Poly(A)-Specific Ribonuclease (PARN)

BY

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Degradation of the mRNA 3'-end located poly(A) tail is an important step for mRNA decay in mammalian cells. Thus, to understand mRNA decay in detail, it is important to identify the catalytic activities involved in degrading poly(A). We identified and purified a 54-kDa polypeptide responsible for poly(A)-specific 3' exonuclease activity in calf thymus extracts. The 54-kDa polypeptide is a proteolytic fragment of the poly(A)-specific ribonuclease (PARN) 74-kDa polypeptide. PARN is a divalent metal ion dependent, poly(A)-specific, oligomeric, processive and cap interacting 3' exonuclease. An active deadenylation complex, consisting of the poly(A)-tailed RNA substrate and PARN, has been identified. The interaction with the 5'-end cap structure stimulates PARN activity and also amplifies the processivity of the deadenylation reaction. Furthermore, the cap binding site and the active site of PARN are separate from each other. To characterise the active site of PARN, we performed side-directed mutagenesis, Fe2+-mediated hydroxyl radical cleavage and metal ion switch experiments. We have demonstrated that the conserved acidic amino acid residues D28, E30, D292 and D382 of human PARN are essential for PARN activity and that these amino acid residues are directly involved in the co-ordination of at least two metal ions in the active site of PARN. Phosphorothioate modification on RNA substrates revealed that the pro-R oxygen atom of the scissile phosphate group interacts directly with the metal ion(s). Based on our studies, we propose a model for the action of PARN. Similarly to what has been observed for ribozymes, aminoglycoside antibiotics inhibit PARN activity, most likely by the displacement of catalytically important divalent metal ions. Among the aminoglycoside antibiotics tested, neomycin B is the most potent inhibitor. We speculate that inhibition of enzymes using similar catalytic mechanisms as PARN could be a reason for the toxic side effects caused by aminoglycoside antibiotics in clinical practice.

Key Words: Poly(A)-specific ribonuclease, PARN, deadenylation, processive, oligomeric, cap interacting, aminoglycoside, active site, Fe2+-mediated cleavage, metal ion switch.
To my family
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Martinez J*, Ren YG*, Thuresson AC, Hellman U, Astrom J and Virtanen A. A 54-kDa fragment of the poly(A)-specific ribonuclease is an oligomeric, processive, and cap-interacting poly(A)-specific 3’ exonuclease. J. Biol. Chem. 2000; 275(31): 24222-24230.

II. Martinez J, Ren YG, Nilson P, Ehrenberg M and Virtanen A. The mRNA m\(^7\)GpppG cap structure stimulates poly(A) removal and makes the reaction highly processive. J. Biol. Chem. 2001; 276(30): 27923-27929.

III. Ren YG, Martinez J and Virtanen A. Identification of the active site of poly(A)-specific ribonuclease by site-directed mutagenesis and Fe\(^{2+}\)-mediated cleavage. (submitted)

IV. Ren YG, Kirsebom LA and Virtanen A. Direct evidence for coordinated divalent metal ions in the active site of poly(A)-specific ribonuclease. (manuscript).

V. Ren YG, Martinez J, Kirsebom LA and Virtanen A. Inhibition of Klenow DNA polymerase and poly(A)-specific ribonuclease by aminoglycosides. (submitted)

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# TABLE OF CONTENTS

## ABBREVIATIONS

**THESIS**

- The mRNA 5’- and 3’-ends 7
- Poly(A) Tail - a Dynamic Structure 7
- Deadenylation 9

**Poly(A) Degrading Activities** 9

- Deadenylating Activities in Yeast 10
- Poly(A)-Specific Ribonuclease (PARN) 10
  - Purification and initial characterisation of PARN 10
  - Properties of PARN 11
    - Divalent metal ion dependence 11
    - Poly(A) specificity 12
    - Oligomeric structure 12
  - Processive mode of degradation 12
  - Interaction with the mRNA cap structure 13
- The active site of PARN 14
  - The conserved acidic amino acid residues are essential 14
  - Co-ordination of divalent metal ions 15
  - Interference of phosphorothioate modifications 16
- A model for PARN action 17

**Coupling Poly(A) Removal with mRNA Transport, Translation and Decay** 18

**Inhibition of PARN Activity by Aminoglycoside Antibiotics** 19

**REFERENCES** 22

**ACKNOWLEDGEMENTS** 28
ABBREVIATIONS

AMP  adenosine monophosphate
ATP  adenosine triphosphate
BSA  bovine serum albumin
cDNA complementary DNA
DMP  dimethyl pimelimidate
DNA  deoxyribonucleic acid
DTT  dithiothreitol
eIF  eukaryotic translation initiation factor
EMSA electrophoretic mobility shift assay
mRNA messenger RNA
kDa  kilo Dalton
NLS  nuclear localisation signal
PABP cytoplasmic poly(A) binding protein
PAN  poly(A) nuclease
PARN poly(A)-specific ribonuclease
PCR  polymerase chain reaction
Pol  DNA polymerase I
RNA  ribonucleic acid
RNase ribonuclease
RRM  RNA-recognition motif
SDS  sodium dodecyl sulphate
The flow of genetic information in all cells goes almost one way (not exclusively) from DNA via messenger RNA to protein as "The central dogma" presented by Francis Crick in 1958. Therefore, mRNA plays an important role in the regulation of gene expression.

The mRNA 5'- and 3'-ends

Almost all mRNAs in eukaryotic organisms contain the m7GpppG cap structure at the 5'-end and the poly(A) tail at the 3'-end (reviewed by Virtanen and Åström, 1997). These cis-acting elements bear multiple important functions in mRNA export, translation initiation and mRNA stability. It has been found that the rate of transport of mature mRNA from the nucleus to the cytoplasm is stimulated by the cap structure and the poly(A) tail (Brown et al., 1995; Eckner et al., 1991) (reviewed by Nakielny and Dreyfuss, 1999; Nakielny et al., 1997). The nuclear cap binding complex (CBC), comprising the CBP20 and CBP80 subunits, is believed to play a role in mRNA export. During translational initiation (shown in Fig. 1), the cap structure and the poly(A) tail of mRNAs are fundamentally important elements. They synergistically stimulate translational initiation by the engagement of protein-RNA and protein-protein interactions (Gallie, 1991) (reviewed by Sachs et al., 1997; Sachs and Varani, 2000)). The eukaryotic translation initiation factor eIF4F consists of three subunits, eIF4E (Tarun and Sachs, 1995), eIF4G (Hentze, 1997) and eIF4A (Grifo et al., 1984). eIF4E is the cytoplasmic cap binding protein and it interacts with the mRNA 5'-end m7G cap structure (Marcotrigiano et al., 1997). The cytoplasmic poly(A) binding protein (Pab1/PABP), which has four RNA-recognition motifs (RRM) (reviewed by Varani and Nagai, 1998), binds and covers about 25 adenosine long poly(A) tail at the 3'-end of mRNA (Deo et al., 1999). eIF4G contacts PABP, the translation initiation factors eIF3 and eIF4E. The simultaneous association of PABP and eIF4E by eIF4G (Tarun et al., 1997) leads to the circularization of the mRNA and causes the 5'- and 3'-ends of mRNA to interact functionally and physically by forming a "closed loop" (reviewed by Munroe and Jacobson, 1990). This circularization of mRNA, mediated by the eIF4E-eIF4G-PABP interaction, has been observed in vitro by atomic force microscopy (Wells et al., 1998). It is believed that the circularization of the mRNA leads to a more efficient recruitment of the small ribosomal subunit (40S subunit) and thereby stimulates of translational initiation (for further discussion, see Tarun and Sachs, 1996).

Poly(A) Tail - a Dynamic Structure

The modulation of poly(A) tail length is an important mechanism by which mRNA translation is regulated. The newly synthesised poly(A) tails of different transcripts are relatively homogeneous, ranging in length from 200 to 250 adenylate residues in mammals, and from 60 to 80 adenylate residues in yeast (Brawerman and Diez, 1975; Sachs and Davis, 1989; Sheets and Wickens, 1989; Sheiness et al., 1975). When the mRNA is exported to the cytoplasm, the length of the poly(A) tail
becomes modulated (McGrew et al., 1989; Paris and Richter, 1990; Sheets et al., 1994). Different cis-acting sequence elements and trans-acting factors are involved in controlling the poly(A) tail length (Baker and Liggit, 1993; Brawerman, 1981; Wilson and Treisman, 1988).

The regulation of selective adenylation or deadenylation of specific mRNAs has been well characterised during *Xenopus* oocyte maturation and early embryogenesis (reviewed by (Richter, 1991)). In frog oocytes, due to the absence of RNA transcription, translational control of gene expression is particularly important (reviewed in

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*Figure 1.* Coupling poly(A) removal with mRNA transport, translation and decay
(Wormington, 1994)). It has been found that the dynamic changes in poly(A) tail length always correlate with changes in translational activity (Fox and Wickens, 1990; McGrew et al., 1989; Vassalli et al., 1989). In general, poly(A) elongation of translationally dormant mRNAs results in translational activation, whereas deadenylation promotes translational silencing or repression (reviewed by (Richter, 1999)).

