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Cap-dependent deadenylation of mRNA

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Poly(A) tail removal is often the initial and rate-limiting step in mRNA decay and is also responsible for translational silencing of maternal mRNAs during oocyte maturation and early development. Here we report that deadenylation in HeLa cell extracts and by a purified mammalian poly(A)-specific exoribonuclease, PARN (previously designated deadenylating nuclease, DAN), is stimulated by the presence of an m^7-guanosine cap on substrate RNAs. Known cap-binding proteins, such as eIF4E and the nuclear cap-binding complex, are not detectable in the enzyme preparation, and PARN itself binds to m^7GTP–Sepharose and is eluted specifically with the cap analog m^7GTP. *Xenopus* PARN is known to catalyze mRNA deadenylation during oocyte maturation. The enzyme is depleted from oocyte extract with m^7GTP–Sepharose, can be photocross-linked to the m^7GpppG cap and deadenylates m^7GpppG-capped RNAs more efficiently than ApppG-capped RNAs both *in vitro* and *in vivo*. These data provide additional evidence that PARN is responsible for deadenylation during oocyte maturation and suggest that interactions between 5′ cap and 3′ poly(A) tail may integrate translational efficiency with mRNA stability.

Keywords: cap structure/deadenylation/mRNA stability/oocyte maturation/poly(A) tails

Introduction

Deadenylation, i.e. the exonucleolytic degradation of the 3′ poly(A) tracts of eukaryotic mRNAs, plays an important role in both mRNA degradation and translational silencing. In *Saccharomyces cerevisiae*, two general pathways of mRNA decay have been characterized, both of which are initiated by poly(A) removal. In the deadenylation-dependent decapping pathway, removal of the 5′ cap occurs rapidly after the poly(A) tail has been shortened to ~10 nucleotides. Decapping is then followed by 5′ exonucleolytic degradation of the remaining mRNA 'body' (Muhlrad *et al*., 1994; Beelman and Parker, 1995; Caponigro and Parker, 1996). In a second pathway, the 5′ cap is not removed, and 3′-exonucleolytic decay continues after poly(A) tail removal (Jacobs Anderson and Parker, 1998). In higher eukaryotes, the decay of many mRNAs is also initiated by deadenylation (Wilson and Treisman, 1988; Shyu *et al*., 1991; Chen and Shyu, 1995; Ross, 1995), but the subsequent degradation events remain undefined. The conservation of several genes encoding RNA turnover components (Bashkirov *et al*., 1997; Mitchell *et al*., 1997; Tharun and Parker, 1999) and additional indirect evidence (Couttet *et al*., 1997) suggest that the two pathways analyzed in yeast also exist in mammalian cells, but direct evidence is lacking, and their relative contributions to the degradation of specific mRNAs are unknown. In addition to the deadenylation-dependent pathways, mRNA decay can also be initiated by endonucleolytic cleavage within the 3′-untranslated region (3′-UTR) (Ross, 1995; Chernokalskaya *et al*., 1998).

Poly(A) removal not only initiates mRNA turnover in yeast and in somatic metazoan cells, but is also used to silence maternal mRNAs translationally during oocyte maturation and embryogenesis in diverse species (Richter, 1996). During oocyte maturation, deadenylation does not require specific *cis*-elements and is a default pathway for mRNAs that do not undergo compensatory poly(A) elongation (Fox and Wickens, 1990; Varnum and Wormington, 1990). In contrast, certain mRNAs which are polyadenylated during meiotic maturation contain 3′-UTR elements that promote their subsequent deadenylation after fertilization. Thus, the translation of these mRNAs is restricted to mature oocytes (Bouvet *et al*., 1994; Legagneux *et al*., 1995). In both cases, deadenylation does not destabilize mRNAs immediately, but is a prerequisite for their degradation at later stages of development (Audic *et al*., 1997; Gillian-Daniel *et al*., 1998; Voeltz and Steitz, 1998). The uncoupling of deadenylation from mRNA decay in gametes and embryos contrasts with both yeast and metazoan cells in which poly(A) removal rapidly promotes mRNA degradation.

