using an anti-



**FIGURE 2.** Maternal mRNA encoding Type 1 poly(A) polymerase. RNase protection experiments were performed using uniformly labeled RNA probes complementary to either Type 1 (A) or Type 2 (B) sequences. Probes were annealed either to total egg RNA or to synthetic RNA corresponding to Type 1 mRNA. As diagrammed at the top of the figure, Type 1 mRNA should protect 130 nt of the 212-nt Type 1 probe but should protect only 66–70 nt of the 167-nt Type 2 probe (see text and Fig. 1 sequences for details). **A:** Type 1 probe. Lane 1, probe prior to hybridization or RNase digestion; lane 2, probe annealed to egg RNA; lane 3, probe annealed to synthetic Type 1 mRNA. **B:** Type 2 probe. Lane 1, probe prior to hybridization and RNase digestion; lane 2, probe annealed to egg RNA.

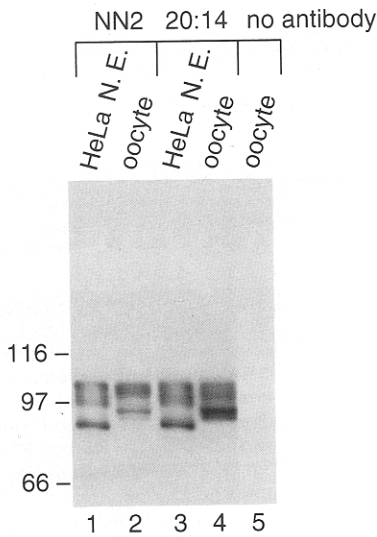
PAP monoclonal antibody, NN2, as the probe (Fig. 3). This antibody was raised against, and specifically reacts with, the amino-terminal half of the human nuclear enzyme (Thuresson et al., 1994). Proteins present in unfractionated oocyte homogenates were separated by SDS-PAGE, transferred to Immobilon P paper, and incubated with the NN2 antibody. Sites to which the NN2 antibody had bound were detected using anti-mouse immunoglobulin coupled to peroxidase and a chemiluminescent substrate.

Several cross-reacting protein species in frog oocyte extracts were detected by the NN2 monoclonal (Fig. 3). These include proteins with apparent molecular weights of 93, 96, 103, and 106 kDa (Fig. 3, lane 2). Immunoblotting of HeLa nuclear extract yields a pattern of bands that are similar, but not identical (Fig. 3, lane 1). A second, independently derived anti-PAP monoclonal, designated 20:14, detects the same bands in both extracts (Fig. 3, lanes 3, 4). No bands are detected in oocyte extracts if the mouse monoclonal is omitted prior to incubation with the anti-mouse immu-

noglobulin (Fig. 3, lane 5). These data strongly suggest that frog oocytes contain multiple forms of PAP that are antigenically related to the mammalian enzyme.

Frog oocytes are surrounded by thousands of somatic follicle cells. To test whether any of the proteins detected by the NN2 monoclonal were somatic in origin, extracts were prepared from oocytes that had been treated with collagenase under conditions in which greater than 99% of the follicle cells were removed (as judged by nuclear staining with Hoechst 33258). The distribution of bands in the immunoblot was unaffected by this treatment (data not shown; see Fig. 4).

The protein encoded by the Type 1 cDNA sequence comigrates with endogenous PAPs (Fig. 4A). PAP1 mRNA (see Fig. 1) was incubated in a rabbit reticulocyte lysate in vitro translation system (Fig. 4A, lane 2) or injected into frog oocytes (Fig. 4A, lane 4). Translation products were detected by immunoblotting using the NN2 monoclonal antibody. In the in vitro translation system, PAP1 mRNA produces a prominent band, detected by the antibody, that co-migrates with the

