Allosteric Regulation of mRNA Metabolism

-Mechanisms of Cap-Dependent Regulation of Poly(A)-specific Ribonuclease (PARN)

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Abstract

Degradation of mRNA is a highly regulated step important for proper gene expression. Degradation of eukaryotic mRNA is initiated by shortening of the 3’ end located poly(A) tail. Poly(A)-specific ribonuclease (PARN) is an oligomeric enzyme that degrades the poly(A) tail with high processivity. A unique property of PARN is its ability to interact not only with the poly(A) tail but also with the 5’ end located mRNA cap structure. A regulatory role in protein synthesis has been proposed for PARN based on its ability to bind the cap that is required for efficient initiation of eukaryotic mRNA translation. Here we have investigated how the cap structure influences PARN activity and how PARN binds the cap. We show that the cap activates PARN and enhances the processivity of PARN. Further we show that the cap binding complex (CBC) inhibits PARN activity through a protein-protein interaction. To investigate the cap binding property of PARN, we identified the cap binding site at the molecular level using site-directed mutagenesis and fluorescence spectroscopy. We identified tryptophan 475, located within the RNA recognition motif (RRM) of PARN, as crucial for cap binding. A crystal structure of PARN bound to cap revealed that cap binding is mediated by the nuclease domain and the RRM of PARN. Tryptophan 475 binds the inverted 7-Me-guanosine residue through a stacking interaction. Involvement of the nuclease domain in cap binding suggests that the cap site and the active site overlap. Mutational analysis showed that indeed amino acids involved in cap binding are crucial for hydrolytic activity of PARN. Taken together, we show that PARN is an allosteric enzyme that is activated by the cap structure and that the allosteric cap binding site in one PARN subunit corresponds to the active site in the other PARN subunit.

Keywords: mRNA degradation, deadenylation, cap, poly(A), PARN, allosteric regulation

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urn:nbn:se:uu:diva-8647 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-8647)
To my Karlsson Nilsson family
List of Publications:

This thesis is based on the following papers, which are referred to in the text by their roman numerals:


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Abbreviations

DNA  deoxyribonucleic acid
RNA  ribonucleic acid
mRNA  messenger RNA
NMD  nonsense-mediated decay
NGD  no-go decay
NSD  non-stop decay
PARN  poly(A)-specific ribonuclease
RNA pol  RNA polymerase
CTD  C-terminal domain
GMP  guanosine monophosphate
GTP  guanosine triphosphate
CPSF  cleavage and polyadenylation specificity factor
PAP  poly(A) polymerase
snRNA  small nuclear RNA
RNP  ribonucleic particle
PABPII  nuclear poly(A) binding protein
PABPI  cytoplasmic poly(A) binding protein
RRM  RNA recognition motif
UTR  untranslated region
Lsm  Sm-like protein
CBC  cap binding complex
PAN  poly(A) nuclease
PTC  pre-mature termination codon
CPE  cytoplasmic polyadenylation element
CPEB  CPE-binding protein
Dcp1/Dcp2  decapping protein 1/2
Xrn1  exoribonuclease 1
DcpS  scavenger decapping protein
RNase  ribonuclease
CCR4  carbon catabolite repressor 4
Caf1  CCR4 associated factor
EMSA  electrophoretic mobility shift assay
ARE  AU-rich element
kDa  kilodalton
MDa  Megadalton
Don’t waste clean thoughts on dirty enzymes.
- Efraim Racker

A good prep is half the way to success.
- The poly(A) group

Omnia mirari etiam tritissima.
Find wonder in all things, even the most commonplace.
- Carl von Linné
Introduction

In the early 1960’s the idea of the presence of a molecule that mediates the genetic information stored in DNA to translated proteins started to form. Sidney Brenner, Francois Jacob, Jacques Monod and Mathew Meselson among others could elegantly show that this molecule consisted of RNA with a short life and which had the same base composition as the corresponding gene in DNA. The concept of messenger RNA (mRNA) was established.