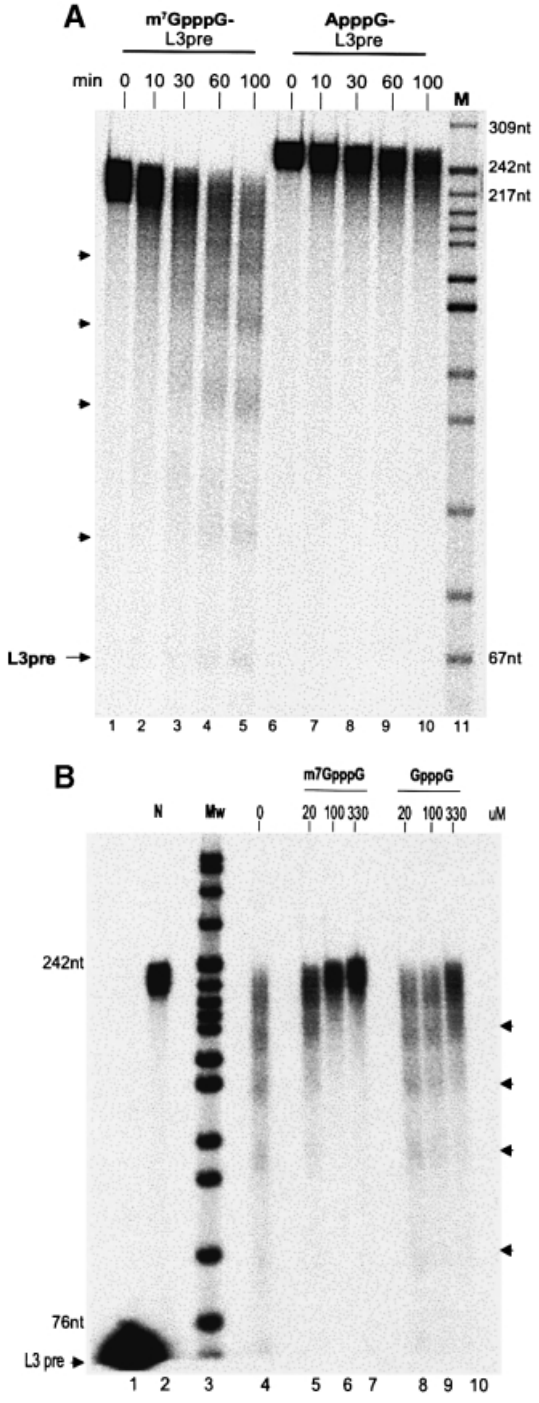
Both the dependence of decapping on prior deadenylation and the inhibition of translation initiation by poly(A) removal reflect interactions between the 5′ cap and the 3′ poly(A) tail of the mRNA. An increasing number of such interactions have been described recently (Wickens *et al*., 1997). The deadenylation dependence of RNA decapping is due to the ability of the poly(A)-binding protein 1 (Pab1p in yeast or PABP1 in metazoans) to inhibit decapping by an unknown mechanism (Muhlrad *et al*., 1994; Caponigro and Parker, 1995; Coller *et al*., 1998). Deadenylation removes the PABP1-binding sites and thus relieves the inhibition of decapping. Deadenylation concomitantly inhibits translation initiation presumably due to the involvement of Pab1p in ribosome loading

at the 5′ end. The central player in translation initiation is the eIF4G subunit of the multimeric eIF4F complex. 40S ribosomal subunit recruitment is facilitated by the synergistic binding of eIF4G to both the cap–eIF4E complex and the poly(A)–Pab1p complex, leading to juxtaposition of both ends of the mRNA into a 'closed loop' (Jacobson, 1996; Tarun and Sachs, 1996; Tarun *et al*., 1997; Imataka *et al*., 1998; Piron *et al*., 1998; Preiss and Hentze, 1998; Wells *et al*., 1998; De Gregorio *et al*., 1999; Otero *et al*., 1999).

As cytoplasmic, translationally active mRNA is the substrate for degradation, and the two main targets of the degradation pathways, the 5′ cap and 3′ poly(A) tail, are also determinants of efficient translation initiation, translation and mRNA turnover would be expected to be integrated. Indeed, the most compelling example illustrating this linkage is the nonsense-mediated decay pathway, which leads to premature degradation of mRNAs containing inappropriate nonsense codons within their coding sequences (Jacobson and Peltz, 1996; Ruiz-Echevarria *et al*., 1996). In yeast, these RNAs undergo deadenylation-independent decapping (Muhlrad and Parker, 1994).

The 3′ exonucleases responsible for mRNA deadenylation *in vivo* remain largely unidentified. In *S.cerevisiae*, one deadenylase, PAN, has been characterized. Poly(A) shortening by PAN is involved in specifying nuclear poly(A) tail length (Brown and Sachs, 1998). The phenotype of PAN mutants indicates that a role for PAN in cytoplasmic deadenylation, if any, cannot be exclusive; additional enzymes must contribute to cytoplasmic poly(A) removal (Boeck *et al*., 1996; Brown *et al*., 1996). In vertebrates, one poly(A)-specific 3′ exonuclease has been purified and cloned. This enzyme, initially described as the deadenylating nuclease, DAN (Körner and Wahle, 1997; Körner *et al*., 1998), and subsequently renamed poly(A)-specific ribonuclease, PARN (Körner *et al*., 1998; Buiting *et al*., 1999), catalyzes deadenylation during the meiotic maturation of *Xenopus* oocytes (Körner *et al*., 1998). It is not known whether PARN is involved in mRNA deadenylation in somatic cells. The identity of another poly(A)-specific 3′ exonuclease (Astrom *et al*., 1991, 1992) and its relationship to PARN remain to be determined.

Here we report that deadenylation by PARN is stimulated by the presence of an N^7-methyl guanosine cap. This cap dependence was found in cytoplasmic extracts from both HeLa cells and *Xenopus* oocytes, with purified bovine PARN and in *Xenopus* oocytes *in vivo*. Recognition of the cap appears to be an intrinsic property of PARN itself.