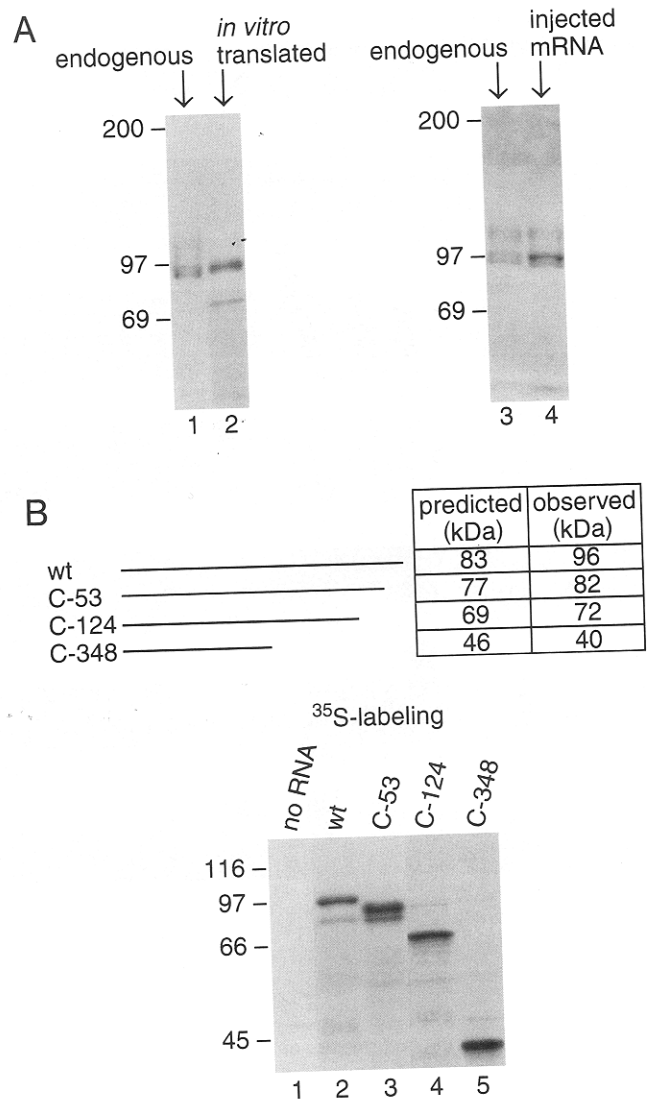


**FIGURE 3.** Immunoblot of poly(A) polymerase proteins in frog oocytes. Two different anti-poly(A) polymerase monoclonal antibodies, designated NN2 and 20:14, were used in immunoblotting (procedure described in the Materials and methods). Sites to which the antibodies had bound were detected using peroxidase-conjugated secondary antibody and a luminescent substrate. Lane 1, HeLa nuclear extract (40  $\mu$ g) probed with the NN2 monoclonal; lane 2, total protein ( $\sim$ 20  $\mu$ g) from a single frog oocyte probed with the NN2 monoclonal; lane 3, HeLa nuclear extract probed with the 20:14 monoclonal; lane 4, total protein ( $\sim$ 20  $\mu$ g) from a single frog oocyte probed with the 20:14 monoclonal; lane 5, total protein ( $\sim$ 20  $\mu$ g) from a single oocyte incubated with no monoclonal, but with the secondary antibody and luminescent substrate.

96-kDa species detected in oocyte extracts (Fig. 4A, lanes 1, 2). Similarly, oocytes injected with this mRNA contain more of the 96-kDa species than do mock-injected cells (Fig. 4A, lanes 3, 4), presumably due to the synthesis of additional 96-kDa protein. This result is confirmed below using synthetic mRNAs encoding an epitope-tagged protein (see Fig. 7).

The electrophoretic mobility of the protein produced from PAP1 mRNA indicated a molecular weight of 96 kDa, whereas the protein predicted by conceptual translation should be 83 kDa. The anomalously slow mobility is due to the carboxy-terminal region of the protein (Fig. 4B). Synthetic mRNAs were prepared that lacked varying lengths of the C-terminal region of the open reading frame. These were translated in vitro, and their mobility was determined by comparison to molecular weight standards. Newly synthesized protein was detected either using  $^{35}$ S-Met/Cys in the translation mixture (Fig. 4B, lanes 1–4) or by immunoblotting (data not shown). The carboxy-terminal truncations restored electrophoretic mobility to that predicted from the DNA sequence. With extreme truncations, the mobility is, in fact, slightly faster than predicted.

Each of the proteins detected by  $^{35}$ S-Met labeling (Fig. 4B, lanes 1–4) is also recognized by the NN2 anti-



**FIGURE 4.** Type 1 poly(A) polymerase protein comigrates with an endogenous poly(A) polymerase present in oocytes and has a mobility slower than predicted. Capped PAP1 mRNA was prepared by transcription in vitro and used for translation in a rabbit reticulocyte lysate or injected oocytes. **A:** Proteins produced by translation of PAP1 mRNA were compared to the endogenous poly(A) polymerases present in oocytes by immunoblotting with the NN2 antibody as in Figure 3. Lanes 1 and 3, total protein from a single oocyte; lane 2, poly(A) polymerase ( $\sim$ 10 fmol) obtained by translation of the PAP1 mRNA in vitro using a rabbit reticulocyte lysate; lane 4, total protein from a single oocyte previously injected with the translatable type 1 mRNA. **B:** Fluorograph comparing the electrophoretic mobilities of full-length and C-terminal deletions of the Type 1 poly(A) polymerase. Proteins were prepared by in vitro translation of PAP1 mRNAs ending at various restriction sites within the ORF (see Materials and methods). Lane 1, no mRNA; lane 2, *Sac* II run-off mRNA; lane 3, *Afl* II run-off mRNA; lane 4, *Alw*NI run-off mRNA. As diagrammed at the top of the figure, deletion of increasing amounts of the C-terminus restores the observed electrophoretic mobility to that predicted from the conceptual translation.

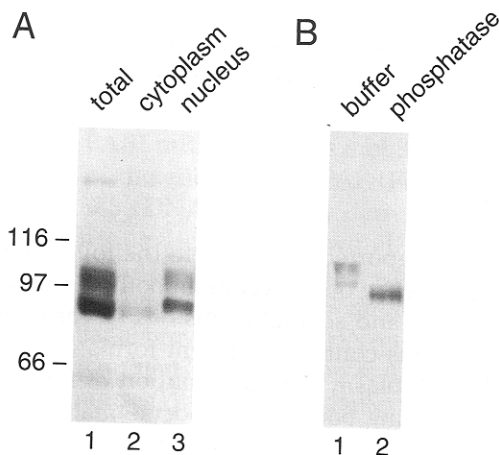
body (data not shown). These results provide further evidence for the specificity of the NN2 antibody and confirm that the epitope is present in the first 425 amino acids of the protein.

To estimate the amount of PAP protein present in an oocyte, various amounts of an *in vitro* translation mixture were used to generate a standard curve for the immunoblotting assay. The results (not shown) indicate that a single oocyte contains approximately 10 fmol of PAP, corresponding to approximately  $10^9$  molecules.

From these results, we conclude that oocytes contain several forms of PAP that differ in electrophoretic mobility, and that the 96-kDa species co-migrates with the protein encoded by Type 1 cDNA.

### Different forms of PAP in the nucleus and cytoplasm

Frog oocytes contain sequence-specific polyadenylation activities in both their nucleus and cytoplasm (Wickens & Gurdon, 1983; Fox et al., 1989; see Introduction). To determine whether PAPs differ in these two compartments, we performed immunoblotting experiments using manually isolated nuclei and cytoplasm (Fig. 5). Oocytes were dissected under mineral oil to minimize leakage of nuclear contents. Isolated nuclei contained primarily the slower migrating forms of PAP, corresponding to the 96-, 103-, and 106-kDa species (Fig. 5A, lane 3), whereas cytoplasm contained primarily the 93-kDa species (Fig. 5A, lane 2). In some experiments, we detect both the 93- and 96-kDa species in isolated cytoplasm.