The process that yields pre-mRNA, transcription, and especially initiation of transcription, was for a long time regarded as the main important step in how gene expression was regulated. But over the time, data have accumulated showing that a significant amount of regulation of gene expression takes place at the mRNA level and especially through mRNA degradation. Large scale analysis has revealed that as much as 50% of altered mRNA levels is due to mRNA degradation rather than transcription (1,2). Recent findings of RNA interference (RNAi), nonsense mediated decay (NMD), no-go decay (NGD) and non-stop decay (NSD) (reviewed in (3-5)) as systems for regulating gene expression as well as quality control at the mRNA level has further emphasized the importance of elucidating the mechanisms of mRNA degradation and the enzymes involved in those processes. This thesis describes properties of one of the enzymes involved in eukaryotic mRNA degradation, the poly(A)-specific ribonuclease (PARN).

Eukaryotic mRNA

The concentration of a specific mRNA at a certain time in the cell is determined by the ratio between the rate of transcription and degradation of the mRNA. The half-life of eukaryotic mRNA varies between minutes and up to more than 24 hours and is in general longer than in prokaryotes. This is mainly due to the shorter generation time of bacteria which implies that a faster turnover of mRNA is required for the cell to be able to quickly correlate gene expression with surrounding physiological conditions and to adapt cell growth and division accordingly. In eukaryotes some tissue-specific mRNA has to have an extremely short half-time, for example cytokines that should only be expressed at a very specific time during the cell cycle. This
type of fast mRNA degradation requires certain elements within the mRNA and the proteins binding to them. It should be noted that the term half-time is not the best terminology to use when quantifying mRNA stability, since the decay is not a stochastic process but rather highly regulated. Interestingly, it has been found that mRNAs encoding for proteins that together function in a process follow the same rate of decay (6).

The eukaryotic pre-mRNA is transcribed by RNA polymerase II (RNA pol II) (7) and it is further processed and modified in the nucleus in a number of steps before export to the cytoplasm. A cap structure is added to the 5´-end and a poly(A) tail is synthesized at the 3´-end. A third process, splicing, removes non-coding introns and splices the pre-mRNA to a mature mRNA. These processes are all coupled and occur to a large extent co-transcriptionally (8). The 52 times-repeated heptapeptide sequence in the C-terminal domain of RNA polymerase II plays an important role in orchestrating these coupled processes. For example the transfer of transcription complex to elongation phase is dependent on the assembly of the 3´-end processing machinery to the pre-mRNA (9). Some of the factors are already associated at the stage of transcription initiation e.g. cleavage and polyadenylation specificity factor (CPSF). Phosphorylation of two serines, Ser 2 and Ser 5, within the repeat of the C-terminal domain (CTD) plays a crucial role in the regulation of this process (10). Further, the process of editing can modulate the mRNA which will give rise to gene products (11).

The 5´-cap

The RNA polymerases yield a transcript with a 5´- triphosphate end. During initiation of transcription a capping enzyme is associated with the phosphorylated CTD of RNA pol II when the transcript has reached a length of 25-30 nucleotides. The first step, catalyzed by the capping enzyme is to remove the γ-phosphate from the first transcribed nucleotide, most commonly a guanosine but can also be an adenosine. Thereafter a guanosine monophosphate (GMP) moiety is transferred from guanosine triphosphate (GTP) to create a 5´Gppp5´G-cap structure after the creation of an unusual inverted 5´ to 5´ triphosphate linkage to the first transcribed nucleotide of the mRNA (Fig. 1). The inverted guanosine residue is further methylated at the N7 position of the guanosine residue by a guanylyltransferase which results in the 7-Me-GpppG cap structure. The cap structure in all animal cells and higher plants are further methylated at the 2´ position of the ribose of the first transcribed nucleotide (cap 1). In vertebrates the 2´ position of the ribose of the second transcribed nucleotide is methylated (cap 2) (12). Methylation of the cap enhances translation and an increase in the concentration of guanylyltransferase during oocyte maturation stimulates translation of certain mRNAs (13). Further, methylation of the 2´ position of the ribose of the first and second transcribed nucleoside of early expressed mRNA such as c-mos
is also increased during oocyte maturation and is coupled to enhanced translation (14,15).