Results

The 7-methyl guanosine cap stimulates deadenylation in vitro and in vivo

In agreement with previous results, a polyadenylated RNA with a 7-methyl guanosine cap (m^7GpppG) at its 5′ end was deadenylated upon incubation in a HeLa cell cytoplasmic extract. The characteristic pattern of decay intermediates differing in length by ~30 nucleotides has

Fig. 1. (**A**) Cap-dependent deadenylation in a HeLa cytoplasmic extract. Two reaction mixtures were assembled on ice with 36 fmol of either m^7GpppG-capped or ApppG-capped, polyadenylated L3 pre-RNA in 70 µl of reaction buffer. The reaction was started by the addition of 1.7 µl of a HeLa cell cytoplasmic extract and incubation at 37°C. Aliquots were taken at the times indicated and RNAs analyzed on an 8% denaturing polyacrylamide gel. Substrate, decay products and completely deadenylated L3 pre-RNA are indicated by arrowheads. Sizes (in nucleotides) of the DNA marker are shown on the right. (**B**) Competition with free cap in HeLa cell cytoplasmic extract. The 20 µl reaction mixtures were assembled on ice with 10 fmol of m^7G-capped and polyadenylated (A_{160}) L3 pre-RNA, 0.5 µl of HeLa cell cytoplasmic extract and 0, 20, 100 or 330 µM m^7GpppG or GpppG. The deadenylation reaction was carried out at 37°C for 80 min. Products were separated on an 8% denaturing polyacrylamide gel and analyzed on a PhosphorImager. Lane 1, substrate RNA lacking a poly(A) tail; lane 2, polyadenylated substrate RNA without incubation; lane 3, DNA size marker; the sizes of two fragments are indicated on the left. Deadenylation intermediates are indicated by arrowheads on the right.

been described previously and is caused by partial protection of the poly(A) tail through the binding of PABP1 (Körner and Wahle, 1997). A small amount of completely deadenylated RNA accumulated upon longer incubation times (Figure 1A). In contrast, ApppG-capped substrate RNA generated neither the periodic pattern of decay intermediates nor an accumulation of fully deadenylated RNA (Figure 1A). Also, the full-length polyadenylated substrate was lost at a 2-fold lower rate (Figure 1A; quantitation not shown). These results suggested not only that RNA decay was retarded in the absence of a bona fide cap structure, but that the majority of ApppG-capped RNA was degraded by a different pathway. RNA lacking any cap was degraded at a rate similar to m^7GpppG-capped substrate RNA, but again the partially deadenylated intermediates characteristic for deadenylation by PARN were not detected (data not shown).

No difference in deadenylation efficiencies was observed between RNA substrates synthesized with either m^7GpppG or non-methylated GpppG caps. As this lack of distinction may reflect cap methylation in the extract, a requirement for the 7-methyl group was tested further by inhibition experiments: free m^7GpppG inhibited deadenylation at the lowest concentration tested, 20 μM, whereas a 5- to 10-fold higher concentration of unmethylated GpppG was required to achieve a similar effect (Figure 1B).

As PARN is the nuclease responsible for poly(A) shortening in HeLa cell cytoplasmic extracts (Körner and Wahle, 1997), the cap dependence of deadenylation catalyzed by purified PARN was examined. The calf thymus PARN preparation described earlier (Körner and Wahle, 1997) exhibited cap-dependent deadenylation activity with two different substrate RNAs tested (Figure 2; data not shown). m^7GpppG-capped RNA directed a 4-fold higher rate of product accumulation compared with GpppG-capped RNA in reactions containing purified PARN (Figure 2). Interestingly, PARN was also able to invade the non-adenylate portion of the m^7GpppG-capped RNA, which was resistant to degradation in GpppG-capped RNA substrates (Figure 2A). Presumably, this reflects a higher affinity of the enzyme for the m^7GpppG-capped RNA rather than a reduced specificity for poly(A). Substrates that either contained an ApppG cap or lacked a 5′ cap entirely were deadenylated at the same rate as GpppG-capped substrates.

When purified PARN was assayed by the release of acid-soluble nucleotides from a radiolabeled poly(A) tail, the m^7GpppG-capped RNA was also deadenylated at a 4-fold higher rate relative to RNA containing an unmethylated cap (data not shown). Purified PARN was also inhibited by free cap, the methylated form again being more efficient (Figure 3). In the reaction lacking free cap, the amount of PARN used was sufficient to deadenylate all of the accessible substrate and degrade most of it further; only a small amount of completely deadenylated RNA remained, in addition to a completely resistant fraction. With increasing inhibition by m^7GpppG, both the completely deadenylated RNA and partially deadenylated intermediates became visible. At the highest concentration of m^7GpppG, little deadenylation was observed. In contrast, 250 μM unmethylated GpppG was less inhibitory than 15 μM m^7GpppG (Figure 3).