**FIGURE 5.** Different forms of poly(A) polymerase in the nucleus and cytoplasm. **A:** Immunoblot of total, cytoplasmic, and nuclear oocyte extracts. Proteins from the various cell fractions, prepared as described in the Materials and methods, were analyzed by immunoblotting with the NN2 monoclonal as in Figure 3. Lane 1, total protein from a single oocyte; lane 2, total protein from a single oocyte cytoplasm; lane 3, total protein from a single oocyte nucleus. **B:** Immunoblot of oocyte nuclear poly(A) polymerases before and after dephosphorylation with alkaline phosphatase. Nuclear extracts were adjusted to pH 8.5 and incubated without and with alkaline phosphatase as described in the Materials and methods. Protein corresponding to one nucleus was then analyzed by immunoblotting with the NN2 monoclonal. Lane 1, no phosphatase; lane 2, phosphatase.

The nuclear forms of PAP are phosphorylated. Treatment of isolated nuclear proteins with bacterial alkaline phosphatase collapses the collection of PAP forms into a single, faster migrating electrophoretic species (Fig. 5B, lane 2 versus lane 1). Control incubations containing buffer but no enzyme have no effect (Fig. 5B, lane 1).

Based on these data, we conclude that the forms of PAP detected in nuclear and cytoplasmic extracts differ. These differences could, in principle, arise artifactually during extract preparation (i.e., by dephosphorylation or proteolysis in cytoplasmic homogenates). However, neither the amount nor the distribution of proteins detected is affected by the inclusion of high concentrations of a collection of protease and phosphatase inhibitors throughout extract preparation (not shown). These data suggest that the differences we detect in nuclear and cytoplasmic fractions exist *in vivo*.

### Changes in PAP during maturation and early embryogenesis

During early development, cytoplasmic polyadenylation first occurs during oocyte maturation. To determine whether PAP changes during this interval, or at any time during early development, we performed immunoblotting experiments using crude extracts prepared from oocytes and embryos at various developmental stages.

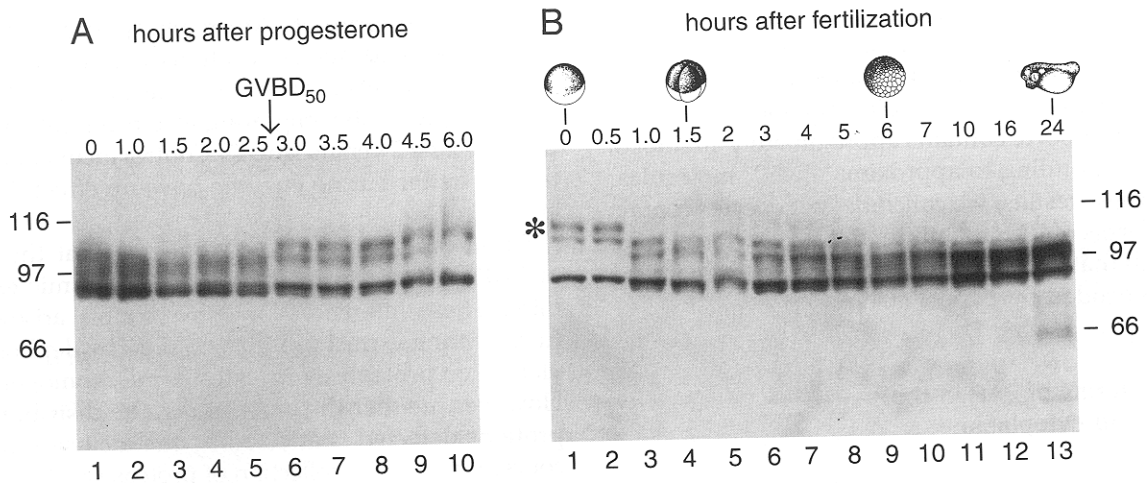
During progesterone-induced maturation, the pattern of proteins detected by the NN2 monoclonal changes, shifting to slower electrophoretic mobilities (Fig. 6A). The amount of the 93-kDa species decreases concomitant with an increase in the amount of the 96-kDa and very slow migrating species. The total amount of PAP detected does not change dramatically. Alterations in electrophoretic mobility first appear at the time the nucleus breaks down (2.5–3 h in Fig. 6). By the end of maturation (Fig. 6A, lane 10), as in the unfertilized egg (Fig. 6B, lane 1), forms of PAP with much slower mobility than any in the non-mature oocyte were detected (Fig. 6, asterisk).

During early postfertilization development, the slowest migrating forms present in the egg progressively disappear, with concomitant increases in faster-migrating species (Fig. 6B). The onset of the mobility shift is remarkably rapid, first being detectable only 0.5 h after fertilization, well before first cleavage. It is complete by 3–4 h. The total amount of PAP does not increase significantly until 7–10 h after fertilization, coinciding approximately with the time at which the zygotic genome is activated (ca. 7 h).

### Phosphorylation of PAP during oocyte maturation

To determine whether the changes in electrophoretic mobility during oocyte maturation were due to phos-





**FIGURE 6.** Changes in poly(A) polymerase during maturation and early embryogenesis. Immunoblot of NN2-detectable poly(A) polymerases present at various times during meiotic maturation (A) and early embryogenesis (B). **A:** Meiotic maturation of isolated oocytes was initiated by addition of progesterone to the incubation media. At the indicated times, cells were frozen and subsequently analyzed immunoblotting with the NN2 monoclonal. Each lane contains one oocyte equivalent of protein. Nuclear envelope breakdown was scored by the appearance of a white spot at the animal pole. The time at which 50% of the isolated cells showed such a spot is indicated by GVBD<sub>50</sub>. **B:** Frog eggs were fertilized in vitro (procedure described in the Materials and methods). At the indicated times, eggs or embryos were dejellied, frozen, and subsequently analyzed by immunoblotting with the NN2 monoclonal. Each lane contains one egg or embryo equivalent of protein. Morphology and synchrony of the developing embryos were monitored by microscopy. Lanes corresponding to stage 0 (1-cell), 1.5 (4-cell), 6 (>4,000-cell), and 22–24 (tail-bud) embryos (Nieuwkoop & Faber, 1956) are indicated at the top of the figure.