It has been proposed that there are at least two classes of mRNAs present in early *Xenopus* development. Class I mRNAs carry long poly(A) tails and are translationally active in *Xenopus* oocytes. During oocyte maturation, these messengers are deadenylated as a default reaction and dissociate from polysomes, thereby becoming translationally silent (Dworkin et al., 1985; Fox et al., 1989). The deadenylated RNAs are stored in an inactive state and may even be readenylated and used again at a later time (Wormington, 1994). In contrast, Class II mRNAs, having short oligo(A)-tails, receive long poly(A) tails by polyadenylation and consequently become translationally active (Dworkin and Dworkin-Rastl, 1990; Huarte et al., 1987; Hyman and Wormington, 1988).

In summary, poly(A) tail length influences translational efficiency. To determine the exact length of the poly(A) tail on specific mRNAs, the processes of polyadenylation and deadenylation must be highly regulated and balanced (reviewed by (Virtanen and Åström, 1997)).

### Deadenylation

The mechanism of mRNA decay pathways has successfully been dissected in yeast. Two major pathways of mRNA turnover have been identified (shown in Fig. 1), the deadenylation-dependent decapping pathway and the 3’-5’ decay pathway (reviewed by (Caponigro and Parker, 1996)). Both pathways are initiated by the rate-limiting step of deadenylation. In the deadenylation-dependent decapping pathway, decapping of the 5’-end cap structure, catalysed by the decapping enzyme (Dcp1p), occurs rapidly after the poly(A) tail has been shortened to A_{10} or less. After decapping, the mRNA is degraded 5’-exonucleolytically by the 5’ exonuclease (Xrn1p) (Decker and Parker, 1993; Muhlrad et al., 1995; Muhlrad and Parker, 1994). In the alternative pathway, the initial and fast deadenylation reaction is followed by 3’-exonucleolytic degradation of the mRNA by the exosome (Jacobs-Anderson and Parker, 1998). Thus, this pathway does not depend on decapping.

In higher eukaryotes, similar to the case in yeast, the degradation of many mRNAs is initiated by poly(A) tail shortening (Shyu et al., 1991; Wilson and Treisman, 1988). However, the subsequent degradation events remain undefined although recent indirect evidence favours decapping as the second step (Couttet et al., 1997). Recently, the decapping activity was found in mammalian extracts (Gao et al., 2001), and a mouse homolog of the XRN1 5’–3’ exonuclease was also reported (Bashkirov et al., 1997).

### Poly(A) Degrading Activities

To elucidate the mechanisms of deadenylation it is crucial to identify the poly(A)-specific exonuclease. Exonuclease activities degrading poly(A) have been extensively investigated in several eukaryotic species (reviewed by (Ross, 1995; Virtanen and Åström, 1997)). However, most of the activities identified in earlier reports were not poly(A)-specific exonucleases.
Deadenylating Activities in Yeast

In yeast, poly(A) nuclease (PAN) has been characterised and found to be a poly(A)-binding protein-dependent poly(A)-specific nuclease, since poly(A) tail shortening by PAN requires the poly(A) binding protein (PABP), either directly or indirectly (Brown and Sachs, 1998; Caponigro and Parker, 1995; Lowell et al., 1992; Sachs and Davis, 1989). PAN is composed of at least two polypeptides, Pan2p and Pan3p (Boeck et al., 1996; Brown et al., 1996). Pan2p contains a conserved RNase D exonuclease domain (Moser et al., 1997) and is most likely responsible for the catalytic activity. The function of PAN was proposed to define the poly(A) tail length of nascent mRNAs in the nucleus before export (Brown and Sachs, 1998). Because it is not a major activity for cytoplasmic mRNA decay (Tucker et al., 2001), additional enzymes must contribute to cytoplasmic poly(A) removal (Boeck et al., 1996; Brown et al., 1996). Recently, a major cytoplasmic deadenylating activity has been identified and this activity consists of at least two previously defined transcriptional regulators (carbon catabolite repression 4 (Ccr4) and Ccr4-associated factor (Caf1)) (Chang et al., 1999; Tucker et al., 2001). However, although Ccr4/Caf1 encode critical components required for optimal poly(A) nuclease activity both in vivo and in vitro, it still remains unclear whether any of these components harbours the deadenylating activity.

Poly(A)-Specific Ribonuclease (PARN)

In mammalian cells a number of different poly(A) degrading activities have been characterised. Lazarus and Sporn first reported in 1967 that a divalent cation-dependent nuclear exoribonuclease was present in Ehrlich tumour cells. This activity degrades single stranded RNA with some specificity for poly(A) (Lazarus and Sporn, 1967). Åström et al. (1991) defined and partially purified a poly(A)-specific 3’ exonuclease activity in HeLa cell-free extracts (Åström et al., 1991; Åström et al., 1992). The important properties of this activity were its high specificity for degrading only 3’-end located poly(A) tails, its requirement for a 3’-end hydroxyl group, and the release of 5’-AMP as the mononucleotide product. Recently, a poly(A)-specific ribonuclease (PARN) from calf thymus extracts, originally referred as DAN (deadenylating nuclease), was purified and biochemically characterised (paper I) (Körner and Wahle, 1997). PARN activity is associated with either a 74-kDa or a 54-kDa polypeptide. The 54-kDa polypeptide is a proteolytic fragment of the 74-kDa PARN. By cloning and protein expression of the corresponding human cDNA, PARN activity has been recovered from both the 74-kDa and 54-kDa PARN polypeptides (paper I) (Körner et al., 1998). The human PARN gene has been mapped on chromosome 16 (position 16p13), while an inactive copy which lacks the 5’-end of the gene is present on chromosome 15 (position 15q11-q13) (Buiting et al., 1999). PARN is ubiquitously expressed in all tissues (Buiting et al., 1999).

Purification and initial characterisation of PARN

To purify the poly(A)-specific 3’ exonuclease activity, we applied a dual assay strategy based on a "gel assay" and a "TLC" assay (paper I). i) Gel assay: consumption of the body-labeled L3(A30) RNA substrate and accumulation of the deadenylated RNA product were investigated by fractionation of the reacted RNAs using analytical polyacrylamide gel electrophoresis, and ii) TLC assay: the released mononucleotide 5’-AMP from the poly(A) tail-labeled L3(A30) RNA substrate was detected by thin-layer chromatography. The TLC assay was crucial for the identification of PARN activity and the
determination of the kinetic parameters of PARN.

The purified exonuclease activity from calf-thymus extracts was similar to the activity identified in HeLa extracts (Åström et al., 1991). After the final purification step (5’-AMP-Sepharose and 7-methyl-GTP-Sepharose chromatography), a prominent 54-kDa polypeptide was copurified with the 3’ exonuclease activity. Immunoblotting using a polyclonal antibody against the C-terminal part of the 74-kDa PARN (Körner and Wahle, 1997) demonstrated that the 74-kDa PARN was only present in fractions obtained during early purification steps. MALDI-TOF mass spectrometry analysis of the trypsin-digested 54-kDa polypeptide showed that six out of ten peptides matched the amino acid sequences of human PARN. The C-terminal end of 54-kDa PARN was proposed to be an asparagine at position 470.

Similarly, two forms of the PARN homologue (62-kDa and 74-kDa polypeptides) were also identified (Körner et al., 1998) and purified from Xenopus oocytes (Copeland and Wormington, 2001). Although the 62-kDa Xenopus PARN was found to be a proteolytic fragment of the 74-kDa Xenopus PARN polypeptide, this isoform was most likely present in vivo during certain developmental stages of Xenopus oocytes. More interestingly, the two forms differed in subcellular distribution; the 62-kDa isoform was cytoplasmic, whereas the 74-kDa isoform was exclusively nuclear. This observation was consistent with the finding that the conserved exonuclease domain (Mian, 1997; Moser et al., 1997) and the RNA recognition motif (RRM) (reviewed by Siomi and Dreyfuss, 1997) are present in the 62-kDa Xenopus PARN (Copeland and Wormington, 2001) as well as in the 54-kDa human PARN (only RNP-2, half of RRM), whereas two putative nuclear localisation signals (NLS) close to the C-terminal end of the 74-kDa human PARN are absent in the 54-kDa human PARN (paper II) (shown in Fig. 2). Therefore, it is possible that the 54-kDa human PARN isoform is present in vivo although we could not rule out the possibility that the 54-kDa polypeptide of bovine PARN was generated by proteolysis during purification.

Properties of PARN activity

**Divalent metal ion dependence** -- PARN activity could be modulated in vitro by changes in reaction conditions, and divalent metal ions were a prerequisite for catalysis. The optimal pH for PARN activity was around seven. Monovalent cations, potassium or sodium, were required for activity, and the optimal concentration was around 100-150 mM (paper I) (Körner and Wahle, 1997). At low salt

![Figure 2. The schematic structure of PARN](image-url)
concentrations, PARN activity depended on the presence of polycationic spermidine (Körner and Wahle, 1997).

