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The mechanism and regulation of deadenylation: Identification and characterization of *Xenopus* PARN

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ABSTRACT

In *Xenopus* oocytes, the deadenylation of a specific class of maternal mRNAs results in their translational repression. Here we report the purification, characterization, and molecular cloning of the *Xenopus* poly(A) ribonuclease (xPARN). xPARN copurifies with two polypeptides of 62 kDa and 74 kDa, and we provide evidence that the 62-kDa protein is a proteolytic product of the 74-kDa protein. We have isolated the full-length xPARN cDNA, which contains the tripartite exonuclease domain conserved among RNase D family members, a putative RNA recognition motif, and a domain found in minichromosome maintenance proteins. Characterization of the xPARN enzyme shows that it is a poly(A)-specific 3′ exonuclease but does not require an A residue at the 3′ end. However, the addition of 25 nonadenylate residues at the 3′ terminus, or a 3′ terminal phosphate is inhibitory. Western analysis shows that xPARN is expressed throughout early development, suggesting that it may participate in the translational silencing and destabilization of maternal mRNAs during both oocyte maturation and embryogenesis. In addition, microinjection experiments demonstrate that xPARN can be activated in the oocyte nucleus in the absence of cytoplasmic components and that nuclear export of deadenylated RNA is impeded. Based on the poly(A) binding activity of xPARN in the absence of catalysis, a model for substrate specificity is proposed.

Keywords: 3′exonuclease; deadenylase; oocyte maturation; poly(A); translational silencing

INTRODUCTION

The role of poly(A) as a determinant of maternal mRNA translation during the meiotic maturation or subsequent fertilization of *Xenopus* oocytes is well established (Richter, 2000). This regulatory system discriminates between classes of mRNAs that are either polyadenylated or deadenylated during maturation. One class of mRNAs, exemplified by G10, *c-mos*, and B4, contains the 3′ UTR localized *cis*-sequences required for polyadenylation and subsequent translational activation (Dworkin & Dworkin, 1985; Fox et al., 1989; McGrew et al., 1989; Wormington, 1994). The *cis* sequences required for polyadenylation are the U-rich cytoplasmic polyadenylation element (CPE) and the ubiquitous nuclear polyadenylation element (AAUAAA) (McGrew & Richter, 1990; Paris & Richter, 1990). The deletion or mutational inactivation of either of these elements prevents both polyadenylation and translation (Fox et al., 1989; McGrew et al., 1989). In contrast, poly(A) removal is a default reaction deadenylating messages that lack a CPE such as those encoding ribosomal proteins and actin (Fox & Wickens, 1990; Varnum & Wormington, 1990). Deadenylated messages are dissociated from polysomes, thus preventing further translation. Although the poly(A) tail is not necessarily sufficient for translatability (McGrew et al., 1989), in no case has deadenylation been uncoupled from translational inactivation. For example, the overexpression of poly(A)-binding protein (PABP) in *Xenopus* oocytes inhibits both maturation-specific deadenylation and translational silencing (Wormington et al., 1996). The activity responsible for deadenylation in mature oocytes is initially nuclear associated, as poly(A) removal is a late maturation event that cannot be detected prior to nuclear envelope breakdown and is prevented if oocytes are enucleated prior to maturation (Varnum et al., 1992).

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Importantly, *Xenopus* is the only system for which an in vivo function for deadenylation has been described (Fox & Wickens, 1990; Varnum & Wormington, 1990).

The role of poly(A) in translation has been under intense scrutiny over the past few years (Sachs et al., 1997; Sachs & Varani, 2000). The closed loop model of mRNA translation originally proposed by Munroe and Jacobson (1990) has been validated by subsequent biochemical and genetic evidence of interactions between the PABP and the cap-binding complex in yeast and mammalian systems (Tarun & Sachs, 1996; Imataka et al., 1998). This mechanism is also important for translation in *Xenopus* oocytes, as shown by the expression of a mutant eIF4G, which is unable to bind PABP, and which reduces the translation of polyadenylated mRNAs, and inhibits oocyte maturation (Wakiyama et al., 2000).

Poly(A) removal has been linked to translational silencing in other systems as well. In primary mouse oocytes, the initial poly(A)$_{\sim 300}$ tracts on nascent tissue-type plasminogen activator mRNAs are shortened to p(A)$_{\sim 40}$ soon after their cytoplasmic export concomitant with their translational silencing. The U-rich elements required for the cytoplasmic polyadenylation and translational activation of these mRNAs at oocyte maturation also promote their initial deadenylation and translational quiescence (Huarte et al., 1992; Paynton & Bachvarova, 1994). In addition, the 3′ UTRs of certain *Xenopus* CPE-mRNAs (e.g., *c-mos*, *cdk2*, and *Eg2*) contain additional elements that promote their deadenylation after fertilization thereby restricting their translation to mature oocytes (Bouvet et al., 1994; Sheets et al., 1994; Stebbins-Boaz & Richter, 1994; Legagneux et al., 1995).

Poly(A) removal is also involved in mRNA destabilization. In *Saccharomyces cerevisiae*, one of the general pathways involved in mRNA decay requires deadenylation-dependent decapping followed by rapid 5′ exonucleolytic degradation (Muhlrad et al., 1994; Beelman & Parker, 1995; Caponigro & Parker, 1995). Differences in message half-lives are determined by the rates of the deadenylation and decapping reactions (Decker & Parker, 1993; Muhlrad et al., 1994, 1995). This process is quite distinct from maturation-specific deadenylation in *Xenopus*, where message stability is not altered by the addition or removal of poly(A) (Hyman & Wormington, 1988; Duval et al., 1990). Recent work, however, has shown that deadenylation in *Xenopus* oocytes does not induce decapping (Gillian-Daniel et al., 1998), but is required for mRNA degradation later in development (Audic et al., 1997; Voeltz & Steitz, 1998). Deadenylation also precedes decay in mammalian systems (Wilson & Treisman, 1988; Chen et al., 1995; Ford & Wilusz, 1999), but the role of decapping in these systems remains unclear. The mechanism of deadenylation has been extensively studied in mammalian systems (Astrom et al., 1992; Korner & Wahle, 1997; Ford et al., 1999; Dehlin et al., 2000; Gao et al., 2000), and recently the human poly(A) ribonuclease (hPARN, formerly referred to as DAN) has been identified (Korner et al., 1998). This enzyme specifically degrades poly(A) and requires the 5′ cap for full activity, presumably through its ability to bind specifically to the m^{7}GpppG cap (Dehlin et al., 2000). Although the in vivo function of hPARN remains to be determined, it has recently been shown to be required for deadenylation-dependent mRNA decay in HeLa extracts (Gao et al., 2000) and is functionally equivalent to the deadenylase that catalyzes poly(A) removal in mature *Xenopus* oocytes (Korner et al., 1998). Using antibodies directed against recombinant hPARN, it has been reported that *Xenopus* oocytes contain two forms of PARN (xPARN), p62 and p74, both of which cross-link to the m^{7}GpppG cap (Dehlin et al., 2000). In addition, these antibodies block deadenylation in *Xenopus* oocytes, providing evidence for an in vivo function for xPARN.