RNA pol I and III lack the CTD and therefore they will not be associated with capping enzymes and consequently, the transcripts produced by these enzymes do not possess a cap structure. Therefore, the cap serves as a tag to discriminate RNA pol II transcripts (e.g. mRNA) from other RNA molecules transcribed by RNA pol I and III within the cell. Hence, the cap will influence mRNA specific events that will determine the fate of the eukaryotic mRNA.

![Figure 1. The eukaryotic mRNA 5'-cap structure. The N7 position of the inverted guanosine is methylated. In mammalian cells the 2' position of the first transcribed guanosine is methylated (not shown).](image)
The 3´-poly(A) tail

Most eukaryotic mRNA harbor a poly(A) tail that is added to the 3´-end of the pre-mRNA by a complex machinery. The exception is replication dependent histone mRNA which do not have poly(A) tails but rather a stem loop that is expected to fulfill the roles of the poly(A) tail in transport, translation and stability (16). As mentioned earlier, the 3´-processing machinery is recruited co-transcriptionally and transcription termination will rely on the proper formation of the 3´-end processing machinery of both poly(A) tail containing mRNA and histone mRNA (17).

The machinery for processing the 3´-end of mRNA with poly(A) tails is a multi-subunit complex. The process that yields the mature 3´-end is a two-step reaction (18). First the pre-mRNA is cleaved at the poly(A) site, an activity most likely carried out by (CPSF-73) (19,20). This is dependent on the recognition of a number of cis elements in the mRNA i.e. the conserved hexanucleotide AAUAAA that is recognized by CPSF, a 5-subunit complex, and a downstream GU-rich sequence element that is recognized by the heterotrimeric cleavage stimulatory factor (reviewed in (18)). A third element of the form UGUA is associated with cleavage factor 1 (21). The cleavage reaction is followed by the addition of adenosine residues to the cleaved pre-mRNA at the poly(A)-site by poly(A) polymerase (PAP), yielding a poly(A) tail with the length of 200-250 adenosines in mammals and 60-80 adenosines in yeast. Poly(A) tails do exist in prokaryotes as well, but with slightly different biological functions. In prokaryotes, polyadenylation works as a signal for mRNA degradation and occurs prior to an exonucleolytic attack. Since most prokaryotic mRNA ends with a stable 3´-end stem loop, the addition of a poly(A) tail may serve as an anchor for the degradation machinery (22).

Recently it has been shown that transcription termination of pol II transcripts lacking poly(A) tails is dependent on the cleavage of pre-mRNA independent of the assembly of the 3´-end processing machinery, indicating that cleavage is the important step for transcription termination (23). This has given rise to the “torpedo model” which suggests that after cleavage, the RNA polymerase is chased by exonucleases leading to termination of transcription (24,25). Although cleavage is a fundamental prerequisite for proper 3´-end formation of both poly(A) tail containing mRNA as well as histone mRNA, the two machineries are unique. Although some recent findings indicate shared components, e.g. it seems that CPSF-73 is the endonuclease that cleaves both pre-mRNA to be polyadenylated and histone pre-mRNA (26). One striking difference is the need for U7 small nuclear RNA (snRNA) in histone pre-mRNA processing. Several of the factors involved in 3´-end formation interact with components of the splicing machinery, another example of coupled processes in mRNA metabolism.
Recently, it has been discovered that RNA molecules other than mRNA, such as U snRNA, contains poly(A) at their 3´-end (27). It was first found in yeast that some transfer RNA became polyadenylated upon the deletion of the nuclear exosome (28). Later it was shown that additionally non-coding RNA was polyadenylated (29). In these cases it seems that the non-coding RNA was polyadenylated by a nuclear PAP Trf4p (30) prior to degradation i.e. polyadenylation works as a signal for degradation. This type of nuclear degradation is very similar to that of prokaryotic mRNA degradation and might reflect an ancient degradation machinery. There are also examples of poly(A) tracts at the 5´-end of certain orthopoxvirus mRNA (31). Interestingly, it seems that these 5´-located poly(A) tracts are bound by Sm-like proteins (Lsm) and that the Lsm-binding influence the stability of the mRNA, another example of communication between the 5´- and the 3´-end of mRNA. Recently, it has been found that some histone mRNA are oligouridylated prior to degradation, once again an example that resembles the prokaryotic mRNA degradation pathway (32). The finding of poly(A) tails in a large repertoire of RNA molecules suggests that the 7-Me-GpppG cap structure contributes more to the mRNA identity than the poly(A) tail. On the other hand there are examples of RNA molecules bearing cap-structures such as some of the U snRNA. Nevertheless the combination of a cap, a poly(A) and the proteins they are bound to clearly and specifically mark the boundaries of the mRNA.