PARN was initially identified as a Mg$^{2+}$-dependent exonuclease (Copeland and Wormington, 2001; Körner and Wahle, 1997; Åström et al., 1991). In contrast with the previous reports, we found that it was possible to replace Mg$^{2+}$ with Mn$^{2+}$ and still recover PARN activity (paper I). We further demonstrated that both the 74-kDa and 54-kDa human PARN polypeptides could carry out the deadenylation reaction in the presence of Fe$^{2+}$ (paper IV), Mn$^{2+}$, Zn$^{2+}$, Co$^{2+}$ and Cd$^{2+}$, using L3(A$_{30}$), A$_2$, A$_3$ or A$_5$ as substrates (paper IV). Therefore, we conclude that PARN is a divalent metal ion-dependent metalloenzyme.

Poly(A) specificity -- Three sets of experiments were performed to define the RNA substrate specificity of PARN (paper I). First, four different RNA substrates with poly(A), poly(U), poly(C) and poly(G) tails at the 3'-ends of the RNAs were incubated with PARN. The resulting RNA was purified and fractionated by gel electrophoresis. It was found that only the L3(A$_{30}$) RNA substrate was efficiently degraded, and that the deadenylated RNA body accumulated. The RNA substrate ML54(U$_{30}$) was degraded to some extent without the accumulation of the RNA body, whereas the RNA substrates ML43(G$_{14}$) and ML40(C$_{32}$) were almost unaffected by PARN. Then, we investigated the specificity of PARN kinetically using the TLC assay. This analysis revealed that poly(A) was approximately 10, 100 and 200 times more efficient as a substrate for PARN than poly(U), poly(C) and poly(G), respectively. The $K_m$ values for the four different substrates were all in the 10 nM range, suggesting that the $V_{max}$ parameter plays an important role for the PARN specificity. Finally, we investigated RNA substrates with internal poly(A) stretches (L3(A$_{30}$)X$_{15}$, L3(A$_{30}$)X$_{49}$, and L3(A$_{30}$)X$_{165}$). All these substrates were poorly degraded by PARN and only traces of deadenylation could be detected using the L3(A$_{30}$)X$_{15}$ RNA as substrate.

Taken together, the 54-kDa bovine PARN activity is highly specific for degrading 3'-end poly(A) tails. The 74-kDa bovine PARN and Xenopus PARN seem to have the same substrate preference (Copeland and Wormington, 2001; Körner and Wahle, 1997).

Oligomeric structure -- During purification, we consistently observed that PARN activity was fractionated by gel-filtration (SMART Superdex-200) with an estimated molecular weight of about 180-220 kDa (paper I). The significant discrepancy between the molecular weights estimated from gel filtration and SDS-PAGE suggested that the 54-kDa bovine PARN should be oligomeric. To clarify this further, we performed a protein/protein chemical cross-linking experiment using the homobifunctional cross-linkers. After cross-linking, the recombinant 74-kDa or 54-kDa human PARN polypeptides were shifted to two slower migrating forms as revealed by SDS-PAGE (paper I and II). These results strongly suggested that native PARN is oligomeric and most likely consists of three subunits (i.e. homotrimer).

Processive mode of degradation -- PARN acted in a highly processive mode when degrading poly(A) (paper I and II). The predictions for a highly processive mode of degradation were: (i) unreacted RNA substrate and fully deadenylated product should be present at some time points during degradation; (ii) a homogenous population of partially deadenylated RNA substrates should not be visible when the RNA substrate is in excess over the amount of enzyme; (iii) the time point for the appearance of the first fully deadenylated product should be independent on the RNA
substrate concentration (on the other hand, if the enzyme is distributive, the appearance of completely deadenylated substrates should be delayed as more substrates are added above the saturation level of the enzyme); and (iv) since the processive reaction mode allows the exonuclease to stay on the RNA substrate during multiple rounds of catalysis, it should be possible to detect the active enzyme-substrate complex. According to these criteria, we investigated the reaction mode for PARN activity. We found that both unreacted RNA substrate and fully deadenylated RNA product could be detected simultaneously. Thus, homogenous populations of partially deadenylated RNA substrates were not observed. Most importantly, we found that the fully deadenylated product appeared at the same time of incubation irrespectively of the RNA substrate concentration (paper I). Furthermore, an electrophoretic mobility shift assay (EMSA) revealed that the recombinant 74-kDa and 54-kDa human PARN polypeptides formed stable cap-independent complexes with poly(A)-tailed RNA substrates. As predicted, these complexes contained partially deadenylated RNAs (paper II and III). The recombinant 74-kDa human PARN also degraded oligo(A) substrates in a processive mode (paper IV). In conclusion, PARN is a highly processive 3’ exoribonuclease.

Although the processivity factor of PARN has not been determined biochemically, the presence of a putative RNA recognition motif (RRM) found in 74-kDa PARN (half motif in 54-kDa PARN) (Copeland and Wormington, 2001) is expected to be responsible for substrate binding. Multiple RRMs in an oligomeric PARN will most likely stabilise the interaction between PARN and the RNA substrate, making the deadenylation reaction more processive.

Interaction of the mRNA cap structure –
During purification we finally identified the 54-kDa polypeptide of bovine PARN after m7GTP-Sepharose chromatography (paper I). The m7GTP-Sepharose matrix partly resembles a methylated cap structure and has been used to purify cap-binding proteins (Morino et al., 1996). Similarly, the 74-kDa human, bovine and *Xenopus* PARN also bound to the m7GTP matrix (Copeland and Wormington, 2001; Gao et al., 2000; Körner et al., 1998). The strong binding of PARN to this matrix suggests that PARN is able to interact directly with the mRNA cap structure. This interaction affects PARN activity in different ways (paper I and II) (Gao et al., 2000; Körner et al., 1998).

In *cis*, the m7G(5’),ppp(5’)G cap structure at the 5’-end of mRNA stimulates the deadenylation activity and amplifies the processivity of PARN. The conclusion is based on the experiments where we used m7G(5’),ppp(5’)G capped, G(5’),ppp(5’)G capped or uncapped L3(A30) RNAs as substrates for deadenylation. We found that the m7G(5’),ppp(5’)G capped RNA was the most efficient substrate and that the efficiency parameter $V_{max}/K_m$ for the m7G(5’),ppp(5’)G capped substrate was higher than for the G(5’),ppp(5’)G and the uncapped substrates. These data suggested that the cap structure is a stimulatory factor for PARN activity. Moreover, the cap structure did not stimulate the activity, but also increased the processivity of the deadenylation reaction. When different amounts of the m7G(5’),ppp(5’)G capped or uncapped L3(A30) RNA substrates were deadenylated in *vitro*, the deadenylation reaction showed a processive mode. However, in the latter case it was not possible to detect both the L3(A30) RNA substrate and the fully deadenylated RNA product at the same time. When the uncapped L3(A5) RNA substrate was used, the substrate and the fully deadenylated L3 products could be simultaneously observed suggesting that the
processivity of PARN should be reduced in the uncapped cases (paper II). We conclude that the cap structure drives PARN into a highly processive mode of deadenylation without being an essential processivity factor.

In trans, the addition of free cap analogues at micromolar concentrations inhibited PARN activity dramatically, while AMP, GMP as the mononucleotide products inhibited the reaction only at 10 - 100 fold higher concentrations (paper I). The different efficacy of m'G(5')ppp(5')G and G(5')ppp(5')G cap analogues on the deadenylation reaction implied that the 7-methyl group of the cap structure should play a significant role in cap binding. The kinetic studies further showed that the free cap analogues inhibited PARN activity as non-competitive inhibitors suggesting that the binding site for the cap structure is separate from the active site of PARN. Surprisingly, the cap analogue m'G(5')ppp(5')G at low concentrations (<0.5 µM) slightly stimulated rather than inhibited deadenylation of the uncapped substrates or the substrates with G(5')ppp(5')G cap, but not with m'G(5')ppp(5')G cap. Similar observations that deadenylation activity was stimulated in cis by m'G(5')ppp(5')G cap structure and inhibited in trans by the cap analogues in vivo and in vitro were also made by other groups (Dehlin et al., 2000; Gao et al., 2000).

In summary, PARN interacts with both the 5'-end cap structure and the 3'-end poly(A) tail of mRNAs during the deadenylation reaction, and the cap structure binding site is distinct from the active site.

The active site of PARN

Bioinformatic analysis of PARN indicates that PARN belongs to the RNase D family (Körner et al., 1998; Mian, 1997; Moser et al., 1997). Four highly conserved acidic amino acid residues found in the RNase D family members are also present in the human PARN amino acid sequence as D28, E30, D292 and D382. The functional and structural studies on the 3'-5' exonuclease (or proofreading) domain of Escherichia coli DNA polymerase I (reviewed by (Joyce and Steitz, 1994)), a member of the RNase D family, suggested that the conserved acidic amino acid residues are most organised in three exonuclease motifs, Exo I, Exo II and Exo III (Bernad et al., 1989), comprising the catalytic active site of PARN (shown in Fig. 3). In the proofreading domain of Klenow fragment these amino acid residues co-ordinate two metal ions essential for catalysis, either directly or indirectly through bridging water molecules (Beese and Steitz, 1991; Steitz and Steitz, 1993).