In this study, we report the purification, biochemical characterization, and molecular cloning of a poly(A)-specific 3′ exoribonuclease from *Xenopus* ovarian extracts. xPARN removes poly(A) in a Mg^{2+}-dependent manner. Our data suggests that a catalytically active, 62-kDa isoform of xPARN is proteolytically derived from the full-length 74-kDa species. An analysis of substrate specificity and reaction products suggests that the deadenylase activity described here is identical to that responsible for maturation-specific poly(A) removal in vivo, thus providing the first evidence for the in vivo function of purified PARN. This work serves to expand our knowledge of deadenylation in vertebrates in its analysis of xPARN substrate specificity, developmental expression pattern, and its regulation by nuclear localization.

RESULTS

Purification of PARN from *Xenopus* oocytes

To facilitate a biochemical characterization of deadenylation activity in *Xenopus* oocytes, the purification of the poly(A) ribonuclease from *Xenopus* ovary was undertaken. Extracts were made from whole ovarian tissue, spun at 100,000 × *g*, and subsequently fractionated using successive DEAE Sephacel, heparin agarose, and poly(A) chromatography. This protocol yielded a 123,500-fold purification based on an estimation of the quantity of a 62-kDa polypeptide that copurifies with activity (Table 1, Fig. 1). SDS-PAGE analysis of proteins from peak fractions shows a significant reduction in the polypeptide complexity after poly(A) chromatography (Fig. 1A). Separation of the active poly(A) fraction by MonoQ anion exchange chromatography shows a clear correlation between the elution of activity and the presence of the 62-kDa polypeptide (Fig. 1B).

TABLE 1. Purification of PARN from *Xenopus* ovary.

Chromatographic step	Elution	Total protein (mg)	Total units	Units/mg	Fold purification
S100	—	384	3,165	8	—
DEAE	150 mM KCl	90	42,053	467	
Heparin	300 mM KCl	8.8	39,393	4,476	559
poly(A)	1 M KCl	0.001	988	988,000	123,500

One unit of activity corresponds to the conversion of $p(A)_{63}$ to 1 pmol 5′ AMP in 1 min at 25 °C.

The activity of xPARN was assayed using an in vitro deadenylation reaction, where the conversion of uncapped radiolabeled polyadenylated substrate RNA (G52 $p(A)^+$ or $p(A)_{63}$; see Methods) to a deadenylated A^- product and 5′ AMP was assayed by either denaturing gel electrophoresis or thin layer chromatography (TLC; see below). The apparent increase in the total amount of xPARN after DEAE and heparin chromatography suggests that inhibitors of in vitro deadenylation, most likely RNA-binding proteins, are being removed at these steps. Indeed, the addition of nonspecific competitors for RNA-binding proteins (e.g., rRNA) was necessary for optimal activity (data not shown).

It is notable that xPARN activity elutes from MonoQ in two peaks, the latter of which consistently coincides with the elution of a 74-kDa polypeptide in addition to p62. Western blot analysis of the active fractions showed that both p74 and p62 cross-react with anti-hPARN antibody (Korner et al., 1998). Figure 2A shows a western blot analysis of staged *Xenopus* oocytes. These blots were probed with anti-h(PARN) polyclonal antibody and anti-FRGY2 antibody as a positive control. The 74-kDa xPARN protein is first detected in stage IV oocytes and is maximally expressed by stage VI. The p62 version of xPARN is detectable in stage V and VI oocytes (Fig. 2A, lanes 5 and 6) but not in mature oocytes (Fig. 2A, lane 7). Interestingly, p74 appears as a doublet in stage VI and mature oocytes, suggesting that a developmentally regulated modification may be occurring. When the oocyte extracts were prepared in the absence of protease inhibitors, p62 is much more prominent and p74 is correspondingly reduced (Fig. 2B),

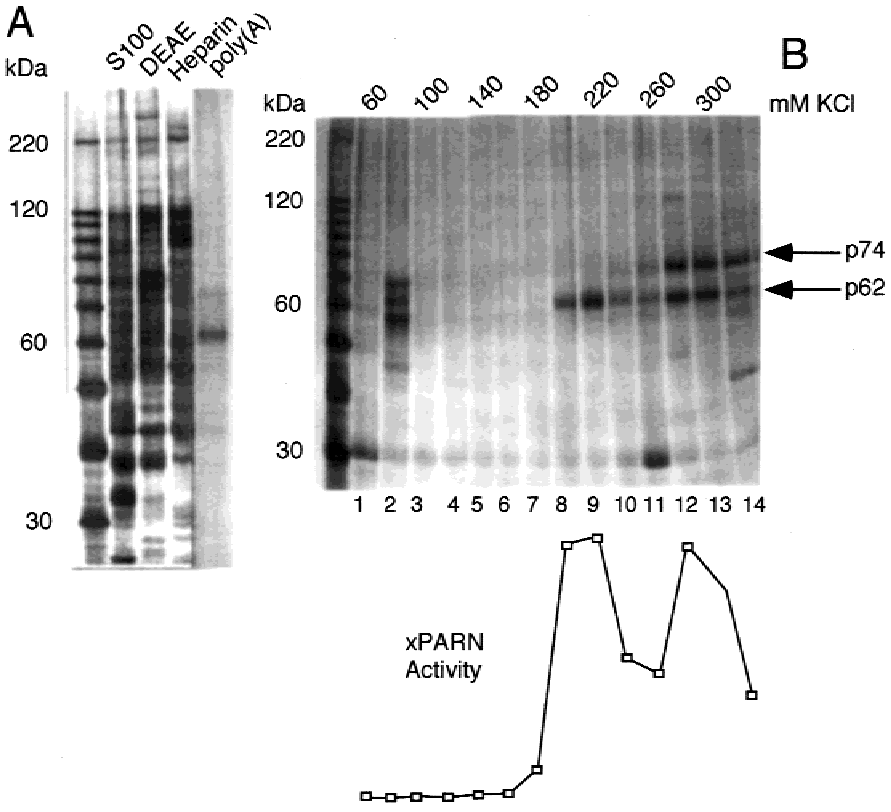

FIGURE 1. Purification of *Xenopus* PARN. **A:** A total of 2.5 μg total protein from S100, DEAE, and heparin active fractions and 50 units xPARN activity from the active poly(A) fraction were resolved by SDS-PAGE (15%) and detected by silver staining. **B:** Fractionation of the active poly(A) material by MonoQ chromatography. Proteins were resolved by SDS-PAGE (15%) and detected by silver staining. The graph below indicates the amount of xPARN activity in corresponding fractions.