phorylation, we injected HA-PAP1 mRNA, in which the PAP1 mRNA sequence is coupled to a sequence encoding a hemagglutinin epitope tag. We first determined whether the tagged protein recapitulated the behavior of the endogenous enzyme. Oocytes injected with the mRNA were incubated in progesterone for various durations. Protein produced by the injected mRNA was analyzed by immunoblotting with an anti-hemagglutinin antibody (Fig. 7A), whereas endogenous PAP present in mock-injected cells was analyzed using the anti-PAP monoclonal (Fig. 7B). Qualitatively, the endogenous and tagged proteins behaved similarly, shifting in mobility at the same time and to similar extents. Most of the tagged protein shifts, whereas only a fraction of the endogenous protein does, implying a distinction between newly synthesized PAP and the PAP that was accumulated during oogenesis (see Discussion). Similarly, the injected mRNA generates little of the most slowly migrating form detected among endogenous proteins.

To determine whether PAP is phosphorylated during maturation, oocytes were injected with HA-PAP1 mRNA and incubated in either <sup>35</sup>S-Met/Cys (Fig. 8A) or <sup>32</sup>P-orthophosphate (Fig. 8B). Oocytes injected with the mRNA produced an <sup>35</sup>S-labeled protein, which is immunoprecipitated by the hemagglutinin antibody (Fig. 8A, lane 3). This protein, which is not detected in mock-injected oocytes (Fig. 8A, lanes 1, 2), shifts in mobility during maturation (Fig. 8A, lanes 3, 4). The shifted protein is labeled by <sup>32</sup>P-orthophosphate

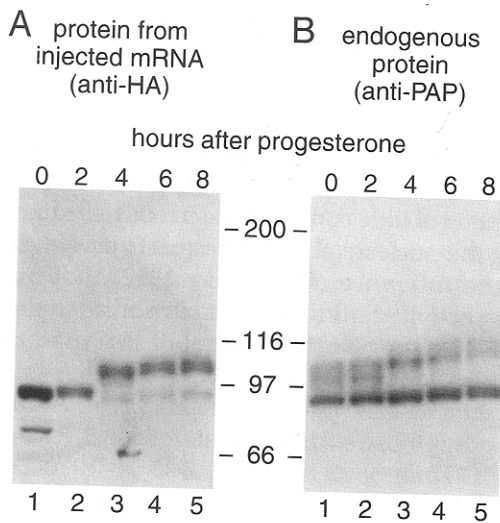
present during progesterone-induced maturation but not in the absence of progesterone (Fig. 8B, lanes 3, 4). This protein is not detected in oocytes that were not injected (Fig. 8B, lanes 1, 2). We conclude that, during oocyte maturation, PAP undergoes phosphorylation. This conclusion is corroborated by the finding that treatment of crude egg extracts with alkaline phosphatase accelerates the electrophoretic mobility of endogenous PAPs (not shown).

## DISCUSSION

The studies reported here lead to the following main conclusions. Frog oocytes contain both an mRNA that encodes PAP and several forms of PAP protein. The oocyte nucleus contains different forms of PAP than does the cytoplasm. The distribution of forms is dynamic, changing both during oocyte maturation and shortly after fertilization. These changes are due, at least in part, to regulated phosphorylation. Maternally encoded PAP is strikingly similar in sequence to mammalian nuclear PAPs.

### Poly(A) polymerases in frog oocytes

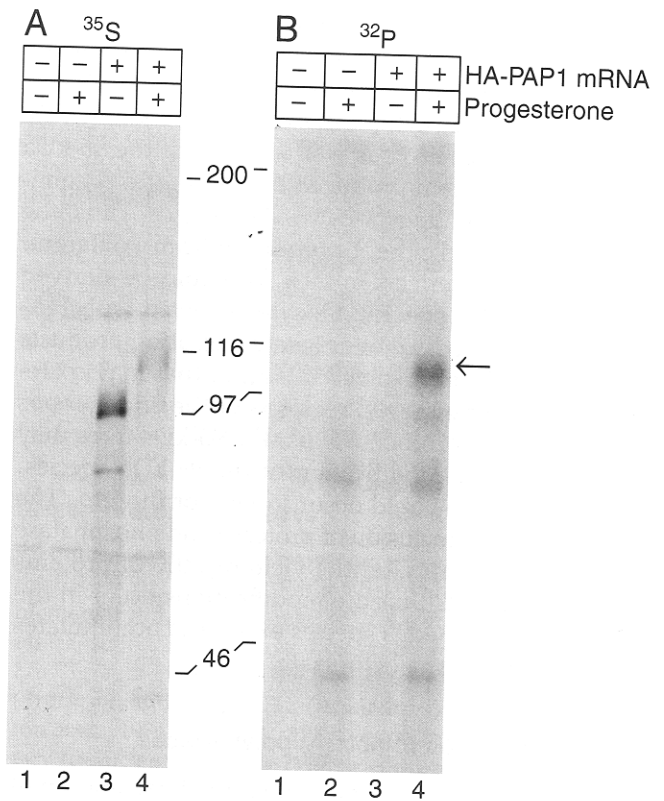
Several findings support the view that the Type 1 cDNA described in Figure 1 represents a functional PAP homolog. First, RNase protection experiments confirm the presence of Type 1 mRNA in total egg RNA (Fig. 2).