The conserved acidic amino acid residues are essential -- By site-directed mutagenesis, we generated single point mutations at each of the conserved acidic amino acid residue D28, E30, D292 and D382 of human PARN by
substituting the corresponding residues with alanine or cysteine. In the presence of Mg\(^{2+}\), all the recombinant mutant polypeptides of PARN(D28A), PARN(E30A), PARN(D292A), PARN(D382A), PARN(D28C), PARN(E30C), PARN(D292C) and PARN(D382C) were catalytically inactive since RNA substrates L3(A\(^{30}\)) (paper III) and adenosine trinucleotide (A\(^{3}\)) (paper IV) were not consumed, and neither of the products, the deadenylated RNA or 5'-AMP, appeared. However, the recombinant alanine mutant polypeptides were able to form the same enzyme-substrate complexes as the wild type PARN, and their apparent RNA binding constants were very similar. Taken together our data showed that the conserved acidic amino acid residues are indispensable for PARN activity, but not crucial for RNA substrate binding.

**Co-ordination of divalent metal ions in the active site** -- Taking advantage of PARN being a metalloenzyme, we used Fe\(^{2+}\)-mediated hydroxyl radical cleavage and metal ion switch experiments to investigate the roles of the conserved acidic amino acid residue D28, E30, D292 and D382 for PARN activity. In the presence of reductants, Fe\(^{2+}\) can generate highly active hydroxyl radicals, which specifically cleave the polypeptide backbones or phosphodiester bonds close to the ion binding site (reviewed by (Draganescu and Tullius, 1996)). By resolving the cleaved bands of the protein in combination with site-directed mutagenesis, the amino acid residues involved in co-ordination of the metal ions can be identified without the crystal structures available. Fenton chemistry has successfully been applied to map the divalent metal ion binding sites in many proteins (Cao and Barany, 1998; Ermacora et al., 1992; Ettner et al., 1995; Farber and Levine, 1986; Godson et al., 2000; Grodsky et al., 2000; Hlavaty et al., 2000; Hlavaty and Nowak, 1997; Huang et al., 2000; Kim et al., 1999; Lykke-Andersen et al., 1997; Mustaev et al., 1997; Soundar and Colman, 1993; Wei et al., 1995; Wei et al., 1994; Zaychikov et al., 1996).

In the presence of both Fe\(^{2+}\) and DTT, the N-terminally \(^{32}\)P-labeled recombinant 74-kDa human PARN polypeptide was cleaved and generated two distinct \(^{32}\)P-labeled fragments. One cleaved fragment (cleavage I) was about 35 kDa, and the other (cleavage II) about 48 kDa in size. A difference between the two corresponding apparent Fe\(^{2+}\) binding constants (app\(K_d\)) of the two cleavages suggested that at least two Fe\(^{2+}\) ions should be involved in generating the cleavage products. We also found that a substitution of D28 to alanine dramatically weakened the intensities of the two cleaved footprint bands by affecting the app\(K_d\) values. Alanine substitution at E30 and D382 decreased the Fe\(^{2+}\) binding affinity and increased the app\(K_d\) values of cleavage II, but did not affect the app\(K_d\) values for cleavage I. The alanine substitution at D292 showed minor or no effects on the app\(K_d\) values at cleavage sites I and II. Taken together, the data of Fe\(^{2+}\)-mediated cleavage suggested that acidic amino acid residue D28, E30 and D382 are directly involved in the co-ordination of at least two Fe\(^{2+}\) metal ions in the active site of PARN. D28 is the most crucial residue and co-ordinates most likely two Fe\(^{2+}\) ions at the same time, whereas D28, E30 and D382 most likely share the same single metal ion for the co-ordination.

Chemically, Mn\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\) and Cd\(^{2+}\) behave as soft metal ions which preferentially co-ordinate soft ligands such as sulfur, while Mg\(^{2+}\) which is a hard metal ion prefers to chelate hard ligands such as oxygen (Jaffe and Cohn, 1978) (reviewed by (Verma and Eckstein, 1998)). Thus, the effect of shifting oxygen to sulfur can to some extent be compensated by changing Mg\(^{2+}\) to a soft metal ion such as Mn\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\).
or Cd$^{2+}$. This is referred as a metal ion switch experiment. Metal ion switch experiments have frequently been used to locate metal ions in ribozymes (reviewed by (Vortler and Eckstein, 2000)).

By means of the cysteine substitution of the conserved acidic amino acid residues of human PARN, we could show that the cysteine mutations severely impaired PARN activity in the presence of Mg$^{2+}$. Interestingly, the presence of soft metal ions such as Mn$^{2+}$, Zn$^{2+}$, Co$^{2+}$ and Cd$^{2+}$, could partially restore PARN activity of the mutant PARN(D28C), PARN(D292C) and PARN(D382C) polypeptides. This result suggested that the conserved D28, E30, D292 and D382 acidic amino acid residues of human PARN are present in the active site of PARN and are directly involved in the co-ordination of catalytically essential metal ion(s).

Interference of phosphorothioate modifications -- To investigate the chemical mechanism catalysed by PARN, we performed phosphorothioate interference analysis using 5'-32P labeled Rp- or Sp-phosphorothioate adenosine trinucleotides (A$_3$) (A$_3$s-Rp and A$_3$s-Sp) as RNA substrates. In these substrates, one of the nonbridging oxygens was substituted by sulfur in the phosphorate between the second and third adenosines from the 5'-end. In the presence of Mg$^{2+}$, Rp-phosphorothioate modified A$_3$ could be deadenylated. However, PARN activity was severely impaired. In contrast, in the metal ion switch experiments, the deadenylation reaction of A$_3$s-Rp was significantly improved in the presence of Cd$^{2+}$, Mn$^{2+}$, Zn$^{2+}$ or Co$^{2+}$. However, Sp-phosphorothioate A$_3$ could not be hydrolysed. These data suggested that the pro-R oxygen atom of the scissile phosphate in the transition state of deadenylation is involved in the co-ordination of the divalent metal ions. Surprisingly, the cysteine substitution mutant PARN(D382C) polypeptide could perform deadenylation to some extent in the presence of Mn$^{2+}$ using the Sp-phosphorothioate A$_3$ as the substrate.

Supporting the sequence alignment, we conclude that PARN shares the similarities in the active site with the 3'-5' exonuclease of Klenow fragment and uses the two-metal ion mechanism for catalysis (Freemont et al., 1988) (shown in Fig. 3). The 3'-5' exonuclease active site of Klenow fragment is the paradigm for the two-metal-ion mechanism in which the reaction proceeds by an S$_2$N2 mechanism (Steitz and Steitz, 1993). The activated nucleophile (shown as HO$^-$), which is oriented and positioned by the cation and protein side chains, attacks the scissile phosphate resulting in the formation of a pentaco-ordinated transition state. The two metal ions stabilize this transition state. After breaking the covalent bond, the metal ion B neutralizes the negative charge on the leaving group (Beese and Steitz, 1991; Steitz and Steitz, 1993). A number of other phosphoryl transfer enzymes have been proposed to utilize a similar mechanism, e.g. DNA polymerases (Brautigam and Steitz, 1998a; Doublie et al., 1998; Pelletier et al., 1994; Wang et al., 1996), adenylyl cyclase (Tesmer et al., 1999), alkaline phosphatase (Kim and Wyckoff, 1991), the RNase H domain of HIV-1 reverse transcriptase (Davies et al., 1991) and ribozymes (Lott et al., 1998; Warnecke et al., 1996; Weinstein et al., 1997).

Our results demonstrated that the phosphorothioate modification of the substrate disturbed the catalytic reaction and that the activity could in some cases be rescued by the use of soft metal ions and/or specific mutations on the protein. Brautigam et al (1998) reported that, in addition to the change of metal ion preference, the phosphorothioate modification of the substrate could affect the structure of the substrate itself and influence its binding to the
enzyme. The arrangement of the active site including the existence and positions of the metal ions was disturbed (Brautigam and Steitz, 1998b). Based on an S\(_2\)2 mechanism, the correct orientation of the scissile bond of the RNA substrate with the attacking nucleophile group is critical for the reaction. Any rearrangement of the substrate or the attacking nucleophile that improves an in-line position is expected to facilitate the formation of the transition state (Derbyshire et al., 1991). We further speculate that proper compensation from some certain different changes, which restores the nucleophile at the in-line position, should make it possible to regain the impaired activity caused by a single change. The compensation can be a result from the rearrangement of the different changes such as the structure of substrate(s) (Brautigam and Steitz, 1998b), the amino side chains (paper IV), the position or the kinds of divalent metal ions (Brautigam and Steitz, 1998b) and the conformations of the protein or RNA enzymes. The fact that the PARN activity was restored for the Sp-RNA substrate using the recombinant mutant human PARN(D382C) polypeptide could be interpreted in this way.