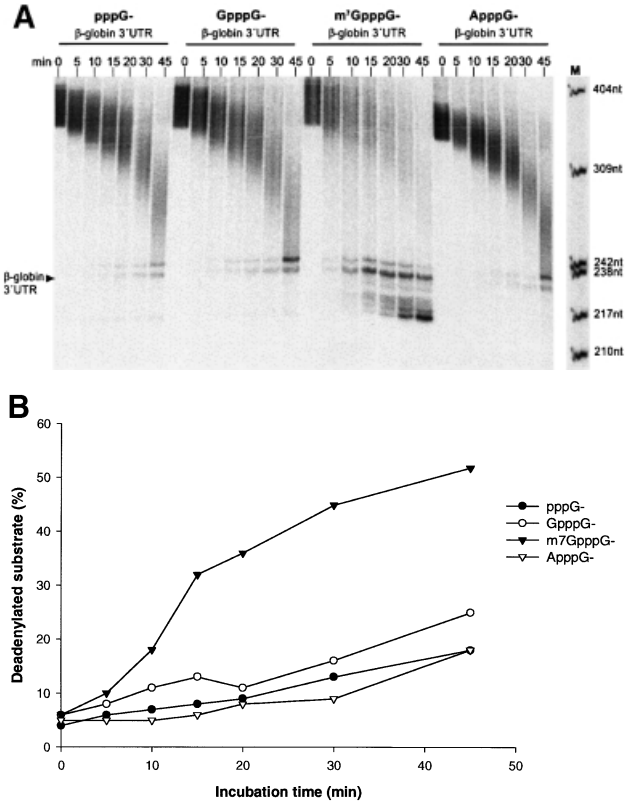


Fig. 2. Purified PARN prefers correctly capped RNA. (**A**) The 50 μl reaction mixtures were assembled on ice containing 15 fmol of bovine PARN and 40 fmol of polyadenylated (A_{130}) β-globin 3′-UTR RNA with an m^7GpppG, GpppG, ApppG dinucleotide or pppG at the 5′ end of the transcript. The reactions were started by the addition of enzyme and incubation at 37°C. Then 7 μl aliquots were withdrawn at the time points indicated in the figure, and decay products were separated on a 6% denaturing polyacrylamide gel and analyzed on a PhosphorImager. Fully deadenylated β-globin RNA is indicated by an arrowhead, and the sizes of the DNA marker are shown at the side of the M lane. (**B**) Quantitation of the accumulation of fully deadenylated RNA in (A). The appearance of deadenylated substrate at each time point, as part of the total radioactivity in each lane, was quantitated on a PhosphorImager and plotted versus incubation time.

It was demonstrated previously that anti-PARN antibody microinjected into *Xenopus* oocytes inhibits default deadenylation during meiotic maturation and that human PARN can substitute for the amphibian nuclease that catalyzes this reaction *in vivo* (Körner *et al*., 1998). In order to determine the relevance of the 5′ cap for deadenylation *in vivo* and at the same time provide an additional test for the role of *Xenopus* PARN in default deadenylation, we examined the cap dependence of default deadenylation *in vivo* and of poly(A) removal directed by the *Xenopus* PARN *in vitro.*

Radiolabeled substrate RNAs containing 5′ m^7GpppG, GpppG or ApppG caps were microinjected into progesterone-matured oocytes and assayed for deadenylation. ApppG was used to generate a stable 'uncapped' RNA as transcripts containing free 5′-terminal phosphates are degraded rapidly in microinjected oocytes (Drummond *et al*., 1985). As shown in Figure 4A, >50% of m^7GpppG-capped RNA was deadenylated 2 h after injection and >80% was deadenylated by 6 h. In contrast, only 15% of

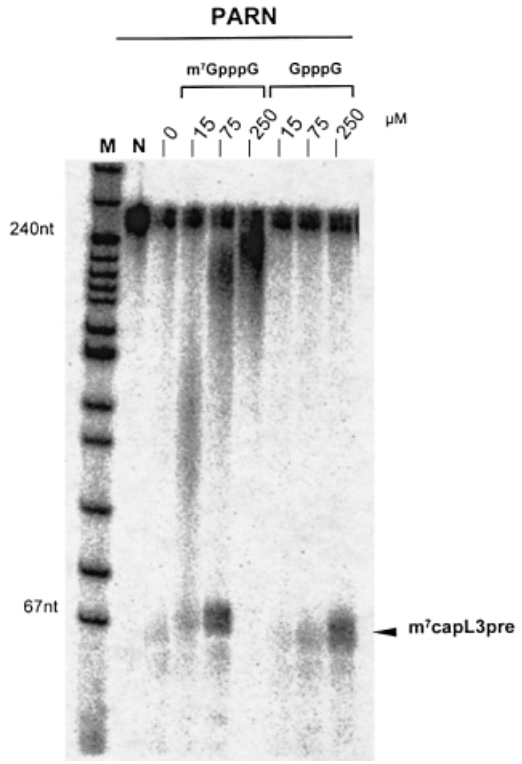


Fig. 3. Inhibition of purified PARN by free m^7GpppG. A reaction mixture was assembled on ice containing 95 fmol of m^7G-capped and polyadenylated (A_{160}) L3 pre-RNA and 60 fmol of bovine PARN in 160 μl of reaction buffer. The mix was divided into 20 μl aliquots and m^7GpppG or GpppG was added at the concentrations indicated. The reactions were started by incubation at 37°C. After 90 min, they were stopped and products analyzed on a denaturing 8% polyacrylamide gel. The sizes of DNA markers are indicated. The position of fully deadenylated L3 pre-RNA (m^7capL3pre) is indicated by an arrowhead. Note that the amount of PARN used was sufficient to degrade most of the body of the RNA; fully deadenylated RNA became visible upon partial inhibition by intermediate concentrations of free cap (see the text).