**FIGURE 7.** Epitope-tagged Type 1 poly(A) polymerase changes in mobility during oocyte maturation coincident with changes in the endogenous proteins. Immunoblots comparing the mobilities of exogenous (A) and endogenous (B) poly(A) polymerases during oocyte maturation. A: Synthetic mRNA was prepared containing the sequence of PAP1 mRNA connected to an N-terminal hemagglutinin epitope recognized by the anti-HA antibody (12CA5) (see Materials and methods). Oocytes injected with this mRNA were incubated for 2 h, then treated with progesterone. At the indicated times, cells were frozen and subsequently analyzed by immunoblotting with the anti-HA (12CA5) monoclonal. B: Uninjected oocytes were treated with progesterone. At the indicated times, cells were frozen and subsequently analyzed by immunoblotting with the NN2 monoclonal. Nuclear envelope breakdown was undetectable at 2 h, and complete after 4 h in both groups. One cell equivalent of protein from each sample was analyzed.

Second, of the cDNAs examined, only Type 1 contained an open reading frame capable of producing protein of the size detected immunologically in oocytes (Fig. 3A). Third, during oocyte maturation, the protein encoded by PAP1 mRNA exhibits mobility shifts similar to those of the endogenous PAP (Fig. 7).

Type 2 and 3 cDNAs are likely derived from a second, nonfunctional gene. The two cDNAs differ by only 1 nt over the first 1,171 nt, after which the Type 3 sequence contains a short (77-nt) 3' stretch followed by poly A. Similar abbreviated PAP cDNAs have also been isolated from human (L. Thuresson & A. Virtanen, unpubl.) and mouse (W. Zhao & J. Manley, pers. comm.) libraries. The mouse cDNA likely arises from cleavage and polyadenylation downstream of AAUAAA sequences present within introns (W. Zhao & J. Manley, pers. comm.). The gene from which Type 2 and possibly Type 3 mRNAs arose is likely to be nonfunctional because the Type 2 cDNA contains two deletions, both of which result in frameshifts followed by premature termination codons. Proteins of the size predicted by either the Type 2 or Type 3 ORF have not been detected immunologically in oocytes. Although precedence exists for the existence of duplicated and mutated genes in the partially tetraploid *Xenopus* genome (reviewed



**FIGURE 8.** Phosphorylation of poly(A) polymerase during oocyte maturation. Immunoprecipitation experiments were performed on cells incubated either in <sup>35</sup>S-Met/Cys (A), or <sup>32</sup>P-orthophosphate (B). Oocytes were injected with synthetic mRNA encoding the epitope-tagged variant of PAP1 or left uninjected, as diagrammed. Both control and injected cells were incubated in the presence and absence of progesterone as indicated. Radiolabeled proteins (2.5 cell equivalents) were recovered with the anti-HA monoclonal and analyzed by SDS-PAGE (see Materials and methods). A: <sup>35</sup>S-labeled proteins. Lane 1, uninjected, no progesterone; lane 2, uninjected, progesterone treated; lane 3, injected, no progesterone; lane 4, injected, progesterone treated. B: <sup>32</sup>P-labeled proteins. Lane 1, uninjected, no progesterone; lane 2, uninjected, progesterone treated; lane 3, injected, no progesterone; lane 4, injected, progesterone treated.

in Kobel & Du Pasquier, 1986), the fact that PAP genes from other species yield comparable unusual mRNAs suggests it is characteristic of the PAP gene rather than the species.

Prior to maturation, oocytes contain multiple forms of PAP, with apparent molecular weights ranging from 93 to 106 kDa (Fig. 3). These different forms may arise by posttranslational modification of a single polypeptide, consistent with the observation that, during maturation, protein produced from a single injected mRNA species becomes heterogenous in electrophoretic mobility. Before maturation, however, the slower migrating forms of the endogenous protein (i.e., 103 and 106 kDa) are not detectably produced from injected PAP1 mRNA (Figs. 2A, 7). Nonetheless, these slow migrating forms may be derived from the same polypeptide; the necessary modifications may occur only early in oogenesis and so not take place in a fully grown oocyte.

The nature of the cytoplasmic form(s) of PAP is particularly germane to the polyadenylation and translational activation of mRNAs prior to nuclear breakdown. Extracts of isolated cytoplasm contain predominantly a 93-kDa form of PAP, as well as some of the 96-kDa species. The protein produced by PAP1 mRNA comigrates with the endogenous 96-kDa species. Thus, the 93-kDa form could either be produced from a different, as yet uncharacterized mRNA, or could be derived from the 96-kDa species. Oocytes injected with HA-PAP1 mRNA produce two closely migrating products on SDS-PAGE (unresolved in Fig. 7) that may correspond to epitope-tagged versions of the endogenous 93- and 96-kDa proteins. Thus, the 93-kDa form may be generated by modification of the 96-kDa species. The modification could occur *in vivo* or *in vitro*. The observation that inclusion of protease and phosphatase inhibitors does not alter the yield or ratio of proteins detected suggests that the modifications occur in the cell, and that a 93-kDa species exists and accumulates preferentially in the cytoplasm.

#### Dynamic changes in poly(A) polymerases

During oocyte maturation, neither the total amount of PAP protein (Fig. 6A) nor the level of detectable PAP activity (Fox et al., 1992) changes dramatically. However, our data demonstrate two qualitative changes in PAP that may be significant in regulating cytoplasmic polyadenylation, namely, subcellular distribution and regulated modification.

#### Subcellular distribution

We previously suggested that both nuclear and cytoplasmic polyadenylation may be catalyzed by similar, or identical, enzymes present in both compartments before maturation (Fox et al., 1992). Our present results demonstrate that related proteins are indeed found in the nucleus and cytoplasm. Similarly, in somatic cells, different forms of the enzyme are detected in nuclear and cytoplasmic fractions; however, in contrast to the situation in oocytes, in somatic cells the slower migrating, modified forms appear to be cytoplasmic, not nuclear as in oocytes (Thuresson et al., 1994).