**A model for PARN action**

The key features of the PARN-RNA substrate complex are the oligomeric nature (most probably homotrimer) of PARN and the simultaneous interaction of PARN with the mRNA 5’-end cap structure and 3’-end poly(A) tail (paper I and II) (Dehlin et al., 2000; Gao et al., 2000). Although the cap structure binding site has not been molecularly identified, our kinetic analysis showed that the active site should be separate from the cap structure binding site, since the cap analogues acted as non-competitive inhibitors. The active site composed of four identified essential acidic amino acid residues is located in the N-terminal part of the amino acid sequence of PARN. Therefore, we propose that three cap binding sites and three active sites are present within the oligomeric PARN. The opposing roles of the cap structure, i.e. stimulating PARN activity when acting in cis and inhibiting PARN activity when acting in trans can be explained by the presence of multiple cap structure binding and active sites. We propose that the binding of a cap structure induces a conformational change of PARN that (i) inactivates the subunit that binds the cap structure and (ii) enhances the activity of the remaining subunits. According to this activation/inactivation model, additional binding of a second and third cap structure would inactivate the active sites of the second and third subunits, thereby inhibiting the exoribonuclease activity. Furthermore, this model explains why the methylated cap analogue at low concentration in trans activated deadenylation of the uncapped or G(5')ppp(5')G capped RNA substrates. The activation in trans is probably caused by mimicking the role of the single cap structure provided in cis. In the activation step, the 7-methyl group of the cap structure must play a very important role to contribute the cap binding affinity. Even though each subunit contains an RNA binding site formed by the putative RRM (Copeland and Wormington, 2001), we suggest that the oligomeric PARN is only engaged in deadenylating one single polyadenylated mRNA molecule at the time, but not multiple mRNA molecules. We could also speculate that PARN first interacts with the poly(A) tail, and then intramolecularly with the 5’-end cap structure of the RNA substrate. This speculation was initially based on the assumption that the \( K_m \) value (about 10 nM) for RNA substrates reflects the dissociation ability of the PARN-RNA substrate complex and that the \( K_i \) values (about 4 \( \mu \)M or 220 \( \mu \)M) for the cap analogues resemble the binding affinity for the PARN-cap interaction (paper II). Later, we showed that the
K_d for PARN interacting with the RNA substrates was about 7 nM (paper III).

The highly processive lambda-exonuclease, which degrades one strand of a double-stranded DNA from a 5' end, possesses a trimeric toroidal structure. The processive mode of the lambda-exonuclease was recently described in a model where the enzyme rotates and thereby brings a new site into action for each cleavage step (Kovall and Matthews, 1997). In much the same way as the lambda-exonuclease, a new catalytic site of the oligomer of PARN may be brought into action at each step of cleavage and with the cap moving in synchrony from subunit to subunit.

### Coupling Poly(A) Removal with mRNA Transport, Translation and Decay

The functions of PARN in vivo have not yet been well understood. However, the functional link between poly(A) tail removal at the 3'-end and the cap structure interaction at the 5'-end with PARN during poly(A) degradation must be important for the cross-talks among mRNA export from the nucleus to the cytoplasm, mRNA decay and translation initiation, since the cap structure and poly(A) tail are involved in all these processes.

In the nucleus, PARN could compete with the nuclear cap binding protein complex (CBC) to interfere with mRNA export efficiency. Polyadenylation and deadenylation at the mRNA 3'-end must be a balanced process to modulate the poly(A) length (shown in Fig. 1). In the cytoplasm, the integration of mRNA decay and translation initiation has been proposed (shown in Fig. 1). PARN could play an important role in this coupling. It has been demonstrated that Xenopus PARN activity is responsible for poly(A) tail removal during mRNA silencing in meiotic maturation of Xenopus oocytes. Human PARN is functionally equivalent to Xenopus PARN (Körner et al., 1998). Gao et al (2000) showed that the deadenylation-dependent mRNA decay required the presence of PARN in HeLa extracts (Gao et al., 2000). For some classes of mRNAs decapping does not occur until the poly(A) tail has been degraded (Couttet et al., 1997; Decker and Parker, 1993; Muhlrad and Parker, 1994), suggesting that the interaction between PARN and the cap structure may prevent the access for the decapping enzyme, and thereby affect mRNA decay. The roles of translation factors in mRNA decay have been investigated in yeast. Mutations of Pab1, eIF4E and eIF4G genes lead to accelerate deadenylation and decapping of mRNA (Schwartz and Parker, 1999). The addition of the recombinant eIF4E inhibited decapping of mRNA in vitro (Schwartz and Parker, 2000). However, the effects of Pab1 on mRNA deadenylation in yeast [Sachs and Davis, 1989; Boeck et al, 1996; Sachs, 1992 #349] are controversial within vertebrate, since PABP inhibits the deadenylating activity of vertebrate PARNs (Gao et al., 2000; Körner et al., 1998).

It has been demonstrated that the closed-loop complex structure of mRNA facilitates translation initiation (reviewed by (Sachs and Varani, 2000)). The complex may simultaneously protect the mRNA against degradation by preventing deadenylation and decapping. The disruption of the tertiary interaction between the factors and the mRNA will affect the translation efficiency and result in mRNA decay. The shortening of the poly(A) tail by PARN may cause the dissociation of PABP from the mRNA (Gallie et al., 1998). The less stably bound translation initiation complex may allow the mRNA cap structure to gain accessibility to PARN and the decapping enzyme. Hence, the mRNA would undergo
In conclusion, PARN, being an enzyme degrading the mRNA poly(A) tail and interacting with the mRNA cap structure, must play a role in all these processes. Further investigations are required to elucidate it.

Inhibition of PARN Activity by Aminoglycoside Antibiotics (paper V)

It has been shown that the function of several ribozymes is inhibited in vitro by aminoglycoside antibiotics most likely through the displacement of catalytically or structurally important metal ions (reviewed by (Schroeder et al., 2000)). This inhibition mechanism led us to speculate that PARN activity might also be impaired by aminoglycoside antibiotics. Furthermore, Hermann and Westhof (1998) had suggested that the active sites of several nucleic acid-metabolising enzymes (e.g. DNA pol I, DNA pol-β, HIV-I reverse transcriptase, T7 RNA pol and gp43 DNA pol) resembled the negatively charged binding pockets critical for aminoglycoside antibiotic binding to RNA. The arrangements of divalent metal ions in these pockets would possibly allow polycationic aminoglycoside antibiotics to compete for similar binding sites to inhibit their activities as in ribozymes (Hermann and Westhof, 1998). However, the possibility of steric hindrance must be taken into account in each case (Mikkelsen et al., 2001).

We found that neomycin B strongly inhibited PARN activity in a concentration-dependent manner (paper V). Paromomycin and lividomycin also belong to the neomycin family, but differ from neomycin B by replacement of the 6'-amino group with a hydroxyl at the R1 position (shown in Fig. 4). Both of these antibiotics were found to be weaker inhibitors than neomycin B. Similarly, the inhibition of PARN activity by the kanamycin family members (kanamycin A, kanamycin B and tobramycin) was tested. Among the ones...
assayed, neomycin B was the most potent inhibitor of PARN activity in vitro. The comparison of the inhibitory potential and the differences between the aminoglycoside antibiotic structures implied that the ammonium group at position R1 was important for the inhibition.

In addition to the R1 amino group, several amino groups of neomycin B and kanamycin B (shown in Fig. 4) are protonated or deprotonated between pH 6 and pH 9. The inhibitory capacity of neomycin B was dramatically affected by changing the pH of the deadenylation reaction. Not unexpectedly, the pH dependence of inhibition was also observed for paromomycin, kanamycin B and kanamycin A. The observation that the protonated amino groups of aminoglycoside antibiotics contributed to the inhibition potential suggested that electrostatic interactions play an important role for the aminoglycoside inhibition of PARN activity.

Since electrostatic interactions play an important role for the inhibition, it is possible that the positively charged aminoglycoside antibiotics could interact unspecifically with negatively charged DNA or RNA (Davey et al., 1970), and thereby forming inappropriate substrates. To clarify the inhibition mechanism, we performed detailed kinetic studies and found that neomycin B acted as a mixed non-competitive inhibitor. This result suggested that neomycin B occupies a binding site in PARN different from the site for RNA substrate binding. Moreover, the inhibition of neomycin B could be released by the addition of increasing Mg^{2+} concentration in a competitive manner indicating that the antibiotic and Mg^{2+} compete with each other. It was found that the K_i value of neomycin B was a parameter that did not depend on the concentrations of Mg^{2+} and RNA substrate. Therefore, we conclude that neomycin B and divalent metal ions bind to the same or overlapping binding sites in the enzyme-substrate complex required for PARN activity.

To verify the kinetic data without the crystal structure of PARN available, we applied Fe^{2+}-mediated cleavage experiments to investigate whether the two protein footprint bands cleaved by Fe^{2+} (s) in the active site could be suppressed by neomycin B, paromomycin and kanamycin A. We found that the intensities of the both cleavage bands were severely attenuated by the addition of neomycin B (app K_i 0.4 µM). In contrast, kanamycin A (app K_i 64.7 µM), a very poor inhibitor of PARN activity, did not influence the cleavage, whereas paromomycin (app K_i 17.3 µM), a weak inhibitor, inhibited Fe^{2+}-mediated cleavage moderately. The data on the Fe^{2+}-mediated cleavages provided strong evidence that neomycin B inhibited PARN activity by displacing Fe^{2+} ions in the active site of PARN. In conclusion, we propose that the mechanism of the inhibition of PARN activity by aminoglycoside antibiotics is that these aminoglycosides displace catalytically essential divalent metal ions in the active site of PARN.