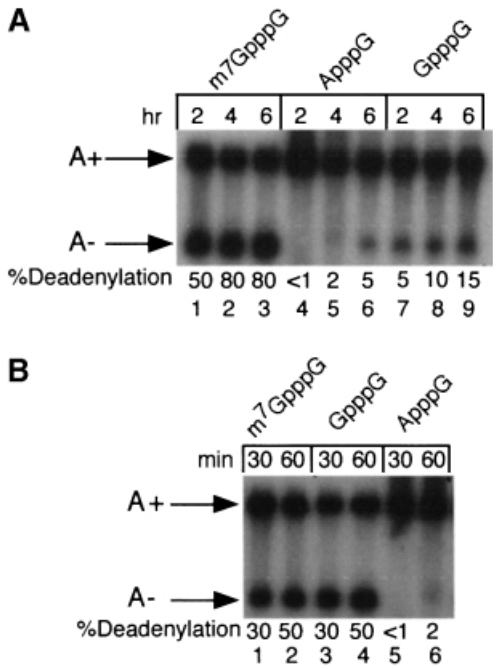


Fig. 4. Cap-dependent deadenylation by *Xenopus* PARN. (**A**) *In vivo* deadenylation in mature oocytes. Gel-purified, radiolabeled G52 RNA containing 5′-terminal m^7GpppG (lanes 1–3), ApppG (lanes 4–6) or GpppG (lanes 7–9) structures was injected into progesterone-matured *Xenopus* oocytes. RNA was isolated from oocytes 2, 4 or 6 h post-injection and analyzed by electrophoresis in 6% polyacrylamide–7 M urea gels. (**B**) *In vitro* deadenylation in *Xenopus* oocyte extracts. Oocyte extracts were incubated at 25°C for the indicated times with gel-purified, radiolabeled G52 RNA containing 5′-terminal m^7GpppG (lanes 1 and 2), GpppG (lanes 3 and 4) or ApppG (lanes 5 and 6) structures.

the non-methylated GpppG-capped RNA was deadenylated 6 h after injection and <5% of RNA containing an ApppG 5′ terminus was deadenylated. Thus, the presence of an m^7GpppG cap significantly stimulates default deadenylation in mature oocytes.

As seen with mammalian extracts, deadenylation in extracts derived from mature *Xenopus* oocytes was also cap dependent (Figure 4B). RNAs containing either methylated m^7GpppG- or non-methylated GpppG-capped RNAs were deadenylated with comparable efficiencies. This lack of distinction was not due to the methylation of GpppG-capped RNAs in these extracts (data not shown). However, a substrate RNA containing the non-physiological ApppG cap was 20-fold less efficient as an *in vitro* deadenylation substrate. We conclude that both mammalian and amphibian PARNs exhibit a marked dependence on the 5′ cap, with most experiments showing a preference for a bona fide m^7GpppG cap structure.

PARN is a cap-binding protein

The cap dependence of deadenylation in both cell-free extracts and in *Xenopus* oocytes *in vivo* could reflect either an intrinsic cap-binding activity of PARN or a requirement for cap-binding proteins as cofactors for this nuclease. Neither of two characterized cap-binding proteins, eIF4E (Gingras *et al.*, 1999) or the 20 kDa subunit of the nuclear cap-binding complex (Izaurralde *et al.*, 1994), were detectable by immunoblot analysis of purified bovine PARN. Less than 1 pmol of eIF4E was present for 7 pmol of PARN, as determined by comparison with a purified eIF4E standard. No standard was available for the 20 kDa subunit of the nuclear cap-binding complex, but strong signals were easily obtained from nuclear extract whereas the protein was not detectable in the purified nuclease (data not shown).

In order to determine whether PARN itself has intrinsic cap-binding activity, the ability of partially purified PARN to bind m^7GTP–Sepharose was assayed. A significant and selective depletion of PARN was evident by a comparison of the loaded and the supernatant fractions after incubation with m^7GTP–Sepharose (Figure 5). A small amount of PARN was released from the m^7GTP resin with buffer containing no nucleotide, and negligible PARN was released by a subsequent wash with buffer containing GTP. In contrast, most of the enzyme was eluted with m^7GTP and some remaining protein with SDS (Figure 5). If binding to the beads had been mediated by an associated cap-binding factor, stoichiometric amounts of this putative protein should have been present in the eluate. This was not the case. Only a small amount of a 60 kDa protein was detectable. In the PARN preparation used for this experiment, a 60 kDa polypeptide reacted with anti-PARN antibody in a Western blot. Thus, the only other protein

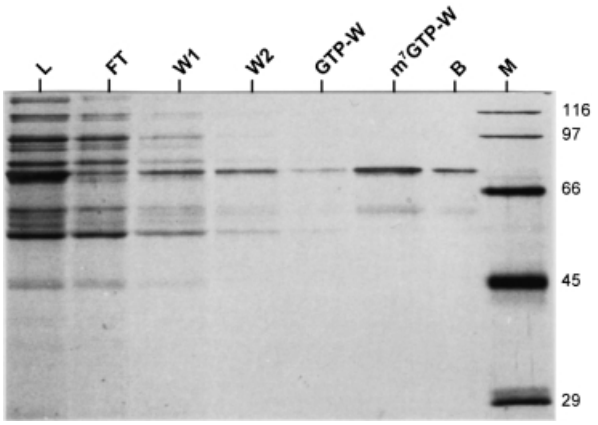


Fig. 5. Binding of bovine PARN to m^{7}GTP–Sepharose. The experiment was carried out with a partially purified fraction of PARN as described in Materials and methods. Lane L (load), starting material; lane FT (flow-through), unbound proteins in the supernatant; lanes W1 and W2, washes with nucleotide-free buffer; lane GTP-W, GTP wash; lane m^{7}GTP-W, m^{7}GTP wash; lane B, beads; lane M, molecular weight markers. Proteins were detected by silver staining. Note that only 50% of the total sample was loaded in lanes L and FT, whereas the entire samples were loaded in the other lanes.