PAP activity is not limiting for the activation of maturation-specific polyadenylation *in vitro* in oocyte extracts (Fox et al., 1992). These extracts are derived from whole cells, containing both nuclear and cytoplasmic factors and so, as noted previously (Fox et al., 1992), may not perfectly reflect the situation *in vivo*. Our results further caution that this may be so. The oocyte nucleus contains different forms of PAP than does the cytoplasm. Thus, changes in the amount or nature of PAP could contribute to the regulation of polyadenylation during maturation. For example, once the nucleus breaks down, previously cytoplasmic mRNAs

will be exposed to more PAP. This might contribute to polyadenylation of those mRNAs, such as cyclin A1, which receive poly (A) late during maturation (Sheets et al., 1994). Alternatively, qualitative changes in the enzyme present in the cytoplasm prior to maturation could have significant effects on polyadenylation *in vivo* but be of little consequence in oocyte extracts containing the nuclear species. These data do not compromise the inference (Fox et al., 1992) that an RNA binding activity limits cytoplasmic polyadenylation in oocytes but suggest that control of PAP may also be important.

#### Modification

Our results demonstrate that the distribution of PAP forms is regulated and changes during development. PAP is phosphorylated both before and during oocyte maturation. Before maturation, in a resting oocyte, the nucleus contains phosphorylated forms of PAP. During maturation, PAP undergoes further phosphorylation, first detectable when the nucleus breaks down. At the end of maturation, as in an unfertilized egg, a very slowly migrating form of PAP accumulates (Fig. 6, asterisk). This form disappears very soon after fertilization, suggesting that it may play a role in the early postfertilization control of polyadenylation. In mammalian cells, PAP also is phosphorylated, most likely in the C-terminal S/T-rich region (Raabe et al., 1994). Regulation of phosphorylation events has not been observed, however.

Newly synthesized and endogenous polymerases may differ. In particular, whereas most of the protein produced from injected PAP1 mRNA undergoes modification, during maturation, only a fraction of the endogenous protein does (Fig. 7). This difference could reflect the preferential accessibility of newly synthesized protein to the phosphorylation apparatus. Alternatively, the endogenous 93-kDa protein detected immunologically may be heterogeneous in sequence, such that only certain species (including the Type 1 form) are able to undergo modification.

The regulated modifications of oocyte and egg polymerases could influence their intrinsic enzymatic activities, or their ability to interact with sequence specificity factors, such as CPSF (Bilger et al., 1994) or CPE-binding proteins (McGrew & Richter, 1990; Paris et al., 1991; Hake & Richter, 1994). Although modification of the mammalian enzyme is not absolutely required for polymerase activity (as bacterially expressed protein is active), quantitative effects on activity may well have escaped notice.

An alternative view of the modifications during oocyte maturation is that they reflect the transition of cells from interphase to metaphase. As oocytes advance through maturation, they leave first meiotic interphase and arrest in second meiotic metaphase. Modification

of PAP during maturation could be a direct and universal consequence of entry into M-phase and might occur in somatic cells as well. In this view, modification would be a consequence of the cell cycle rather than (or in addition to) a mechanism by which to affect control of polyadenylation. The presence of multiple p34<sup>cdc2</sup> kinase sites in the frog Type 1 PAP is consistent with, but only weakly supports, this hypothesis. A second kinase, SRPK1, has recently been implicated in the M-phase specific modification of the SR class of splicing factors and in the redistribution of those factors to daughter cells (Gui et al., 1994). PAP, another nuclear pre-mRNA processing factor, may also be a target for this kinase, as it contains a putative SRPK1 phosphorylation site (S/T-P-X-R/K) within its second nuclear localization signal.

## MATERIALS AND METHODS

Unless indicated, all chemicals were purchased from Sigma or Fisher.

### Cloning and sequencing of frog PAP cDNAs

An alignment of the predicted amino acid sequences of the bovine (Wahle et al., 1991) and yeast (Lingner et al., 1991) poly(A) polymerase was used to identify four blocks of five or more conserved residues. Degenerate oligonucleotides corresponding to the sense strand of the two amino-terminal-most blocks and the anti-sense strand of the two more carboxyl blocks were synthesized with cleavage sites for *Sac* II or *Eco* RI near their 5' ends. The sequences of the oligonucleotides are indicated below, with degenerate positions enclosed by parentheses.

sense I	AACCGCGGT(ACGT)AC(ACGT)GA(CT)GA(AG)AT
sense II	AACCGCGGC(ACGT)AT(ACT)AA(AG)(CT) T(ACGT)TGGGC
anti-sense I	CCCGAATTCTT(ACGT)(GC)(AT)(CT)TC(ACGT) AC(ACGT)A(AG)(ACGT)CC
anti-sense I	CCCGAATTCGG(AG)TA(ACGT)GC(ACGT) GG(ACGT)AGT)AT(ACGT)A(CT)(ACGT)GGCAT

The sense and anti-sense I oligonucleotide pair were used in a PCR-containing template DNA produced by random hexamer-primed reverse transcription of total *Xenopus* egg RNA and the GeneAmp RNA PCR kit (Perkin-Elmer Cetus). Products from this reaction were then used as templates in a second PCR reaction primed with the sense and anti-sense II oligos and yielding a 279-base pair product. This product was subcloned into the *Eco* RI and *Sac* II sites of pBluescript II (Stratagene) to give pSB3.

PAP-homologous cDNAs were isolated from a *X. laevis* ovary cDNA library, constructed in  $\lambda$ gt-10 (Rebagliati et al., 1985). Plating and duplicate filter preparation were as described (Sambrook et al., 1989). A random-primed DNA probe was prepared using agarose gel-purified insert from pSB3, [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham), and the Prime-It labeling kit (Stratagene). The probe was separated from unincorporated nucleotides on a Sephadex G-50 column, quantitated

by scintillation counting, and denatured by incubating at 100 °C for 5 min in 50% formamide. Filters were incubated at 37 °C for >16 h in a hybridization solution (5× SSPE, 1% SDS, 5× Denhardt's, 30% deionized formamide), then at 50 °C for >20 h in the same solution containing  $\sim 1.5 \times 10^6$  cpm/mL of denatured probe. Filters were washed four times for 15 min at 50 °C in solutions containing 0.1% SDS and 2×, 0.5×, 0.2×, and 0.1× SSC, successively.