Our findings are in agreement with the inhibition mechanism proposed for various ribozymes that are inhibited by aminoglycoside antibiotics (Earnshaw and Gait, 1998; Mikkelsen et al., 1999; Rogers et al., 1996; Stage et al., 1995; von Ahsen et al., 1991), but in disagreement with the previous reports that neomycin B inhibited E. coli DNA polymerase I (Lazarus and Kitron, 1973), DNase I (Woegerbauer et al., 2000) and phospholipase C (Burch et al., 1986). It was concluded that the aminoglycoside antibiotics unspecifically bound DNA or phospholipids as counter ions to make them as an inappropriate substrate for the reactions.

Aminoglycoside antibiotics have been widely used in clinical treatments against bacterial
infections. However, they cause many severe toxic side effects, especially on the function of kidney and hearing. So far, the mechanism of toxicity has not been fully understood. In the literature many possible reasons for this have been proposed and discussed. For example, the observations that aminoglycoside antibiotics increase the formation of free radicals (Schacht, 1999), that aminoglycoside antibiotics inhibit essential ribozyme activities (Forge and Schacht, 2000; Schroeder et al., 2000), as well as that neomycin B can block signal transduction by inhibition of phospholipase C activity (Burch et al., 1986), are all potential causes for the toxic side effects. The fact that aminoglycoside antibiotics behave as 'metal mimics' to inhibit PARN activity suggests that the inhibition of divalent metal ion-dependent enzymes (mainly nucleic acid-metabolising enzymes) may be a common mechanism for the toxic side effects of aminoglycoside antibiotics. In support of this, we have preliminary data suggesting that several other nucleic acid-metabolising enzymes, among them E. coli Klenow polymerase, HIV reverse transcriptase, Taq DNA polymerase and T7 RNA polymerase, can be inhibited by neomycin B in vitro (unpublished observations). Interestingly, the metalloenzyme phospholipase C, which cleaves phosphodiester bonds, can be inhibited by neomycin B (Burch et al., 1986). We speculate that the inhibition mechanism for these enzymes may be the same as for PARN.

Several applications for aminoglycoside antibiotics might be possible. i) Aminoglycoside antibiotics can be used as probes to locate catalytically important metal ions and define metal ion dependent active sites (paper V). ii) Small molecules displacing metal ions in enzymes can be developed into drugs against pathogens (animal viruses) or even as anticancer drugs. iii) Novel small molecules could be used as probes to identify metal ion interacting proteins in "functional genomic" screen.

Here is the end of my thesis, but the story of PARN is just started.
REFERENCES

Baker, E. J., and Liggit, P. (1993). Accelerated poly(A) loss and mRNA stabilization are independent effects of protein synthesis inhibition on alpha-tubulin mRNA in Chlamydomonas. Nucleic Acids Res 21, 2237-2246.

Bashkirov, V. I., Scherthan, H., Solinger, J. A., Buerstedde, J. M., and Heyer, W. D. (1997). A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. J Cell Biol 136, 761-773.

Beese, L. S., and Steitz, T. A. (1991). Structural basis for the 3'-5' exonuclease activity of Escherichia coli DNA polymerase I: a two metal ion mechanism. Embo J 10, 25-33.

Bernad, A., Blanco, L., Lazaro, J. M., Martin, G., and Salas, M. (1989). A conserved 3'----5' exonuclease active site in prokaryotic and eukaryotic DNA polymerases. Cell 59, 219-228.

Boeck, R., Tarun, S., Rieger, M., Deardorff, J. A., Müller-Auer, S., and Sachs, A. B. (1996). The yeast Pan2 protein is required for poly(A)-binding protein-stimulated poly(A)-nuclease activity. J Biol Chem 271, 432-438.

Brautigam, C. A., and Steitz, T. A. (1998a). Structural and functional insights provided by crystal structures of DNA polymerases and their substrate complexes. Curr Opin Struc Biol 8, 54-63.

Brautigam, C. A., and Steitz, T. A. (1998b). Structural principles for the inhibition of the 3'-5' exonuclease activity of Escherichia coli DNA polymerase I by phosphorothioates. J Mol Biol 277, 363-377.

Brawerman, G. (1981). The role of the poly(A) sequence in mamalian messenger RNA. Crit Rev Biochem 10, 1-38.

Brawerman, G., and Diez, J. (1975). Metabolism of the polyadenylate sequence of nuclear RNA and messenger RNA in mammalian cells. Cell 5, 271-280.

Brown, C. E., and Sachs, A. B. (1998). Poly(A) tail length control in Saccharomyces cerevisiae occurs by message-specific deadenylation. Mol Cell Biol 18, 6548-6559.

Brown, C. E., Tarun, S. Z., Jr., Boeck, R., and Sachs, A. B. (1996). PAN3 encodes a subunit of the Pab1p-dependent poly(A) nuclease in Saccharomyces cerevisiae. Mol Cell Biol 16, 5744-5753.

Brown, J. A., Bharathi, A., Ghosh, A., Whalen, W., Fitzgerald, E., and Dhar, R. (1995). A mutation in the Schizosaccharomyces pombe rael gene causes defects in poly(A)+ RNA export and in the cytoskeleton. J Biol Chem 270, 7411-7419.

Buiting, K., C., Ulrich, B., Wahle, E., and Horstemke, B. (1999). The human gene for the poly(A)-specific ribonuclease (PARN) maps to 16p13 and has a truncated copy in the Prader-Willi/Angelman syndrome region on 15q11--q13. Cytogenet Cell Genet 87, 125-131.

Burch, R. M., Luini, A., and Axelrod, J. (1986). Phospholipase A2 and phospholipase C are activated by distinct GTP- binding proteins in response to alpha 1-adrenergic stimulation in FRTL5 thyroid cells. Proc Natl Acad Sci U S A 83, 7201-7205.

Cao, W., and Barany, F. (1998). Identification of TaqI endonuclease active site residues by Fe2+-mediated oxidative cleavage. J Biol Chem 273, 33002-33010.

Caponigro, G., and Parker, R. (1995). Multiple functions for the poly(A)-binding protein in mRNA decapping and deadenylation in yeast. Genes & Dev 9, 2421-2432.

Caponigro, G., and Parker, R. (1996). Mechanisms and control of mRNA turnover in Saccharomyces cerevisiae. Microbiol Rev 60, 233-249.

Chang, M., French-Cornay, D., Fan, H. Y., Klein, H., Denis, C. L., and Jaehning, J. A. (1999). A complex containing RNA polymerase II, Paf1p, Cdc73p, Hpr1p, and Ccr4p plays a role in protein kinase C signaling. Mol Cell Biol 19, 1056-1067.

Copeland, P. R., and Wormington, M. (2001). The mechanism and regulation of deadenylation: identification and characterization of Xenopus PARN. Rna 7, 875-886.

Couttet, P., Fromont-Racine, M., Steel, D., Pictet, R., and Grange, T. (1997). Messenger RNA deadenylylation precedes decapping in mammalian cells. Proc Natl Acad Sci U S A 94, 5628-5633.

Davey, P. J., Haslam, J. M., and Linnane, A. W. (1970). Biogenesis of mitochondria. 12. The effects of aminoglycoside antibiotics on the mitochondrial and cytoplasmic protein-synthesizing systems of...
Saccharomyces cerevisiae. Arch Biochem Biophys 136, 54-64.

Davies, J. F., 2nd, Hostomska, Z., Hostomsky, Z., Jordan, S. R., and Matthews, D. A. (1991). Crystal structure of the ribonuclease H domain of HIV-1 reverse transcriptase. Science 252, 88-95.

Decker, C. J., and Parker, R. (1993). A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. Genes Dev 7, 1632-1643.

Dehlin, E., Wormington, M., Körner, C. G., and Wahle, E. (2000). Cap-dependent deadenylation of mRNA. Embo J 19, 1079-1086.

Deo, R. C., Bonanno, J. B., Sonenberg, N., and Burley, S. K. (1999). Recognition of polyadenylate RNA by the poly(A)-binding protein. Cell 98, 835-845.

Derbyshire, V., Grindley, N. D., and Joyce, C. M. (1991). The 3'-5' exonuclease of DNA polymerase I of Escherichia coli: contribution of each amino acid at the active site to the reaction. Embo J 10, 17-24.

Doublie, S., Tabor, S., Long, A. M., Richardson, C. C., and Ellenberger, T. (1998). Crystal structure of a bacteriophage T7 DNA replication complex at 2.2 Å resolution [see comments]. Nature 391, 251-258.

Draganescu, A., and Tullius, T. D. (1996). Targeting of nucleic acids by iron complexes. Met Ions Biol Syst 33, 453-484.

Dworkin, M. B., and Dworkin-Rastl, E. (1990). Functions of maternal mRNA in early development. Mol Reprod Dev 26, 261-297.

Dworkin, M. B., Shrutkowski, A., and Dworkin-Rastl, E. (1985). Mobilization of specific maternal RNA species into polyosomes after fertilization in Xenopus laevis. Proc Natl Acad Sci USA 82, 7636-7640.