Proteins binding to the nascent mRNA

In the nucleus proteins will directly bind to the nascent newly transcribed mRNA. The mRNA specific structures, i.e. the 5´-located cap structure and the 3´-end located poly(A), will be associated with different proteins as will the body of the mRNA to form a messenger ribonucleic particle (mRNP). This will create a more homogeneous pool of mRNP complexes, where different mRNAs with completely different structures have a more similar molecular appearance. Being assembled into an mRNP complex facilitates, for example, the interactions with the established export machinery. The cap is bound by the cap binding complex (CBC) (33) and the poly(A) tail will be bound by multiple copies of the nuclear poly(A) binding protein (PABPII) (34). PABPII consists of 4 RNA recognition motifs (RRM). PABPI binds to 25 adenosine residues per protomer. Further, the mRNA is bound by heterogeneous RNP proteins, some of which has preference for different regions in the mRNA e.g. the polypyrimidine tract in the 3´-untranslated region (UTR).

After splicing, a complex of proteins called the exon-junction complex will be deposited where a correct splicing event has occurred. This complex interacts with a number of proteins, worth mentioning are the Upf proteins
that are involved in both transport and quality control mechanisms of mRNA in the cytoplasm e.g. NMD (35).

Roles of the cap and the poly(A) tail

Transport

The cap structure plays several roles, it protects the mRNA from degradation by 5’ to 3’ exonucleases, it influences splicing and it is involved in the transport of the mRNA to the cytoplasm. Both splicing and transport is regulated through binding of CBC to the 5’-cap structure (36,37). It has been shown that transport of large Balbiani ring mRNP s occurs with directionality. Electron microscopy studies have shown that ribosomes are associated with the 5’-end of the mRNP before transport through the nuclear pore is even accomplished (38,39). That implies that the direction of transport is 5’ to 3’ since translation is initiated at the 5’-end of the mRNA. The large subunit of CBC (CBP80) has been found to interact with the mRNA export machinery through a direct interaction with the phosphorylated adaptor for RNA export (40) as well as to the conserved mRNA export machinery (41). It has therefore been suggested that CBP80 directs the transport which further would explain why the mRNA is exported in a 5’ to 3’ direction (41).

The involvement of the poly(A) tail during mRNA export has been studied and there are indications that the poly(A) tail, through the binding of PABPII, participated in the transport. The homolog of PABPII in yeast PAB1, is a protein of a size that would allow it to freely diffuse through the nuclear pore, yet it interacts directly with transportin, suggesting an active role for PAB1 in transport. Experiments have shown that even though transcription is blocked, PAB1 continues to shuttle between the nucleus and the cytoplasm in an mRNA-independent way (42). Further, recent work has shown a coupling between mRNA metabolism and export. A deletion of the poly(A) nuclease (PAN) leads to a retention of mRNA in the nucleus. It has been suggested that poly(A) tail trimming has to occur before the export of the mRNA can take place (43). A genetic link between the exosome and PAB1 has also been seen in yeast where a Rrp6p (an exosome associated factor) mutation functioned as a suppressor in a PAB1 deletion strain, further emphasizing a functional coupling between export and mRNA processing (44,45). Accordingly, the deletion of Rrp6 leads to an accumulation of poly(A) RNA in the nucleus (46). This suggests that the exosome is important for both the degradation of aberrant RNA molecules and for the correct trimming of transcript that will ensure proper export. Since the export of mRNA is an irreversible process, it is crucial that only proper transcripts are transported out of the nucleus. It is therefore highly possible that the transport involves several quality control mechanisms. Indeed, myosine-like pro-
tein 1 is involved in the process of recognizing mRNA that has not successfully gone through splicing and will retain such mRNA. Further, myosine-like protein 1 interacts with splicing factor 1 indicating that myosine-like protein 1-bound mRNA will remain in the nucleus until splicing is accomplished (47).