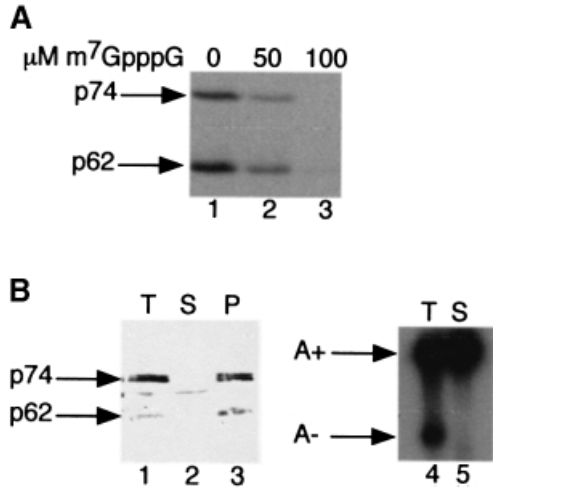


Fig. 6. Binding of *Xenopus* PARN to m^{7}GpppG cap. (**A**) Photochemical cross-linking of *Xenopus* p74 and p62 PARN isoforms to [^{32}P]cap-labeled RNA. Total mature oocyte extract was incubated with 20 fmol of G52 RNA containing a radiolabeled m^{7}GpppG cap in the presence of 0, 50 or 100 μM m^{7}GpppG. After UV irradation and digestion with RNase A, samples were immunoprecipitated with anti-PARN 205-5 antibody and analyzed by 10% SDS–PAGE. (**B**) m^{7}GTP–Sepharose depletion of *Xenopus* PARN. Total oocyte extract was incubated with m^{7}GTP–Sepharose for 2 h at 4°C. Fractions corresponding to two oocyte equivalents of total (T), depleted supernatant (S) and bound (P) protein eluted with 0.5 mM m^{7}GpppG were analyzed by immunoblot probed with anti-PARN 205-5 antibody (lanes 1–3). In addition, total (T) and m^{7}GTP–Sepharose-depleted extract was analyzed for deadenylation activity by incubation for 1 h at 25°C with radiolabeled m^{7}GpppG-capped G52 substrate RNA.

showing specific binding to the m^{7}GTP resin was probably a proteolytic fragment of PARN. Interestingly, deadenylation catalyzed by bacterially expressed recombinant PARN was not cap dependent, and binding of recombinant PARN to m^{7}GTP or m^{7}GpppG was much weaker by several criteria, suggesting that post-translational modifications may be required for cap recognition by this enzyme (data not shown).

The ability of *Xenopus* PARN present in extracts derived from mature *Xenopus* oocytes to bind the 5′ cap was also tested. *Xenopus* oocytes contain two isoforms of PARN: a 74 kDa species that initially is localized to the oocyte nucleus and catalyzes default deadenylation at maturation and a 62 kDa protein that is exclusively cytoplasmic (Körner *et al*., 1998). Both polypeptides could be cross-linked photochemically to RNA containing radioactive label exclusively in the m^{7}GpppG cap (Figure 6A). This cap binding was specific as it was competed by the presence of free m^{7}GpppG. Both p74 and p62 PARN isoforms could be depleted quantitatively from oocyte extracts with m^{7}GTP–Sepharose (Figure 6B, lanes 1–3). This depletion correlated with the loss of default deadenylation activity *in vitro* (Figure 6B, lanes 4 and 5). We conclude that both amphibian and mammalian PARNs not only exhibit a strong cap dependence in catalyzing deadenylation, but that cap binding is an inherent and evolutionarily conserved property of this enzyme.

Discussion

Here we have presented evidence that PARN-dependent deadenylation of mRNA *in vivo* and *in vitro* is stimulated by a 5′ cap structure. PARN itself is responsible for the cap dependence of deadenylation by binding to m^{7}GpppG. While most experiments revealed a preference for the genuine methylated cap structure, this was not seen in *Xenopus* oocyte extracts. A possible explanation might be that m^{7}GpppG-capped RNA is a better substrate for PARN but at the same time is protected more efficiently by other cap-binding proteins. Our results suggest that the ‘closed loop’ configuration in which the 5′ cap and the 3′ poly(A) tail are juxtaposed is the optimal substrate not only for the assembly of a translation initiation complex but also for deadenylation.

In HeLa cell extracts, capped RNA was deadenylated with the previously described pattern of intermediates caused by partial protection through PABP1 binding (Körner and Wahle, 1997), whereas uncapped RNA or RNA containing a non-physiological cap did not show these decay intermediates. This difference might be caused by an increased affinity of PABP1 for the m^{7}GpppG-capped RNA owing to its indirect interaction with the cap mediated by eIF4E and eIF4G. In this case, one would expect the uncapped RNA, being protected less stringently by PABP1, to be deadenylated faster than the capped RNA. However, uncapped and m^{7}GpppG-capped RNAs exhibited comparable stabilities in HeLa cell extracts, and ApppG-capped RNA was, in fact, the most stable. In *Xenopus* oocyte extracts, the ApppG cap had an even stronger stabilizing effect, and deadenylation intermediates were not detected with m^{7}GpppG-capped RNAs, presumably owing to the limited amount of PABP1 present (Wormington *et al*., 1996). Thus, differences in protection by PABP1 are unlikely to account for the differences in decay. Instead, m^{7}GpppG-capped RNA appeared to decay mainly through deadenylation by PARN, whereas uncapped or non-physiologically capped RNA was degraded primarily through other pathways without detectable intermediates. Experiments with purified PARN confirmed that deadenylation is indeed cap dependent.