Earnshaw, D. J., and Gait, M. J. (1998). Hairpin ribozyme cleavage catalyzed by aminoglycoside antibiotics and the polyamine spermine in the absence of metal ions. Nucleic Acids Res 26, 5551-5561.

Eckner, R., Ellmeier, W., and Biernstiel, M. L. (1991). Mature mRNA 3' end formation stimulates RNA export from the nucleus. EMBO J 10, 3513-3522.

Ermacora, M. R., Delfino, J. M., Cuenoud, B., Schepartz, A., and Fox, R. O. (1992). Conformation-dependent cleavage of staphylococcal nuclease with a disulfide-linked iron chelate. Proc Natl Acad Sci U S A 89, 6383-6387.

Ettner, N., Metzger, J. W., Lederer, T., Hulmes, J. D., Kisker, C., Hinrichs, W., Ellestad, G. A., and Hillen, W. (1995). Proximity mapping of the Tet repressor-tetracycline-Fe2+ complex by hydrogen peroxide mediated protein cleavage. Biochemistry 34, 22-31.

Farber, J. M., and Levine, R. L. (1986). Sequence of a peptide susceptible to mixed-function oxidation. Probable cation binding site in glutamine synthetase. J Biol Chem 261, 4574-4578.

Forge, A., and Schacht, J. (2000). Aminoglycoside antibiotics. Audiol Neurootol 5, 3-22.

Fox, C. A., Sheets, M. D., and Wickens, M. P. (1989). Poly(A) addition during maturation of frog oocytes: distinct nuclear and cytoplasmic activities and regulation by the sequence UUUUAU. Genes Dev 3, 2151-2162.

Fox, C. A., and Wickens, M. (1990). Poly(A) removal during oocyte maturation: a default reaction selectively prevented by specific sequences in the 3' UTR of certain maternal mRNAs. Genes Dev 4, 2287-2298.

Freemont, P. S., Friedman, J. M., Beese, L. S., Sanderson, M. R., and Steitz, T. A. (1988). Cocrystal structure of an editing complex of Klenow fragment with DNA. Proc Natl Acad Sci U S A 85, 8924-8928.

Gallie, D. R. (1991). The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. Genes Dev 5, 2108-2116.

Gallie, D. R., Le, H., Caldwell, C., and Browning, K. S. (1998). Analysis of translation elongation factors from wheat during development and following heat shock. Biochem Biophys Res Commun 245, 295-300.

Gao, M., Fritz, D. T., Ford, L. P., and Wilusz, J. (2000). Interaction between a poly(A)-specific ribonuclease and the 5' cap influences mRNA deadenylation rates in vitro. Mol Cell 5, 479-488.

Gao, M., Wilusz, C. J., Peltz, S. W., and Wilusz, J. (2001). A novel mRNA-decapping activity in HeLa cytoplasmic extracts is regulated by AU-rich elements. Embo J 20, 1134-1143.

Godson, G. N., Schoenich, J., Sun, W., and Mustaev, A. A. (2000). Identification of the magnesium ion binding site in the catalytic center of Escherichia coli primase by iron cleavage. Biochemistry 39, 332-339.
Grifo, J. A., Abramson, R. D., Satler, C. A., and Merrick, W. C. (1984). RNA-stimulated ATPase activity of eukaryotic initiation factors. J Biol Chem 259, 8648-8654.

Grodsky, N. B., Soundar, S., and Colman, R. F. (2000). Evaluation by site-directed mutagenesis of aspartic acid residues in the metal site of pig heart NADP-dependent isocitrate dehydrogenase. Biochemistry 39, 2193-2200.

Hentze, M. W. (1997). eIF4G: a multipurpose ribosome adapter? Science 275, 500-501.

Hermann, T., and Westhof, E. (1998). Aminoglycoside binding to the hammerhead ribozyme: a general model for the interaction of cationic antibiotics with RNA. J Mol Biol 276, 903-912.

Hlavaty, J. J., Benner, J. S., Hornstra, L. J., and Schildkraut, I. (2000). Affinity cleavage at the metal-binding site of phosphoenolpyruvate carboxykinase. Biochemistry 39, 3097-3105.

Hlavaty, J. J., and Nowak, T. (1997). Affinity cleavage at the metal-binding site of porcine NAD-specific isocitrate dehydrogenase. Protein Sci 6, 104-111.

Husen, A., and Warwicker, J. (1997). Aminoglycoside binding to the hammerhead ribozyme: a general model for the interaction of cationic antibiotics with RNA. J Mol Biol 276, 903-912.

Jacobs-Anderson, J. S., and Parker, R. (1998). The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SK12 DEVH box protein and 3' to 5' exonucleases of the exosome complex. EMBO J 17, 1497-1506.

Jaffe, E. K., and Cohn, M. (1978). Divalent cation-dependent stereospecificity of adenosine 5'-O-(2-thiotriphosphate) in the hexokinase and pyruvate kinase reactions. The absolute stereochemistry of the diastereoisomers of adenosine 5'-O-(2-thiotriphosphate). J Biol Chem 253, 4823-4825.

Joyce, C. M., and Steitz, T. A. (1994). Function and structure relationships in DNA polymerases. Annu Rev Biochem 63, 777-822.

Kim, D. R., Dai, Y., Mundy, C. L., Yang, W., and Oettinger, M. A. (1999). Mutations of acidic residues in RAG1 define the active site of the V(DJ) recombinase. Genes Dev 13, 3070-3080.

Kim, E. E., and Wyckoff, H. W. (1991). Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis. J Mol Biol 218, 449-464.

Kovall, R., and Matthews, B. W. (1997). Toroidal structure of lambda-exonuclease. Science 277, 1824-1827.

Körner, C. G., and Wahle, E. (1997). Poly(A) tail shortening by a mammalian poly(A)-specific 3'-exoribonuclease. J Biol Chem 272, 10448-10456.

Körner, C. G., Wormalton, M., Muckenthaler, M., Schneider, S., Dehlin, E., and Wahle, E. (1998). The deadenylating nuclease (DAN) is involved in poly(A) tail removal during the meiotic maturation of Xenopus oocytes. Embo J 17, 5427-5437.

Lazarus, H. M., and Sporn, M. B. (1967). Purification and properties of a nuclear exoribonuclease. J Biol Chem 242, 10448-10456.

Lott, W. B., Pontius, B. W., and von Hippel, P. H. (1998). A two-metal ion mechanism operates in the hammerhead ribozyme-mediated cleavage of an RNA substrate. Proc Natl Acad Sci U S A 95, 542-547.

Lowell, J. E., Rudner, D. Z., and Sachs, A. B. (1992). 3'-UTR-dependent deadenylation by the yeast poly(A) nuclease. Genes Dev 6, 2088-2099.

Lykke-Andersen, J., Garrett, R. A., and Kjems, J. (1997). Mapping the catalytic sites of two homing endonucleases. EMBO J 16, 3272-3281.

Marcotrigiano, J., Gingras, A. C., Sonenberg, N., and Burley, S. K. (1997). Co-crystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell 89, 951-961.

McGrew, L. L., Dworkin-Rastl, E., Dworkin, M. B., and Richter, J. D. (1989). Poly(A) elongation during Xenopus oocyte maturation is required for translational recruitment and is mediated by a short sequence element. Genes Dev 3, 803-815.
Mian, I. S. (1997). Comparative sequence analysis of ribonucleases HII, III, II PH and D. Nucleic Acids Res 25, 3187-3195.

Mikkelsen, N. E., Brännvall, M., Virtanen, A., and Kirsebom, L. (1999). Inhibition of RNase P cleavage by aminoglycosides. Proc Natl Acad Sci USA 96, 6155-6160.

Mikkelsen, N. E., Johansson, K., Virtanen, A., and Kirsebom, L. A. (2001). Aminoglycoside binding displaces a divalent metal ion in a tRNA- neomycin B complex. Nat Struct Biol 8, 510-514.

Morino, S., Hazama, H., Ozaki, M., Teraoka, Y., Shibata, S., Doi, M., Ueda, H., Ishida, T., and Uesugi, S. (1996). Analysis of the mRNA cap-binding ability of human eukaryotic initiation factor-4E by use of recombinant wild-type and mutant forms. Eur J Biochem 239, 597-601.

Moser, M. J., Holley, W. R., Chatterjee, A., and Mian, I. S. (1997). The proofreading domain of Escherichia coli DNA polymerase I and other DNA and/or RNA exonuclease domains. Nucleic Acids Res 25, 5110-5118.

Muhlrad, D., Decker, C. J., and Parker, R. (1995). Turnover mechanisms of the stable yeast PGK1 mRNA. Mol Cell Biol 15, 2145-2156.

Muhlrad, D., and Parker, R. (1994). Premature translational termination triggers mRNA decapping. Nature 370, 578-581.

Munroe, D., and Jacobson, A. (1990). Tales of poly(A): a review. Gene 91, 151-158.

Mustaev, A., Kozlov, M., Markovtsov, V., Zaychikov, E., Denissova, L., and Goldfarb, A. (1997). Modular organization of the catalytic center of RNA polymerase. Proc Natl Acad Sci U S A 94, 6641-6645.

Nakielyn, S., and Dreyfuss, G. (1999). Transport of proteins and RNAs in and out of the nucleus. Cell 99, 677-690.