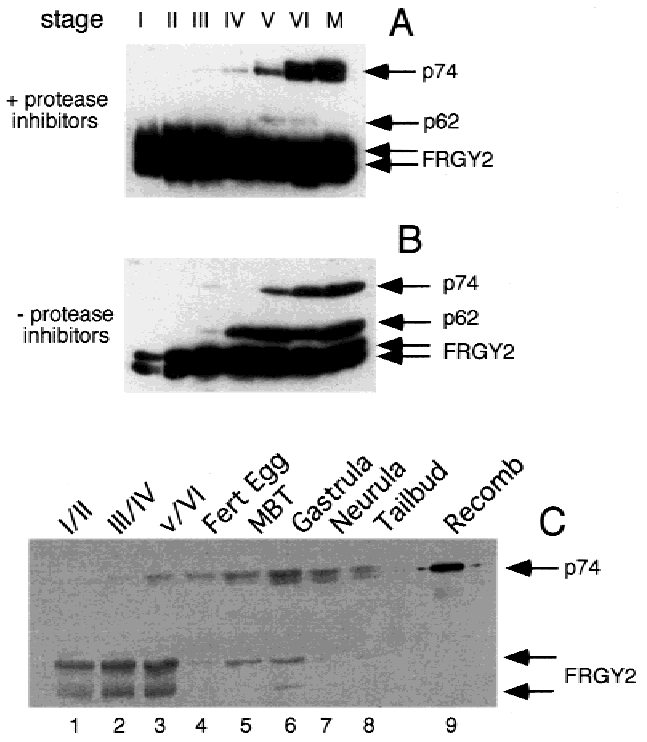

FIGURE 2. Immunoblot analysis of xPARN expression during development. Staged oocytes were homogenized in J Buffer containing protease inhibitors (1 μg/mL aprotinin, chymostatin, leupeptin, and pepstatin) (**A**) or lacking protease inhibitors (**B**). Two oocyte equivalents for each stage and mature oocytes (M) were resolved by 15% SDS-PAGE and probed with anti-PARN and anti-FRGY2 (as a positive control). **C:** Two oocyte or embryo equivalents of the developmental stages indicated. Lane 1: stage I and II oocyte mixture (I/II); lane 2: stage III and IV oocyte mixture (III/IV); lane 3: stage V and VI oocyte mixture (V/VI); lane 4: fertilized egg (Fert egg); lane 5: mid-blastula transition (MBT); lane 6: Gastrula; lane 7: stage 10 embryo (Neurula); lane 8: stage 15 embryo (Tailbud); lane 9: 2 ng recombinant hPARN (Recomb).

suggesting that p62 is derived from the p74 species by proteolytic cleavage. This appears to be a specific cleavage, as the truncated p62 protein is not further degraded. In addition, this proteolysis appears to be insensitive to PMSF, which was used throughout the purification. Interestingly, the putative modification of p74 is also lost in the absence of protease inhibitors. These data indicate that p74 is the precursor of p62 and, because the activity of p74 hPARN is stimulated by the presence of a 5′ cap, we believe that p74 xPARN is responsible for cap-dependent activity observed in mature *Xenopus* oocytes in vivo. Previous analysis of xPARN subcellular localization showed that p62 is cytoplasmic whereas p74 is nuclear (Korner et al., 1998). Analyses of the predicted protein sequence of xPARN using the PORT II Prediction algorithm reveals the presence of a single putative nuclear localization signal (NLS) located in the C-terminal portion (amino acids 522–539). Thus, it is likely that the proteolysis is occurring in the C-terminal domain and that this region is required for nuclear localization. Figure 2C shows a developmental western blot probed with anti-hPARN and FRGY2 antibodies as described above. This blot shows that p74 xPARN protein is readily detectable in later embryonic stages with an apparent peak of expression during gastrulation. These data suggest that xPARN activity persists well after fertilization and therefore may also participate in fertilization-specific deadenylation (Bouvet et al., 1994; Sheets et al., 1994; Stebbins-Boaz & Richter, 1994; Legagneux et al., 1995) as well as post-mid-blastula transition mRNA destabilization (Audic et al., 1997; Voeltz & Steitz, 1998).

Cloning of the xPARN cDNA

Peptide sequence data were obtained from purified p62 xPARN by mass spectrometry (see Fig. 3 for peptide positions). As previously reported, these peptides were highly homologous to the human poly(A) ribonuclease (hPARN) amino acid sequence (Korner et al., 1998). Taking advantage of this similarity, an hPARN cDNA probe was used to screen a *Xenopus* oocyte cDNA library at low stringency. A 1.53-kb clone was obtained that was 75% identical to the 5′ coding region of the human cDNA, but which lacked the 3′ portion and did not contain an in-frame stop codon. To obtain a full-length clone, the partial sequence was used to screen a *Xenopus* kidney λ ZAP II cDNA library. Two clones were obtained from this library from a screen of ~600,000 plaques. The larger of the clones (2 kb) overlapped the original clone and contained all of the coding region except for the first 16 nt. The predicted molecular weight of full-length xPARN is 72.8 kDa. The deduced amino acid sequences for xPARN and hPARN are shown in Figure 3. Although the two proteins share 72% overall identity, there is significantly more heterogeneity in the C-terminal region than elsewhere, suggesting a possible divergence of function. No other proteins of known function are similar to human or *Xenopus* PARN, but related hypothetical proteins from several species (*Arabidopsis thaliana*, *Schizosaccharomyces pombe*, and *Caenorhabditis elegans*) are present in the nonredundant database. In addition to the conserved tripartite RNase D exonuclease domains (Korner et al., 1998), xPARN also contains motifs that were not reported for the human enzyme. One of these is a region that is similar to a functionally undefined domain conserved among helicases known as minichromosome maintenance proteins (MCM; amino acids 132–152). Although the conserved region falls outside the Walker A and Walker B motifs known to be required for nucleotide binding, other conserved regions, which may confer nucleotide specificity, have not been characterized (Tye, 1999). The xPARN sequence also contains a putative RNA recognition motif (RRM; amino acids 446–502) that may contribute to its poly(A)-binding activity (Siomi & Dreyfuss, 1997).