**Initiation of Translation**

Once the mRNA is transported out from the nucleus into the cytoplasm an exchange of the proteins bound to the cap will take place. The two subunits of CBC, CBP20 that binds directly to the cap structure through a stacking interaction and CBP80 that acts as a scaffold protein facilitating CBP20 cap binding, are replaced after export of the mRNA. When the mRNP complex enters the cytoplasm the eukaryotic initiation factor 4F (eIF4F) complex will associate with the 5′-cap structure and CBC will dissociate from the cap. eIF4F consists of three subunits *i.e.* eIF4E, eIF4A and eIF4G (48). The eIF4F complex binds to the 5′-cap structure via a direct binding of eIF4E to the cap through a stacking interaction, similar to how CBP20 binds the cap (49). eIF4A is an RNA helicase and eIF4G a scaffolding protein to which several other proteins will bind. The binding of eIF4F complex to the 5′-cap allows the small 40S subunit of the ribosome to be associated with both eIF4F and translational initiation factor eIF2 to initiate cap-dependent translation. Therefore the cap serves as one of the main molecular determinants for proper initiation of translation in eukaryotic systems.

**Pioneer round of translation-NMD**

NMD is a quality control mechanism that identifies mRNAs with pre-mature termination codons (PTCs). The subsequent analysis of the proteins involved has revealed CBP80 as one of the main components. CBP80 has been found to interact with NMD factors Upf2 and Upf3 as well as eIF4G (50). Further, cyclohexamide-induced inhibition of translation can increase the association of PTC-containing mRNA with CBP80 indicating that a pioneer round of translation is needed to initiate NMD. CBP80 bound to PTC-containing mRNA is involved in active translation as shown by the requirement of eIF4G for NMD. The translation in this case cannot be inhibited by eIF4E binding protein 1 that inhibits eIF4E dependent translation; however, this translation is less efficient than translation involving binding of eIF4E to the cap (51,52). CBP80 also interacts with the translation initiation factors eIF3, eIF4A1 and eIF2a. An intriguing question is how an mRNA designated for NMD is degraded, what the mechanisms are and what enzymes are involved. In *Drosophila* it has been shown that degradation is initiated by an endonucleolytic cleavage (53). In contrast, in mammalian systems degradation of mRNA targeted by NMD, involves both decapping and subsequent 5′ to 3′ degradation and deadenylation and subsequent 3′ to 5′ degradation. PARN
can be immunoprecipitated with Upf NMD factors and additionally down regulation of PARN increases the abundance of PTC-containing mRNA, indicating a role for PARN in NMD (54).

Translation

At the 3´-end, the nuclear PABPII will dissociate from the poly(A) tail upon delivery of the mRNP into the cytoplasm and shuttle back to the nucleus. Instead, the cytoplasmic version of poly(A) binding protein, PABPI, will bind to the poly(A) tail. PABPI interacts with eIF4G and this leads to a circularization of the mRNA, a phenomenon that has been visualized by electron microscopy (55). It has also been shown that the rate of translation of eukaryotic mRNA is synergistically enhanced by the presence of a cap and a poly(A) tail. The synergistical enhancement is due to PABPI interacting with eIF4G, which brings the 5´- and 3´-end of the mRNA in close proximity to each other (56). This will mediate a faster recycling of the ribosomes after the termination of translation has occurred. Hence the ribosome will be able to reinitiate a new round of translation at a faster rate than in the absence of poly(A). In conclusion, the 5´-cap structure together with the 3´-poly(A) tail plays very important roles in eukaryotic translation.