The pattern of deadenylation observed here with capped, polyadenylated substrate RNAs might be considered evidence for a processive reaction mechanism, as full-length substrate and completely deadenylated product co-existed. However, the substrate RNAs are likely to be heterogeneous due to incomplete capping, partially blocked 3′ ends (Körner and Wahle, 1997) and, possibly, conformational heterogeneity. Thus, conclusions regarding the processivity of PARN should not be drawn from the experiments presented here. When homopolymeric poly(A) containing a 5′ monophosphate is used as the substrate, PARN acts distributively (Körner and Wahle, 1997). Additional experiments are required to determine whether this distributive behavior is altered by the interaction of PARN with the cap or with other non-adenylate residues within the substrate.

Cap-dependent deadenylation does not appear to require either of the two known cap-binding proteins, eIF4E or the nuclear cap-binding complex. The sequence of human PARN contains a possible match (amino acids 56–72) to the eIF4E-binding site of eIF4G (Morley *et al.*, 1997), although the invariant tyrosine required for the interaction with eIF4E (Marcotrigiano *et al.*, 1999) is not present. Immunoblot analysis of purified PARN did not reveal the presence of eIF4E, and no detectable eIF4E co-purified with PARN on m^7GTP–Sepharose. Also, the addition of recombinant eIF4E did not enhance the cap dependence of PARN activity (C.G.Körner, unpublished data). Recombinant eIF4E did not inhibit PARN, as would be expected should these two proteins compete directly for cap binding. It is possible that in the absence of eIF4G (Haghigat and Sonenberg, 1997; Ptushkina *et al.*, 1998) and phosphorylation (Minich *et al.*, 1994), the cap-binding affinity of eIF4E is so weak that it cannot compete with PARN.

The two primary targets of mRNA decay, the 5′ cap and 3′ poly(A) tail, also play crucial roles in translation initiation by mediating multiple interactions through their respective binding proteins, eIF4E and PABP1. Thus, one would expect actively translated mRNAs to be stabilized via protection of both targets. A translation-dependent protection of the cap by Pab1p has in fact been demonstrated (Caponigro and Parker, 1995; Wickens *et al.*, 1997), although it is unclear how precisely the protection is mediated (Schwartz and Parker, 1999). Similarly, one would expect translation to protect mRNA against the attack of PARN, by preventing the enyzme's access to the cap and by stabilizing the binding of PABP1, which is inhibitory to PARN under physiological conditions (Wormington *et al.*, 1996; Körner and Wahle, 1997). As the cap accelerates deadenylation but is not absolutely required, binding of the translation initiation complex may be expected to modulate the rate of deadenylation and, thus, mRNA stability. In this respect, it is interesting that destabilizing AU-rich elements in the 3′-UTR of unstable mRNAs, which are known to stimulate deadenylation, have been shown to repress translation (Kruys *et al.*, 1989). A number of additional experiments, primarily in yeast, support the idea of a stabilizing influence of translation (Muhlrad *et al.*, 1995; Muckenthaler *et al.*, 1997; LaGrandeur and Parker, 1999; Schwartz and Parker, 1999). However, as the yeast genome contains no obvious homolog of PARN, it remains to be determined whether cap-dependent deadenylation exists in this organism and contributes to the translation dependence of mRNA stability. Even in mammalian cells, a role for PARN in cytoplasmic mRNA decay has not been demonstrated.

It should be noted that translation-mediated mRNA stabilization is not supported by all experiments. Inhibition of translation by insertion of a stable stem–loop structure into the 5′-UTR of mammalian mRNAs often resulted in the stabilization of an unstable mRNA, although this effect has not been observed uniformly (Jacobson and Peltz, 1996). The reason for these apparent discrepancies is unknown. However, one has to consider the possibility that the insertion of a stable RNA secondary structure in the 5′-UTR may not only inhibit translation but may also affect the extent to which the cap is protected by the translation initiation complex. A stable RNA structure inserted close to the cap might even directly inhibit PARN independently of its influence on translation.

The data presented here are consistent with the idea that the translational status of an mRNA is a major factor in determining its half-life (Schwartz and Parker, 1999). The influence of translation and in particular of the initiation complex on deadenylation has to be tested directly. It will be of particular interest to determine whether destabilizing elements present within the 3′-UTR of mRNAs work by directly recruiting one or more of the enzymes catalyzing mRNA decay or, alternatively, accelerate decay as an indirect consequence of translational repression.

Materials and methods

RNA

pSP6L3pre and pSP6glob (Körner and Wahle, 1997) were linearized with *Rsa*I or *Eco*RI, respectively, and transcribed *in vitro* by SP6 RNA polymerase (Boehringer) in the presence of 50 μCi of [α-^{32}P]UTP, 600 μM GpppG or m^7GpppG (New England Biolabs), 100 μM GTP and UTP, 500 μM CTP and ATP. Polyadenylated transcripts were prepared as described (Körner and Wahle, 1997). Non-radioactive transcripts with homogeneously labeled poly(A) tails, used for the trichloroacetic acid (TCA) precipitation assay, were made with 125-fold molar excess of ATP over transcript and 50 μCi of [α-^{32}P]ATP. Capped and polyadenylated G52 RNAs (Varnum and Wormington, 1990) were synthesized with SP6 RNA polymerase and uniformly labeled with [α-^{32}P]UTP as described above except that reactions contained 50 μM GTP and 1 mM m^7GpppG, GpppG or ApppG. Unlabeled G52 RNA was synthesized as described (Wormington, 1991). A 5 pmol aliquot of gel-purified G52 RNA was incubated for 2 h at 37°C in a 20 μl reaction containing: 5 U of guanylyltransferase, 50 μCi of [α-^{32}P]GTP (800 Ci/mmol), 0.5 mM *S*-adenosyl-L-methionine, 50 mM Tris–HCl pH 7.9, 2 mM $MgCl_2$, 0.1 mM EDTA, 5 mM KCl, 2.5 mM dithiothreitol (DTT), 40 U of RNasin and 0.1 U of pyrophosphatase. RNAs typically were labeled to a specific activity of 2×10^3 d.p.m./fmol. Incorporation of the methylated cap was confirmed by thin-layer chromatography of nuclease P1 digestion products on polyethyleneimine–cellulose plates developed with saturated ammonium sulfate/1 M NaOAc/isopropanol (81:18:2) using m^7GpppG and GpppG as standards detected by UV shadowing. All RNAs were gel purified prior to use.