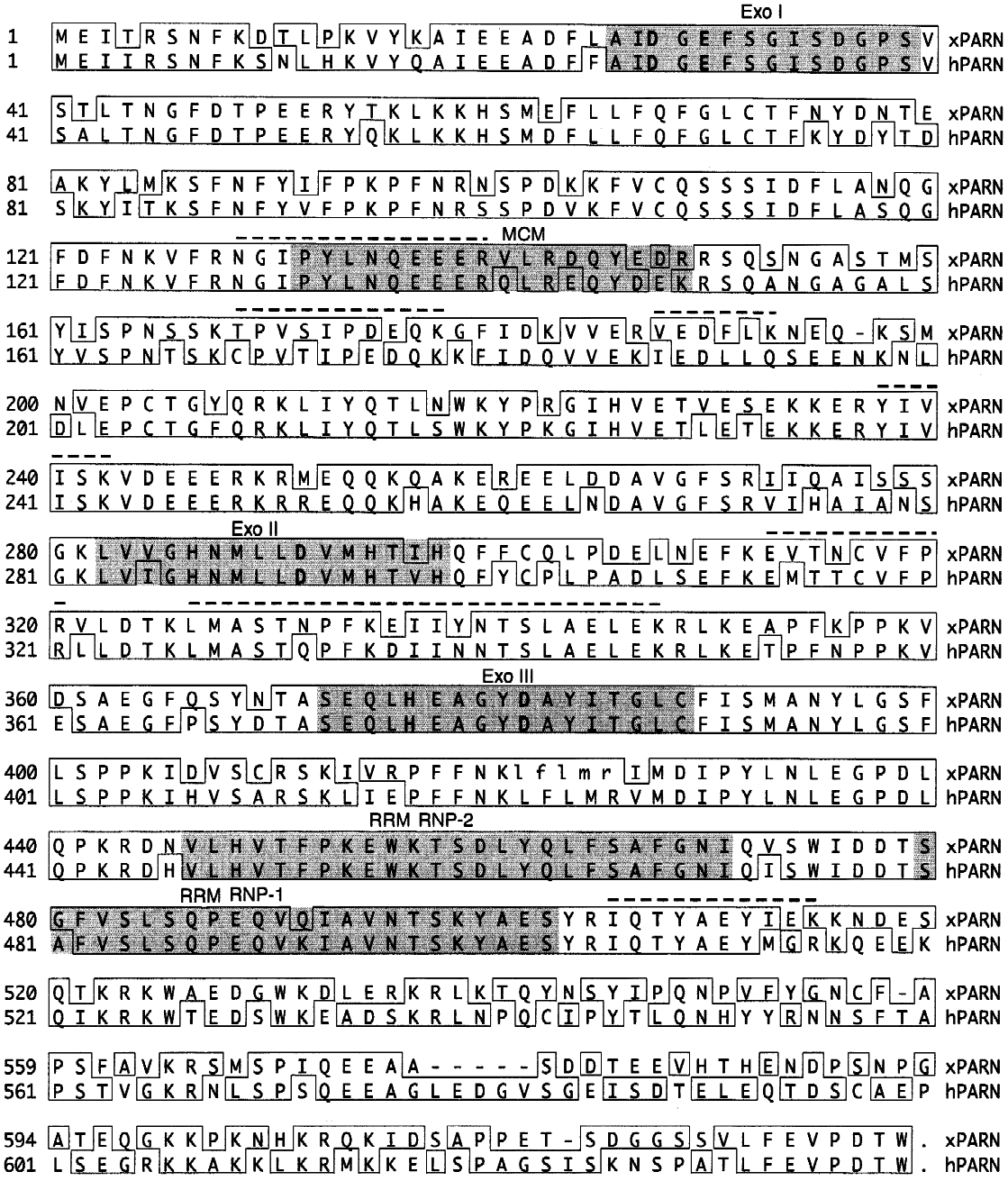

FIGURE 3. Deduced amino acid sequence of xPARN shown with an alignment of human PARN (hPARN). Putative mini-chromosome maintenance (MCM) and RNA recognition motif (RRM) with conserved RNP-2 and RNP-1 domains are shaded. Amino acids conserved among RNase D exonuclease family members are also shaded (Exo I, II, and III), and the conserved residues are in boldface. Peptides from p62 that were identified by mass spectrometry are superscribed by a dashed line.

Biochemical properties of xPARN

To better understand the biochemistry of deadenylation, xPARN was subjected to extensive analysis in vitro. The activity of xPARN in vitro is functionally identical to that responsible for deadenylation in vivo as demonstrated by high-resolution gel electrophoresis of the reaction products. By a comparison of the deadenylation of G52 A^+ in vitro and after injection into stage VI oocytes, Figure 4A demonstrates that the extent of poly(A) removal was precisely the same for activities found in the hydroxyapatite fraction, and in vivo. The extent of poly(A) removal as determined by this assay was 1–2 nt 5′ to the start of the poly(A) tail based on a comparison to a run-off transcript of G52 A^- RNA.

The products of the xPARN deadenylation reaction are 5′ AMP and deadenylated RNA, as demonstrated by one-dimensional TLC (Fig. 4B) and gel electropho-

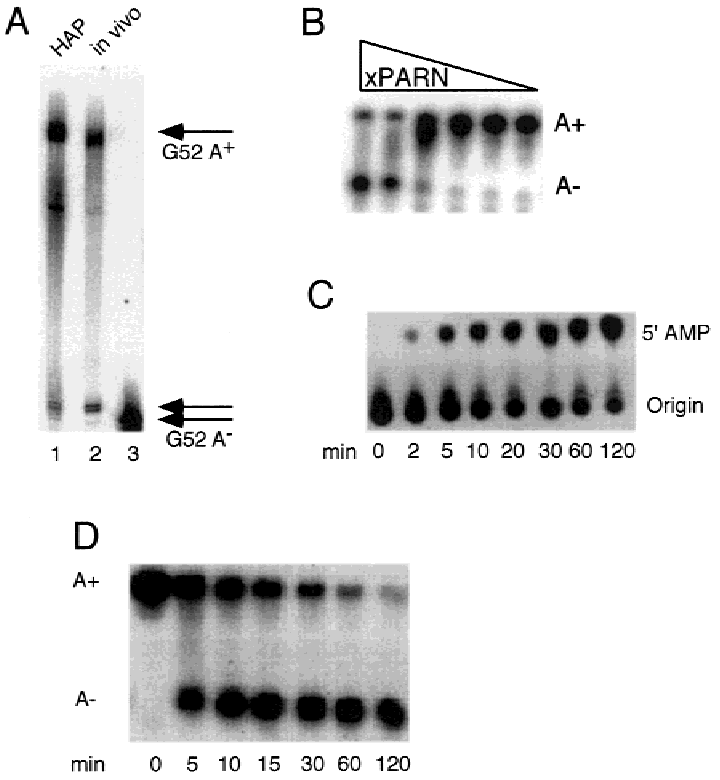

FIGURE 4. Characterization of -xPARN. **A:** xPARN from the active HAP fraction was used in an in vitro deadenylation reaction that was subsequently run on a 6% sequencing gel (lane 1). ^{32}P G52 A$^+$ RNA was injected into a stage VI oocyte, progesterone was added, and RNA was extracted from 5 mature oocytes (lane 2). Lane 3 contains in vitro-transcribed G52 A$^-$ RNA that lacks poly(A) and 6 nt upstream. **B:** Twofold serial dilution of xPARN HAP fraction in a standard deadenylation assay. The products were resolved on a 6% gel. **C:** The deadenylation of ^{32}P A-labeled G52 A$^+$ analyzed by TLC over2 h. Time points are marked below the image. **D:** Same time course as in **C**, except with ^{32}P U-labeled G52A$^+$ substrate resolved by 6% gel.

resis, respectively (Fig. 4C). The identity of 5′ AMP was verified by spotting unlabeled 5′ AMP and comparing its migration with the deadenylation product by UV shadowing. The radioactive deadenylation product migrated only with 5′ AMP and not 3′ AMP or cAMP (data not shown). The results illustrated in Figure 4 demonstrate that xPARN is a 3′ exonuclease by virtue of mononucleotide release as demonstrated by TLC and 3′-5′ directionality as demonstrated by the gel assay in which the ^{32}P-UTP-labeled 5′ body of the substrate is shifted to a deadenylated size by virtue of 3′ poly(A) degradation. The deadenylation reaction requires the presence of Mg^{2+}, which cannot be replaced with Mn^{2+}, Zn^{2+}, or Ca^{2+}. The role of Mg^{2+} appears to be limited to catalysis and is not required for RNA binding. This can be demonstrated chromatographically, as xPARN can bind to and be recovered from poly(A)-Sepharose either in the presence or absence of Mg^{2+} (data not shown).