Nakielyn, S., Fischer, U., Michael, W. M., and Dreyfuss, G. (1997). RNA transport. Annu Rev Neurosci 20, 269-301.

Paris, J., and Richter, J. D. (1990). Maturation-specific polyadenylation and translational control: diversity of cytoplasmic polyadenylation elements, influence of poly(A) tail size, and formation of stable polyadenylation complexes. Mol Cell Biol 10, 5634-5645.

Pelletier, H., Sawaya, M. R., Kumar, A., Wilson, S. H., and Kraut, J. (1994). Structures of ternary complexes of rat DNA polymerase beta, a DNA template-primer, and ddCTP. Science 264, 1891-1903.

Richter, J. D. (1991). Translational control during early development. BioEssays 13, 179-183.

Richter, J. D. (1999). Cytoplasmic polyadenylation in development and beyond. Microbiol Mol Biol Rev 63, 446-456.

Rogers, J., Chang, A. H., von Ahsen, U., Schroeder, R., and Davies, J. (1996). Inhibition of the self-cleavage reaction of the human hepatitis delta virus ribozyme by antibiotics. J Mol Biol 259, 916-925.

Ross, J. (1995). mRNA stability in mammalian cells. Microbiol Rev 59, 423-450.

Sachs, A. B., and Davis, R. W. (1989). The poly(A) binding protein is required for poly(A) shortening and 60S ribosomal subunit-dependent translation initiation. Cell 58, 857-867.

Sachs, A. B., Sarnow, P., and Hentze, M. W. (1997). Starting at the beginning, middle, and end: translation initiation in eukaryotes. Cell 89, 831-838.

Sachs, A. B., and Varani, G. (2000). Eukaryotic translation initiation: there are (at least) two sides to every story. Nat Struct Biol 7, 356-361.

Schacht, J. (1999). Biochemistry and pharmacology of aminoglycoside-induced hearing loss. Acta Physiol Pharmacol Ther Latinoam 49, 251-256.

Schroeder, R., Waldsich, C., and Wank, H. (2000). Modulation of RNA function by aminoglycoside antibiotics. Embo J 19, 1-9.

Schwartz, D. C., and Parker, R. (1999). Mutations in translation initiation factors lead to increased rates of deadenylation and decapping of mRNAs in Saccharomyces cerevisiae. Mol Cell Biol 19, 5247-5256.

Schwartz, D. C., and Parker, R. (2000). mRNA decapping in yeast requires dissociation of the cap binding protein, eukaryotic translation initiation factor 4E. Mol Cell Biol 20, 7933-7942.

Sheets, M. D., Fox, C. A., Hunt, T., Woude, G. V., and Wickens, M. (1994). The 3'-untranslated regions of c-mos and cyclin mRNAs stimulate translation by regulating cytoplasmic polyadenylation. Genes Dev 8, 926-938.

Sheets, M. D., and Wickens, M. (1989). Two phases in the addition of a poly(A) tail. Genes Dev 3, 1401-1412.

Sheiness, D., Puckett, L., and Darnell, J. E. (1975). Possible relationship of poly(A) shortening to mRNA turnover. Proc Natl Acad Sci U S A 72, 1077-1081.
Shyu, A.-B., Belasco, J. G., and Greenberg, M. E. (1991). Two distinct destabilizing elements in the c-fos message trigger deadenylation as a first step in rapid mRNA decay. Genes Dev 5, 221-231.

Siomi, H., and Dreyfuss, G. (1997). RNA-binding proteins as regulators of gene expression. Curr Opin Genet Dev 7, 345-353.

Soundar, S., and Colman, R. F. (1993). Identification of metal-isocitrate binding site of pig heart NADP-specific isocitrate dehydrogenase by affinity cleavage of the enzyme by Fe(2+)-isocitrate. J Biol Chem 268, 5264-5271.

Stage, T. K., Hertel, K. J., and Uhlenbeck, O. C. (1995). Inhibition of the hammerhead ribozyme by neomycin. RNA 1, 95-101.

Steitz, T. A., and Steitz, J. A. (1993). A general two-metal-ion mechanism for catalytic RNA. Proc Natl Acad Sci U S A 90, 6498-6502.

Tarun, S. Z., Jr., and Sachs, A. B. (1996). Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G. Embo J 15, 7168-7177.

Tarun, S. Z., Jr., Wells, S. E., Deardorff, J. A., and Sachs, A. B. (1997). Translation initiation factor eIF4G mediates in vitro poly(A) tail- dependent translation. Proc Natl Acad Sci U S A 94, 9046-9051.

Tarun, S. Z., and Sachs, A. B. (1995). A common function for mRNA 5' and 3' ends in translation initiation in yeast. Genes & Dev 9, 2997-3007.

Tesmer, J. J., Sunahara, R. K., Johnson, R. A., Gosselin, G., Gilman, A. G., and Sprang, S. R. (1999). Two-metal-Ion catalysis in adenylyl cyclase. Science 285, 756-760.

Tucker, M., Valencia-Sanchez, M. A., Staples, R. R., Chen, J., Denis, C. L., and Parker, R. (2001). The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in Saccharomyces cerevisiae. Cell 104, 377-386.

Varani, G., and Nagai, K. (1998). RNA recognition by RNP proteins during RNA processing. Annu Rev Biophys Biomol Struct 27, 407-445.

Vassalli, J.-D., Huarte, J., Belin, D., Gubler, P., Vassalli, A., O’Connell, M. L., Parton, L. A., Rickles, R. J., and Strickland, S. (1989). Regulated polyadenylation controls mRNA translation during meiotic maturation of mouse oocytes. Genes Dev 3, 2163-2171.

Verma, S., and Eckstein, F. (1998). Modified oligonucleotides: synthesis and strategy for users. Annu Rev Biochem 67, 99-134.

Virtanen, A., and Åström, J. (1997). Function and characterization of poly(A)-specific 3’ exoribonucleases. In Prog. Mol. Subcell. Biol., P. Jeanteur, ed. (Berlin Heidelberg, Springer-Verlag), pp. 199-220.

von Ahsen, U., Davies, J., and Schroeder, R. (1991). Antibiotic inhibition of group I ribozyme function. Nature 353, 368-370.

Vortler, L. C., and Eckstein, F. (2000). Phosphorothioate modification of RNA for stereochemical and interference analyses. Methods Enzymol 317, 74-91.

Wang, J., Yu, P., Lin, T. C., Konigsberg, W. H., and Steitz, T. A. (1996). Crystal structures of an NH2-terminal fragment of T4 DNA polymerase and its complexes with single-stranded DNA and with divalent metal ions. Biochemistry 35, 8110-8119.

Warnecke, J. M., Forst, J. P., Hardt, W. D., Erdmann, V. A., and Hartmann, R. K. (1996). Ribonuclease P (RNase P) RNA is converted to a Cd(2+)-ribozyme by a single Rp-phosphorothioate modification in the precursor tRNA at the RNase P cleavage site. Proc Natl Acad Sci U S A 93, 8924-8928.

Wei, C. H., Chou, W. Y., and Chang, G. G. (1995). Identification of Asp258 as the metal coordinate of pigeon liver malic enzyme by site-specific mutagenesis. Biochemistry 34, 7949-7954.

Wei, C. H., Chou, W. Y., Huang, S. M., Lin, C. C., and Chang, G. G. (1994). Affinity cleavage at the putative metal-binding site of pigeon liver malic enzyme by the Fe(2+)-ascorbate system. Biochemistry 33, 7931-7936.

Weinstein, L. B., Jones, B. C., Cosstick, R., and Cech, T. R. (1997). A second catalytic metal ion in group I ribozyme. Nature 388, 805-808.

Wells, S. E., Hillner, P. E., Vale, R. D., and Sachs, A. B. (1998). Circularization of mRNA by eukaryotic translation initiation factors. Mol Cell 2, 135-140.

Wilson, T., and Treisman, R. (1988). Removal of poly(A) and consequent degradation of c-fos mRNA facilitated by 3’ AU-rich sequences. Nature 336, 396-399.

Woegerbauer, M., Burgmann, H., Davies, J., and Graninger, W. (2000). DNase I induced DNA degradation is inhibited by neomycin. J Antibiot (Tokyo) 53, 276-285.
Wormington, M. (1994). Unmasking the role of the 3' UTR in the cytoplasmic polyadenylation and translational regulation of maternal mRNAs. BioEssays 16, 533-535.

Zaychikov, E., Martin, E., Denissova, L., Kozlov, M., Markovtsov, V., Kashlev, M., Heumann, H., Nikiforov, V., Goldfarb, A., and Mustaev, A. (1996). Mapping of catalytic residues in the RNA polymerase active center. Science 273, 107-109.

Åström, J., Åström, A., and Virtanen, A. (1991). In vitro deadenylation of mammalian mRNA by a HeLa cell 3’ exonuclease. EMBO J 10, 3067-3071. Åström, J., Åström, A., and Virtanen, A. (1992). Properties of a HeLa cell 3’ exonuclease specific for degrading poly(A) tails of mammalian mRNA. J Biol Chem 267, 18154-18159.
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