Proteins

PARN was the preparation described previously (Körner and Wahle, 1997). Antibodies to eIF4E and recombinant eIF4E were gifts from Martina Muckenthaler and Matthias Hentze, EMBL, Heidelberg. Antibodies to the nuclear cap-binding complex were a gift from Iain Mattaj, EMBL, Heidelberg.

Deadenylation and photochemical cross-linking assays

Deadenylation assays were performed as described (Körner and Wahle, 1997) with the substrate and enzyme concentrations indicated in the figure legends. The reaction buffers contained 100 mM KCl for assays

with purified enzyme and 120 mM potassium acetate for assays with HeLa cytoplasmic extract. Extracts derived from progesterone-matured *Xenopus* oocytes were prepared as described (Varnum *et al*., 1992). In brief, 100 mature oocytes were homogenized by repeated pipeting in 100 µl of buffer containing: 10% (v/v) glycerol, 10 mM HEPES–KOH pH 7.2, 70 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA. Homogenates were centrifuged at 14 000 r.p.m. for 5 min, to remove yolk platelets and pigment granules. Deadenylation reactions containing 10 µl of clarified supernatant and 5 fmol of uniformly labeled G52 RNA were performed as described (Wormington *et al*., 1996). Reaction products were electrophoresed on 6% polyacrylamide–7 M urea gels, visualized and quantified using a Molecular Dynamics PhosphorImager. Photochemical cross-linking reactions contained 10 µl of clarified supernatant, 5 fmol of [^{32}P]cap-labeled G52 RNA and 0, 50 or 100 µM unlabeled m^7GpppG competitor. After incubation for 10 min at 25°C, reactions were transferred to ice and UV irradiated for 10 min in a Stratalinker 1800. Samples were digested with 40 µg of RNase A for 30 min at 37°C, immunoprecipitated with anti-PARN 205-5 antiserum and analyzed by SDS–PAGE (10% polyacrylamide) as described (Körner *et al*., 1998). Cross-linked proteins were visualized by phosphoimagery.

Oocyte manipulation and microinjection

Stage VI oocytes were obtained and used for microinjection as described (Körner *et al*., 1998). In brief, defolliculated oocytes were isolated from collagenase-treated dissociated ovarian fragments and maintained at 18°C in modified Barth's saline. Oocytes were incubated for 12 h in the presence of 10 µg/ml of progesterone in order to induce maturation, which was ascertained by the appearance of a white spot on the animal hemisphere. Mature oocytes were injected cytoplasmically with 5–10 fmol of uniformly radiolabeled G52 RNA containing either m^7GpppG, GpppG or ApppG caps. Total RNA was isolated at various times after injection and analyzed as described (Körner *et al*., 1998).

Binding of PARN to m^7GTP–Sepharose

A 100 µl aliquot of a side fraction (#34) from the bovine PARN purification described (Körner and Wahle, 1997) was diluted in 500 µl of wash buffer [50 mM Tris–HCl pH 7.9, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2% (v/v) NP-40, 0.4 µg/ml leupeptin, 0.7 µg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Then 300 µl of this mixture were incubated with 150 µl of packed m^7GTP–Sepharose beads (Pharmacia), pre-washed with wash buffer, on a rotating wheel at 4°C for 2 h. The beads were pelleted by a quick centrifugation. They were washed successively twice with 300 µl of wash buffer, then with 300 µl of wash buffer containing 0.1 mM GTP and 300 µl of wash buffer containing 0.1 mM m^7GTP (Sigma). A 150 µl aliquot of each of the loaded material and the first supernatant and the entire volume of each of the washes was TCA precipitated with 10 µg of yeast RNA (Boehringer) as a carrier. Pellets were resuspended in 15 µl of SDS–gel loading buffer, separated on a 9% SDS–polyacrylamide gel and silver stained. To examine cap binding of *Xenopus* PARN, mature oocyte extracts were incubated with m^7GTP–Sepharose beads as described above and depleted supernatants were assayed for deadenylation activity *in vitro*. Fractions corresponding to total extract, depleted supernatant and bound protein eluted with 0.5 mM m^7GpppG were separated by SDS–PAGE (12.5% polyacrylamide) and analyzed by immunoblot analysis with anti-PARN 205-5 antiserum as described (Körner *et al*., 1998).

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Cap-dependence of mRNA deadenylation has independently been observed by Jeff Wilusz and colleagues [M.Gao, D.T.Fritz, L.P.Ford and J.Wilusz (2000) Interaction between a poly(A)-specific ribonuclease and the 5′ cap influences mRNA deadenylation rates *in vitro*. *Mol. Cell*, in press].