Optimal reaction conditions do not require exogenously added ATP and span a pH range from 6.5 to 8 and 50 to 100 mM KCl. The reaction was inhibited by a variety of substances, as summarized in Table 2. It is interesting to note that the reaction product (5′ AMP) is the most inhibitory of the nucleotide monophosphates, and that the inhibition by this class of molecules, with the exception of IMP, is divided into two groups represented by more inhibitory purines on one hand and less inhibitory pyrimidines on the other. These data suggest that xPARN may contain a binding site with preference for a purine structure that may play a role in determining substrate specificity. The presence of up to 40 units of an RNase A inhibitor (RNasin) had no effect on activity. Deadenylation did not take place in the presence of SDS, and activity was not recovered from denaturing gels. Nonionic detergents, however, did not affect activity. The enzyme appears to be cryostable,

TABLE 2. Assay of deadenylation in the presence of various reagents.[a]

Reagent	K_i
5′ AMP	0.6 mM
5′ GMP	0.8 mM
5′ CMP	2.4 mM
5′ UMP	2.2 mM
5′ IMP	2.2 mM
cAMP	7.8 mM
Mg-ATP	>10 mM
EDTA	1 mM[b]
$ZnCl_2$	<0.15 mM
$MnCl_2$	0.15 mM
$CaCl_2$	4.5 mM
oligo d(A)	0.8 mM
oligo (A)	0.8 mM
ssDNA	>2 mM

A twofold serial dilution of each reagent was used in a standard deadenylation assay. The concentration required for 50% inhibition (K_i) was determined by PhosphorImager quantitation.

[a]1.25 units xPARN per assay.

[b]Assayed in the presence of 1 mM $MgCl_2$.

as minimal loss of activity was incurred after several months storage at −80 °C. The aggregate molecular weight of xPARN was determined to be 81 kDa by Superose-12 size exclusion chromatography (data not shown).

Substrate specificity

Two experiments were performed to address the issue of substrate specificity. First, by extended time course analysis, xPARN was found to remove the p(A) tail from G52 A^+ while leaving the remaining nonadenylated portion intact. This same time course applied to G52 A^- RNA leaves the substrate largely unaffected with the exception of the slow accumulation of an intermediate produced by an apparently distributive activity that may or may not be related to xPARN (Fig. 5A). Second, substrate RNAs containing 3′ poly(A, C, U, or G)$_{30}$ tracts were used to further demonstrate poly(A) specificity. A short time course was performed with each substrate in which only the poly(A)-containing RNA was hydrolyzed (Fig. 5B).

To address the 3′-end recognition requirements of xPARN, ^{32}P A-labeled G52 RNA, terminating with the sequence GGGGAUC downstream of the poly(A) tail, was degraded with RNase A, leaving p(A)$_{100}$GGGAUp.

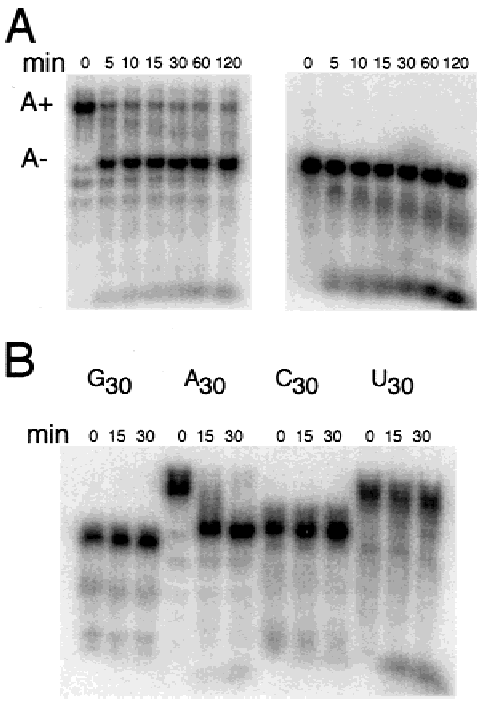

FIGURE 5. Poly(A) specificity of xPARN. **A:** A 2-h time course of activity using excess xPARN with both G52 A^+ (left) and G52 A^- RNA (right). **B:** Four RNAs were constructed with the four possible homopolymeric 3′ ends as noted above the image. RNAs are of different sizes due to varying vector sequence lengths upstream of the homopolymeric ends. Each RNA was subjected to a short time course (0, 15, and 30 min time points) and analyzed by 6% gel.

This substrate was resistant to deadenylation by xPARN unless it was first treated with phosphatase (Fig. 6A). In addition, the ligation of a ^{32}P 3′-pCp residue blocked activity unless the 3′-phosphate was removed with alkaline phosphatase prior to incubation with xPARN. The radiolabeled terminal residue was efficiently removed from the substrate RNA in the presence of xPARN, but not in the presence of xPARN plus EDTA (Fig. 6B). The addition of ^{32}P-cordycepin (3′ deoxyadenosine) to the 3′ end of unlabeled substrate RNA with poly(A) polymerase did not inhibit removal of the terminal residue (not shown). This is in contrast to the results obtained for a HeLa poly(A)-specific nuclease that cannot deadenylate 3′ deoxy terminated substrates (Åstrom et al., 1992). With regard to the substrate backbone, xPARN was not inhibited by the incorporation of α-S-ATP into the poly(A) tail (Fig. 6C). Together these results begin to describe the flexibility of nucleotide structure that can be recognized by xPARN, and should prove beneficial for protein structural studies in the conceptualization of the enzyme's active site.

To investigate the importance of position for the poly(A) tract, four RNAs were synthesized with varying lengths of nonadenylate sequences downstream of a p(A)$_{63}$ tract. Figure 6D shows that degradation of the p(A) tract by *Xenopus* xPARN is prevented by the addition of 25 nt of non-poly(A) sequence 3′ of the poly(A) tract. The RNA bearing one 3′ non-A residue was efficiently processed, and as mentioned above, the G52 construct used in this study contains 7 non-A residues at the 3′ end. These results indicate that although the 3′ most nucleotide does not have to be an A residue, the accessibility of a poly(A) tract is rather sharply determined by the number of downstream non-A residues. These data are consistent with in vivo analyses of deadenylation substrate RNAs possessing internal poly(A) tracts (Fox & Wickens, 1990).