The cDNA present in the phage clones analyzed were isolated as *Eco* RI fragments and subcloned into the corresponding site of pGEM 3Z (Promega). The nucleotide sequences of the cDNAs were determined by the dideoxy method (Sanger et al., 1977), using synthetic oligonucleotide primers and the Sequenase 2.0 kit (USB). Sequence analyses were made using GCG software (Genetics Computer Group, 1994).

### RNase protection assays

Ribonuclease protection experiments were performed using the RPA II kit (Ambion). Radiolabeled anti-sense RNA complementary to Types 1 and 2 cDNA were prepared by run-off transcription of linearized plasmid containing the 5'-most *Eco* RI fragment of each cDNA, using SP6 RNA polymerase (Promega) and [ $\alpha$ -<sup>32</sup>P]UTP (Amersham) as described (Melton et al., 1984; Fox et al., 1989). Type 1 probe, complementary to nucleotides 1991–2119, was prepared from plasmid digested at the *Afl* II site in the Type 1 cDNA. Type 2 probe, complementary to nucleotides 2144–2229, was prepared from plasmid restricted at the *Apa* LI site in the Type 2 cDNA. Solution hybridization of the probes with  $\sim 20$   $\mu$ g of egg RNA or  $\sim 10$  pg of synthetic PAP sense mRNA (see below) was performed at 42 °C for 16 h. RNAs were digested with a 1:2,000 dilution of supplied RNase A and T1 at 37 °C for 30 min. RNA was recovered by precipitation, separated by electrophoresis on 8% denaturing gels, and detected by autoradiography.

### Oocyte and embryo extract preparation

Stage VI oocytes were manually dissected from surgically excised *Xenopus* ovaries. Oocytes were placed in MMR (100 mM NaCl, 2 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH 7.4) supplemented with 0.1 mg/mL each of penicillin and streptomycin, and  $\sim 1$  mg/mL BSA if incubating overnight. Maturation was induced by addition of progesterone to a final concentration of 10  $\mu$ g/mL and confirmed by the appearance of a white spot at the animal pole. Mature oocytes had been incubated for 10 or more hours in progesterone, except for the experiments in Figures 6A and 7, in which oocytes were isolated at the indicated times and frozen in liquid nitrogen. All samples were homogenized in 10  $\mu$ L/cell of ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.5, and 10  $\mu$ g/mL chymostatin, pepstatin, and leupeptin) and centrifuged at 14,000  $\times$  g for 10 min at 4 °C. The soluble phase was withdrawn with a fine pipet.

Freshly laid eggs were fertilized in vitro with sperm from surgically excised testes. The morphology and synchrony of the developing embryos was monitored by microscopy. At the times indicated in Figure 6B, five eggs or embryos were dejellied with 2% cysteine and frozen in liquid nitrogen. Samples were homogenized in 10  $\mu$ L per embryo of homogenization buffer as described for oocytes above.

## Isolation of oocyte cytoplasm and nuclei

Micromanipulations were performed at room temperature. Cytoplasm refers to the material remaining upon removal from the oocyte of both the nucleus and the surrounding follicle cells. Oocytes were enucleated and allowed to heal for ~4 h in MMR containing 1 mg/mL BSA as described (Fox et al., 1989). Healthy cells were then incubated in collagenase (0.2% collagenase in 100 mM sodium phosphate, pH 7.4) for 60–90 min. In some experiments, cells were stained with Hoechst 33258 (2 mg/mL in MMR) and analyzed by fluorescent microscopy to confirm the removal of associated follicle cells. Nuclei were isolated from oocytes under mineral oil as described (Lund & Paine, 1990), homogenized in 10  $\mu$ L per nuclei of ice-cold homogenization buffer, centrifuged at 14,000  $\times$  g and 4 °C for 10 min, and the resulting extract withdrawn by pipet. Phosphatase treatment was performed in 50- $\mu$ L reactions containing extract from three nuclei (30  $\mu$ L), 1 $\times$  dephosphorylation buffer (10 $\times$  is 0.5 M Tris-HCl, pH 8.5, 1.0 mM EDTA), and 90 units of alkaline phosphatase (Boehringer Mannheim), incubating at 15 °C for 16–18 h.

## Translation of synthetic PAP1 mRNA in vitro

Intact Type 1 cDNA insert was isolated as a *Hind* III to *Sac* I fragment from purified phage DNA, and cloned into the corresponding sites of a modified pSP64(polyA) vector (Promega) containing a *Cla* I site in place of the unique *Eco* RI site, and resulting in the plasmid, pXlpap. A portion of the 5' end of the Type 2 cDNA was fused to the Type 1 cDNA to complete the open reading frame. The *Hind* III to *Kpn* I fragment of the plasmid containing the 5'-most *Eco* RI fragment of Type 2 cDNA was inserted into the same sites in pXlpap1, giving the plasmid pXlpap1. pXlpap1 was used as a template for transcription of RNA by SP6 RNA polymerase (Promega) as described (Melton et al., 1984). <sup>me7</sup>GpppG was included to produce a capped mRNA (Melton et al., 1984).