xPARN activation during oocyte maturation

A model for the activation of deadenylation during oocyte maturation has been proposed in which xPARN is released into the cytoplasm at nuclear envelope breakdown, thereby allowing the enzyme access to cytoplasmic poly(A) tracts (Varnum et al., 1992). Indeed, the p74 xPARN isoform has been shown by western analysis to be exclusively nuclear (Korner et al., 1998). However, as nuclear extracts lacked deadenylation activity in vitro unless supplemented with a crude cytoplasmic fraction (Varnum et al., 1992), it remained to be determined if nuclear xPARN is indeed catalytically active in vivo. Figure 7A shows that deadenylation can occur in the oocyte nucleus when poly(G) is co-injected into the nucleus with the substrate G52 A^+. In contrast, no deadenylation is observed if G52 A^+ is co-injected with the same amount of tRNA instead of poly(G), and deadenylation is not observed if the substrate is injected

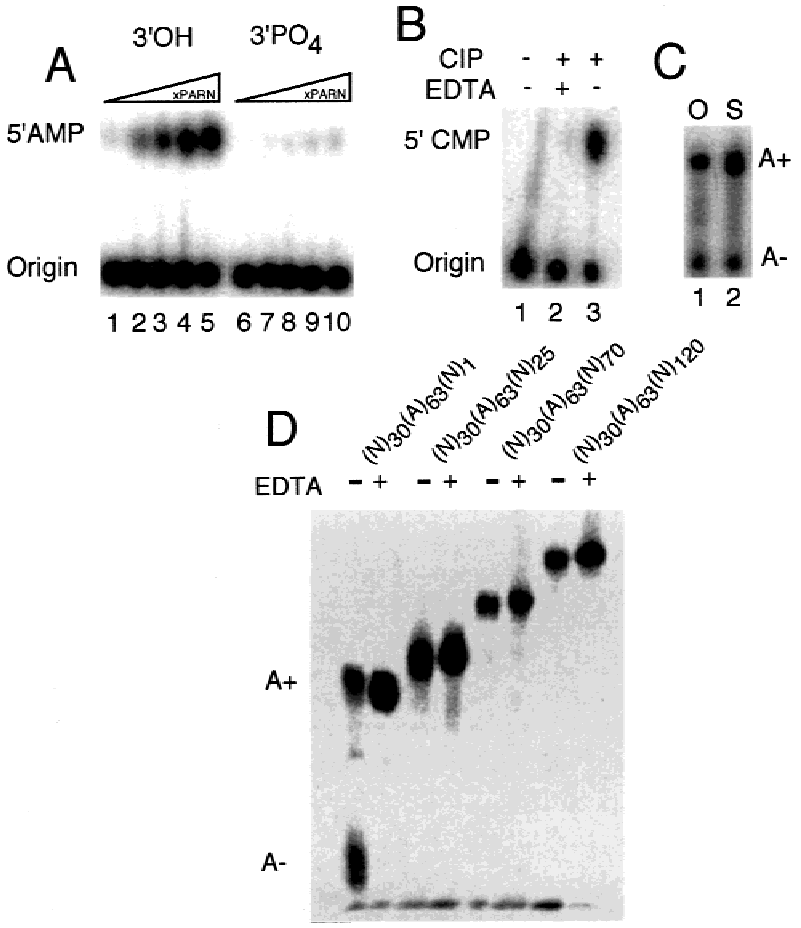

FIGURE 6. 3′ end requirements. **A:** ^{32}P A-labeled G52 A^+ RNA was treated with RNase A leaving radiolabeled poly(A) with a 3′ PO_4. Lanes 1–5 show increasing concentration of xPARN with phosphatase treated (3′ OH) substrate; lanes 6–10 show same levels of xPARN with nonphosphatase-treated substrate. Products were resolved by TLC. **B:** ^{32}P pCp was added to the 3′ end of G52 A^+ with RNA ligase. A portion of this substrate was then treated with phosphatase to remove the 3′ terminal phosphate. Lane 1: xPARN/pCp; lane 2: xPARN/pC-OH/EDTA; lane 3: xPARN/pC-OH. **C:** G52 A^+ was synthesized in the absence (O; lane 1) or presence (S; lane 2) of α-thio-ATP and subject to deadenylation analysis. **D:** The $p(A)_{63}$ substrate (see Methods) was synthesized with varying amounts of 3′ non-poly(A) sequence as indicated in the figure and subjected to a standard deadenylation assay. Each reaction was performed in the presence and absence of EDTA.

into the cytoplasm. The simplest explanation for the results shown in Figure 7A would be that nuclear retention is sufficient to promote deadenylation. Because poly(G) is known to inhibit the export of RNAs from the nucleus when injected into either the nucleus (Jarmolowski et al., 1994) or the cytoplasm (P.R. Copeland & M. Wormington, unpubl. observation), we chose to examine the role of nuclear export in regulating the deadenylation of injected substrate RNAs. Substrate RNA in the presence or absence of poly(G) was injected into oocytes in which nuclear export had been inhibited by a prior cytoplasmic injection of poly(G). Figure 7B shows that deadenylation only occurred when poly(G) was co-injected into the nucleus, and minimal deadenylation was found when the substrate RNA was retained in the nucleus in the absence of nuclear poly(G) (compare lanes 1–3 with lanes 4–6). This indicates that nuclear retention of the substrate RNA is not sufficient to confer susceptibility to deadenylation, but that the presence of poly(G) in the nucleus is also required. Although the mechanism by which poly(G) activates nuclear deadenylation is unknown, it is likely due to its titration of nuclear RNA-binding proteins that bind the poly(A) tail during export, thus preventing xPARN access (P.R. Copeland & C.A. Hurney, unpubl. observations). It should also be noted that deadenylation is apparently inhibitory to export as the majority of the exported RNA retained poly(A) (Fig. 7B, lanes 7–9). These results confirm that nuclear-localized p74 xPARN is indeed catalytically active, but that nuclear RNA-binding proteins preclude its access to substrate RNAs. These proteins must presumably dissociate in the process of remodeling nuclear ribonucleoprotein complexes in the cytoplasm.

DISCUSSION

The identification of xPARN reported here establishes a function for this ribonuclease in the developmental regulation of poly(A) stability. As regulated deadenylation is evolutionarily conserved, it is likely that PARN is responsible for deadenylation in many metazoans. This hypothesis is underscored by the high level of identity, at both the nucleic acid and protein levels, between human and *Xenopus* PARN. It is likely, therefore, that the regulation of this enzyme may be key in determining both the translatability and the stability of mRNAs. This analysis of the *Xenopus* PARN has addressed key issues in substrate specificity, developmental expression, and the regulation of its activity by nuclear local-

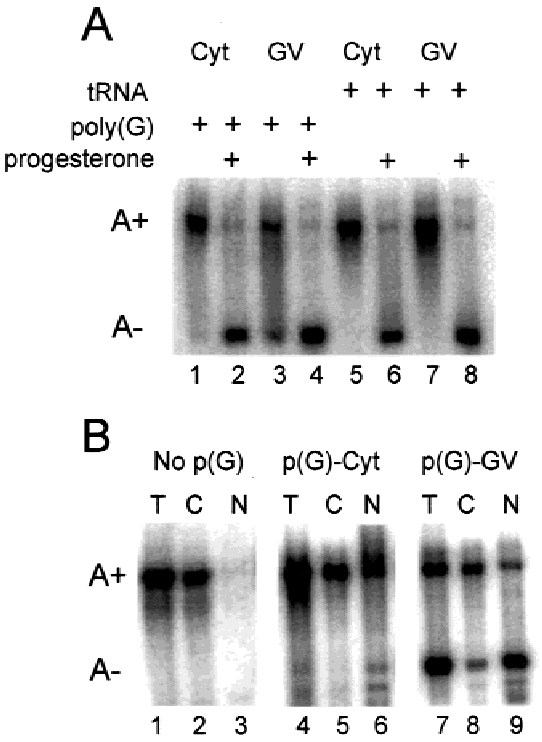

FIGURE 7. In vivo analysis of deadenylation. **A:** A total of 40 fmol of capped, ^{32}P-labeled G52 A^+ RNA was injected into an oocyte nucleus or cytoplasm either in the presence of 125 ng tRNA or poly(G). The addition of progesterone to nuclear-injected oocytes provided a control for maturation induced deadenylation (lanes 2, 4, 6, 8). **B:** A total of 40 fmol ^{32}P-labeled G52 A^+ was injected into the nuclei of oocytes that had been cytoplasmically preinjected with water or 250 ng poly(G). For lanes 7–9, G52 A^+ was co-injected with 250 ng poly(G) into the nucleus. Following a 3–4 h incubation, nuclei were isolated and RNA extracted from whole oocytes (T), oocyte cytoplasms (C), and isolated nuclei (N), and the RNA was analyzed by electrophoresis.

ization. In addition, the study of the *Xenopus* enzyme is important as it will allow a detailed investigation of the role of deadenylation in silencing translation in the absence of mRNA degradation.

Two proteins copurified with xPARN activity, both of which cross-react with anti-PARN antibody. The smaller form (p62) eluted in two peaks that corresponded exactly with the elution of xPARN activity. The larger form (p74) eluted exclusively in the second peak and likely represents the full-length xPARN protein. Although it is clear from the western analysis in Figure 2 that p62 is a proteolytic fragment of p74, the fact that some p62 is detectable in the oocyte under all conditions tested suggests that this cleavage occurs in vivo. That the cleavage affects subcellular localization is further evidence that xPARN processing may be important in vivo. In addition, the analysis of p62 xPARN described here suggests that the NLS is most likely located near the C-terminus of the protein. Unfortunately the distribution of peptide sequences obtained by mass spectrometry do not allow us to definitively distinguish which end was cleaved, but the presence of a bipartite NLS in the C-terminal region supports this hypothesis, and this NLS is downstream of the most distal p62 peptide that we recovered (IQTYAEYIEK).

Our characterization of xPARN activity has revealed that deadenylation results in a complete removal of poly(A) despite the fact that all of the substrates tested contained 1–7 nt of nonadenylate sequence at the 3′ end. The addition of 25 nt to the 3′ end, however, completely eliminated activity. This phenomenon raises interesting questions about specificity and mechanism. Clearly the enzyme does not have an absolute requirement for A residues, so the basis of specificity may lie in its ability to bind poly(A) even under conditions where catalysis is blocked. It is tempting to hypothesize that xPARN is bound to cytoplasmic poly(A), and that free xPARN must interact with the 3′ end and the bound xPARN simultaneously for deadenylation to occur. This raises the question of where the PABP might fit into this model. As PABP is known to be limiting in *Xenopus* oocytes (Stambuk & Moon, 1992), it is possible that xPARN has sufficient access to cytoplasmic poly(A) to be active. It is also an intriguing possibility that PABP may specifically bind to newly polyadenylated messages, thus preventing xPARN access and providing the discrimination between messages destined for deadenylation versus those destined for polyadenylation. Because PABP is known to inhibit deadenylation (Wormington et al., 1996; Ford & Wilusz, 1999), this hypothesis already has preliminary support.

Our analysis of deadenylation in vivo reveals an important aspect of xPARN regulation. It has long been known that the deadenylase activity is sequestered in the oocyte nucleus (Varnum et al., 1992), but the mode of activation has only been a matter of speculation. The fact that poly(G) injected into the nucleus simultaneously stimulated deadenylation and blocked transport suggests that these two processes might be coregulated. It is likely, therefore, that the process of mRNA export is responsible for protecting nascent poly(A) from being degraded by xPARN in the nucleus. By demonstrating that xPARN retains significant activity within the nucleus, we have shown that a cytoplasmic factor is not required for activation, and that release into the cytoplasm is probably sufficient to initiate cytoplasmic deadenylation. In addition, this suggests that xPARN may be involved in some regulated poly(A) tail length determination in the nucleus, as has been shown to be the case for the yeast deadenylases (Tucker et al., 2001). Because blocking export is not sufficient for xPARN activation, however, it is likely that RNP remodeling during maturation is also required. Whether other factors are involved in regulating the amount of activity remains unknown. Indeed, it is clear from the western blot in Figure 2 that the migration of p74 during SDS-PAGE is altered during oogenesis, suggesting that a specific modification is occurring. Future work will fo-

cus on the regulation of xPARN both in terms of its activity and regulation during early development.

METHODS

Extract preparation and chromatography

Xenopus ovaries (one ovary = 8–12 g) were surgically removed from mature female animals and rinsed with Deadenylase Buffer (DB) (10 mM KPO_4, pH 7.2, 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM PMSF, 5% (v/v) glycerol, 0.05% (v/v) Tween). DB was added at 1 mL per gram of tissue, PMSF was added to 0.4 mM, and the mixture was homogenized with a Biospec Bio Homogenizer. All subsequent procedures were carried out at 0–4 °C. Crude extract was spun at 10,000 × *g* (8,000 rpm in a Sorval SS34 rotor) for 20 min to remove debris, and the supernatant was transferred to 70-mL bottles for 100,000 × *g* (29,000 rpm) centrifugation in a Beckman 45Ti ultracentrifuge rotor.

Ten milliliters of S100 (~400 mg protein) were loaded onto a 2.6 × 26 cm column, packed with DEAE-Sephacel (Pharmacia), fitted with a flow adapter and washed at 1 mL/min with DB until A_{280} reached baseline. The column was eluted with a 180-mL gradient from 40 to 400 mM KCl in DB. Fractions containing xPARN were identified by TLC-assay (see below) using 1 μL of every fifth fraction. Active fractions were pooled, then diluted with one volume of DB (~50 mL). All buffer and sample delivery was performed by peristaltic pump. Diluted DEAE fractions (~100 mL) were loaded onto a 2.6 × 26 cm column containing heparin agarose (Sigma). The column was washed, then eluted with a 1800-mL gradient from 40 to 500 mM KCl in DB. xPARN activity was assayed as above, using 5 μL of every fifth fraction.

For polypeptide identification, active heparin fractions were pooled and loaded (0.25 mL/min) directly onto a 0.5 × 5 cm poly(A)-Sepharose (Pharmacia) column equilibrated with 500 mM KCl in DB on a Beckman HPLC dual-pump system with a 5-mL sample loop. The column was washed until the cessation of baseline drift and eluted with a step to 1.5 M KCl in DB at 1 mL/min. Eight 2-mL fractions were collected starting 4 min after start of elution. Activity was recovered by concentrating and desalting in a Centricon C-30 ultrafiltration device (Amicon). Apparently homogeneous xPARN (p62) was obtained by loading desalted and concentrated activity from poly(A)-Sepharose onto a 0.5 × 5 cm MonoQ column developed with a gradient to 400 mM KCl in DB over 20 min. Silver staining of protein gels was performed as described previously (Heukeshoven & Kernick, 1985).