PAP1 mRNA, which contains the sequence given in Figure 1B, is 2,601 nt in length. Its structure, diagrammed in Figure 1A, is as follows. The 5'UTR, AUG, and first 14 amino acids of coding sequence, which are missing from our Type 1 cDNA, are derived from Type 2 cDNA and are very similar to the corresponding regions of mammalian PAPs (Fig. 1C). The fusion of Type 1 and 2 cDNAs also introduces 8 amino acid substitutions over the next 64 amino acids, most of which are conservative. Downstream of the junction, the sequence of PAP1 mRNA is Type 1.

mRNAs capable of producing either full-length proteins, or those lacking defined amounts of the C-terminus were produced from different restriction digests of the template plasmid pXlpap as full length (*Stu* I),  $\Delta$ 53 (*Afl* II),  $\Delta$ 124 (*Bst*E II), and  $\Delta$ 348 (*Alu* NI). mRNAs were translated in a nuclease-treated rabbit reticulocyte lysate (Promega) as described by the manufacturer. Where indicated, ~0.5 mCi/mL [<sup>35</sup>S]methionine and cysteine (Translabel, Amersham) were included. The quantity of translated protein was determined by TCA precipitation (Promega) and the integrity monitored by SDS-PAGE followed by fluorography.

## Immunoblotting

Extracts and synthesized proteins were prepared for electrophoresis by adding an equal volume of 2 $\times$  sample buffer

(125 mM Tris-HCl, pH 6.8, 20% glycerol, 6% SDS, 0.025% bromophenol blue) and 0.1 volume of  $\beta$ -mercaptoethanol, followed by boiling for 5 min and centrifugation at 14,000  $\times$  g for 5 min. Samples were separated by 6.5% SDS-PAGE (Laemmli, 1970) and transferred to Immobilon-P (Millipore) in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol). All incubations were performed at room temperature on a slowly rotating orbital platform. Blots were incubated for 1 h in blocking buffer (5% non-fat dry milk [Carnation] in TBS [20 mM Tris-HCl, pH 7.6, 137 mM NaCl]), followed by a dilution of the primary antibody in blocking buffer. The two PAP antibodies (Thureson et al., 1994) were used at a 1:10 dilution, the 12CA5 antibody (Berkeley Antibody Co.) was diluted 1:2,000. Blots were washed four times for 15 min in blocking buffer, then incubated for 1 h in a 1:2,000 dilution of anti-mouse immunoglobulin coupled to horseradish peroxidase (Amersham). Blots were washed four times for 15 min in TBS containing 0.05% Tween-20. Detection was performed with the LumiGLO chemiluminescent substrate kit (Kirkegaard and Perry) as described by the manufacturers.

Two types of molecular weight standards were used for all SDS-PAGE. Prestained standards were detectable on immunoblots without staining (Rainbow markers, Amersham). Unstained standards (Bio-Rad) were detected in gels using Coomassie staining, or visualized on immunoblots using Amido black staining.

## Epitope-tagged PAP

Two copies of the epitope recognized by the 12CA5 anti-hemagglutinin monoclonal antibody were introduced at the N-terminus of the PAP1 cDNA. An *Nco* I site was introduced at the translational start site of pXlpap1 by PCR directed mutagenesis, using the oligonucleotides GCGAAGCTTAAACAGACACCATGGCGTTTCCACTTGCAAGCCAGG (sense) and GACTGGTACAAATGCTTC (anti-sense). The resulting product was subcloned, sequenced, and inserted into the original pXlpap1 plasmid as a *Hind* III to *Sac* I fragment, giving pXlpap-N. Introduction of the *Nco* I site alters the predicted amino acid at position two of the protein from proline to alanine. Sequence coding for the two serial copies of the 12CA5 epitope was isolated from the plasmid pMR2307 (Roof et al., 1992) as a *Not* I to *Bam* HI fragment, filled-in with the Klenow fragment of DNA polymerase I (Promega) and dNTPs, and ligated into *Nco* I-digested and filled-in pXlpap-N to give pXlpap-HA.

## Microinjection of RNA into oocytes and labeling of proteins

Stage VI oocytes were microinjected with ~50 nL of in vitro transcribed RNA at a concentration of 1 mg/mL in water. Injected oocytes were incubated at room temperature in MMR and maturation was induced with progesterone as described above. In the experiments presented in Figures 3 and 7, extracts of injected oocytes were compared to those of parallel uninjected oocytes by western blotting.

In the experiments presented in Figure 7, injected oocytes were incubated in MMR containing either 1.0 mCi/mL of <sup>35</sup>S-methionine and cysteine (Translabel, Amersham), or 2.5 mCi/mL of <sup>32</sup>P-orthophosphate (Amersham). Three hours

postinjection, 10 oocytes were placed in each labeling solution, either containing or lacking progesterone. Cells were collected 11 h postinjection (complete GVBD having occurred at ~7 h postinjection), washed three times in MMR, and the cells homogenized in 400  $\mu$ L of lysis buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 80 mM  $\beta$ -glycerophosphate, 50 mM NaF, 20 mM EGTA, and 10  $\mu$ g/mL chymostatin, pepstatin, and leupeptin). Lysates were centrifuged at 14,000  $\times$  g for 10 min at 4  $^{\circ}$ C to pellet the yolk. Labeled supernatants were subjected to immunoprecipitation with the 12CA5 monoclonal antibody.

### Immunoprecipitations

Protein A-Sepharose CL-4B beads containing ~2 mg protein A/mL gel were swollen in water and then diluted 1:1 with RIPA (5 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Supernatants were pre-cleared by incubation with 50  $\mu$ L of protein A beads for 30 min on ice, followed by centrifugation at 14,000  $\times$  g for 15 min at 4  $^{\circ}$ C. Ten micrograms of purified 12CA5 antibody was added to the cleared supernatant and the mixture incubated for 2 h on a rocking platform at 4  $^{\circ}$ C. The immune complexes were collected with 50  $\mu$ L of protein A beads, incubating for an additional 1 h. The beads were washed four times with 1 mL of RIPA, each time collecting by centrifugation at 3,000  $\times$  g for 5 min at 4  $^{\circ}$ C. Recovered proteins were released from the beads by boiling in 40  $\mu$ L of 1 $\times$  sample buffer, separated by 6.5% SDS-PAGE, dried, and visualized by autoradiography.

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