As a source of partially purified xPARN, heparin agarose fractions were pooled and loaded directly onto a 0.5 × 5 cm HAP (Bio-Rad ceramic hydroxyapatite) column that was eluted with a linear gradient to 150 mM KPO_4 in 20 min at 1 mL/min. Fractions were then analyzed by TCA precipitation/SDS-PAGE or deadenylation assay or both. For some experiments, active HAP fractions were further purified on MonoQ as described above.

Peptide sequencing

The stained gel was sent to the W.M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia for capillary LC/MS peptide sequencing. The purified protein (p62) was excised from the gel and digested in situ with trypsin (Shevchenko et al., 1996). The digest was analyzed by capillary LC-electrospray MS, and peptide amino acid sequences were characterized by collisionally activated dissociation (CAD) using LC-electrospray-tandem MS.

Deadenylation substrates

G52 A^+ RNA, which is comprised of the last 96 nt of the globin 3′ UTR followed by $p(A)_{100}$ (Varnum & Wormington, 1990), was synthesized in vitro from pG52/SP65 linearized with *Bam*HI using SP6 RNA polymerase as previously described (Wormington, 1991). G52 A^- RNA was synthesized as described for G52A^+, except that template DNA was linearized with *Dde*I to terminate transcription 6 nt upstream of the poly(A) tract. $p(A)_{63}$ RNA was made from $p(A)_{63}$ cloned into pGEM3Z (de Melo Neto et al., 1995), linearized with *Xba*I (leaving one nonadenylate base downstream of the poly(A) tract) and synthesized with T7 RNA polymerase. This construct was linearized with *Hin*dIII, *Bst*NI, and *Pvu*II to add progressively more non-poly(A) sequence downstream of the $p(A)_{63}$ tract. To synthesize radiolabeled poly(A), $p(A)_{63}$ was transcribed with ^{32}P-ATP (1 h at 37 °C), digested with RNase A in the presence of one unit of calf intestinal alkaline phosphatase (30 min at 37 °C), and gel purified. For specificity studies, RNAs with A, C, U, or G homopolymeric ends were synthesized from pBluescript containing $(N)_{30}$ tracts with T7 or T3 RNA polymerase. RNAs labeled by ^{32}P-UTP incorporation were gel purified (6% acrylamide, 7 M urea) and the gel slices eluted overnight in TNES at room temperature (0.1 M Tris-HCl, pH 7.5, 0.3 M NaCl, 0.01 M EDTA, 2% SDS). Eluents were phenol/chloroform extracted, chloroform extracted, and ethanol precipitated. For oocyte injections, the G52A^+ RNA was synthesized in the presence of a 5′ GpppG cap analog.

Deadenylation assays

A standard deadenylation assay contained 10,000 dpm (~0.5 ng) radiolabeled substrate RNA (U-labeled for gel analysis, A-labeled poly(A) for TLC), 100–200 units xPARN (one unit represents the release of 1 pmol AMP per minute at 25 °C), and DB to a final volume of 10 μL. Reactions to be assayed by gel included 2 mM DTT and 10–20 units of RNasin (Promega). Reactions were stopped with 2× formamide stop buffer (95% formamide, 10 mM EDTA, 0.5% each of xylene cyanol and bromophenol blue), heated at 80 °C for 3 min and loaded onto a 6% polyacrylamide, 7 M urea gel. RNAs were visualized by a Molecular Dynamics PhosphorImager. For TLC assays, 0.3 μL of each reaction were spotted on a PEI cellulose TLC plate (Sigma), which was developed in 15:1:10 isobutyric acid:NH_4OH:H_2O, dried, and exposed to a PhosphorImager screen. Specific activity was determined by assaying twofold serial dilutions of xPARN by TLC and quantitating conversion from $p(A)_{63}$ to 5′ AMP.

Western analysis

Immature oocytes were obtained from whole ovary and sorted according to stage. Oocytes were extracted by pipetting in

Buffer J (10 mM HEPES, pH 7.3, 70 mM KCl, 1 mM MgCl, 0.1 mM EDTA, 2 mM DTT, 10% glycerol) with or without added protease inhibitors (1 μg/mL each aprotinin, chymostatin, peptstatin A, and leupeptin). Two oocyte equivalents were resolved by 15% SDS-PAGE and blotted to nitrocellulose. For the detection of xPARN, a 1:1,000 dilution of anti-hPARN polyclonal antibody (Korner et al., 1998) was used, and for the detection of FRGY2 (Tafuri & Wolffe, 1993), a 1:10,000 dilution of anti-FRGY2 polyclonal antibody (provided by M. Murray, Wayne State University) was used (Wormington et al., 1996). HRP-conjugated secondary antibodies were detected by enhanced chemiluminescence (Amersham) for Figure 2A,B. Alkaline phosphatase/NBT-BCIP (Promega) detection was used for Figure 2C.

cDNA cloning

Using peptide sequences derived from purified bovine PARN and p62 xPARN, a full-length human PARN (hPARN) cDNA was obtained from the IMAGE consortium. A ^{32}P-labeled DNA probe derived from this clone was used to screen a *Xenopus* oocyte (St. VI) plasmid library (provided by Nancy Standart, Cambridge, UK). Low stringency hybridization conditions yielded several clones, one of which showed significant sequence homology to the hPARN cDNA. Because this clone lacked the 3′ 357 nt found in the human clone, we used a fragment of the xPARN clone to screen a *Xenopus* kidney λ ZAPII cDNA (Stratagene). Out of 600,000 clones screened, two positives were obtained, one of which contained a complete 3′ end as indicated by multiple in-frame stop codons. These overlapping clones were used to derive the deduced amino acid sequence shown in Figure 3. This sequence has been assigned GenBank accession number AF309688.

Injections and nucleus isolation

We injected 10,000 cpm (30 nL) ^{32}P-labeled G52 A$^+$ (as described above) into oocyte nuclei and cytoplasm. Incubations were performed at 20 °C for 3–4 h. Maturation of oocytes was achieved by a 12-h incubation in 10 μg/mL progesterone (Sigma). Oocytes were incubated in 1× priming buffer (Birkenmeier et al., 1978) for 30 min, then a small incision was made with a 26G3/8 gauge syringe needle at the top of the animal pole and the nucleus was gently extruded by squeezing the oocyte. Nuclear injections were standardized by injecting blue dextran, isolating nuclei, and verifying the injection of dye. RNA was isolated from intact oocytes, oocyte cytoplasms, and nuclei (with an equal number of uninjected oocytes) as described (Varnum et al., 1992). RNAs were analyzed on denaturing 6% acrylamide gels and detected by a Molecular Dynamics PhosphorImager.

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