Figure 2. During *Xenopus* oocyte maturation and early development the poly(A) tail length of different mRNA is modulated. The poly(A) tail length is regulated by cis-regulatory elements. The time is not to scale. Adopted from Colegrove-Otero et al. (57).
The role of poly(A) in early development

A short poly(A) tail of ~50 adenosines is associated with translationally silent mRNA while a long poly(A) tail will induce translation. This feature has been well studied in maturing *Xenopus* oocytes as well as in early development where maternally inherited mRNAs are the first to be translated before zygotic transcription is initiated later in development (58). The spatial and temporal regulation of translation of maternally inherited mRNA is controlled by poly(A) tail length (Fig. 2). In *Drosophila*, the spatial distribution of mRNA regulates the development of the embryo. For example, localization of *bicoid* mRNA determines where in the embryo the encoded transcription factor will be expressed. This determines the anterior-posterior development of the embryo (59). Polyadenylation and deadenylation are therefore crucial processes in order to regulate gene expression in a way that will ensure correct development of the organism. Some of the maternal mRNAs are translated into proteins that are themselves regulators of translation (60).

Two important *cis*-elements in the 3´-UTR of *Xenopus* mRNA control cytoplasmic deadenylation, polyadenylation and activation of translation. These are the cytoplasmic polyadenylation element (CPE) (61) and the hexanucleotide AAUAAA. One mRNA that contains the two *cis* elements is the cyclin B1 mRNA. It receives a long poly(A) tail in the nucleus but once exported to the cytoplasm it is rapidly deadenylated (Fig. 2), an activity dependent on the CPE (reviewed in (57)). It is not clear why an mRNA bearing a long poly(A) tail is more efficiently translated but one possibility could be that several PABP1 bind to a longer poly(A) tail facilitating PABP1 interaction with eIF4G. Translational repression during oocyte maturation is not only due to the short poly(A) tail but it also depends on maskin, a protein that binds to CBE-binding protein (CPEB) and eIF4E (62). This interaction will prevent the formation of eIF4F and subsequent the binding of 40S. The CPEB-maskin interaction decreases during oocyte maturation which, together with an extension of the poly(A), will induce translation.

Three proteins have been characterized as key components for the modulation of the poly(A) tail length during *Xenopus* oocyte maturation; CPEB, the poly(A) polymerase Gld2, and PARN (63). In order to elucidate if PARN was involved in CPE dependent deadenylation, immunoprecipitation experiments using a PARN antibody was performed and indeed cyclin B1 mRNA could be recovered. In the complex CPEB, CPSF and Gld2 were also found. Furthermore, injecting PARN antibodies into oocytes stimulated longer poly(A) tails on CPE containing mRNA. If an mRNA coding for a dominant negative PARN(D28A) mutant was injected, the fast deadenylation normally obtained upon entrance to the cytoplasm was abolished. This indicates that PARN(D28A) replaced PARN in the complex and that the replacement released the deadenylating activity. From these experiments and others it was concluded that PARN activity is robust and will dominate over
poly(A) polymerase activity of Gld2 and therefore the poly(A) tail will remain short.

Upon progesterone stimulation, which induces oocyte maturation, the poly(A) tail of cyclin B1mRNA extends and translation initiates. Oocyte maturation includes activation of Aurora A kinase that phosphorylates CBEP at Ser 174 (64). As a result, the interaction between CPEB and CPSF, is enhanced and can direct Gld2 to proper elongation of the poly(A) tail (65). This occurs at the expense of PARN which is then expelled from the complex. PABP1 will bind to the newly synthesized poly(A) tail and make contact with eIF4G so that maskin will no longer interact with eIF4G and thereby releasing translational repression. A recent study has shown that an interplay of CPE and the hexanucleotide exists. Specifically, the distance between and the number of copies of each in the 3’-UTR determines more precisely the exact onset of polyadenylation (66).

**Allosteric regulation**

Proteins can be regulated in many different ways. Regulation can be induced by either covalent or non-covalent modifications. In the 1960s, Francois Jacob and Jacques Monod investigated the regulation of the expression of the lac-operon in *E. coli* (67-69). They could show that the expression of the lac-operon was modulated depending on whether the concentrations of glucose or lactose were high within the cell. Later on it was shown that the regulation was dependent on proteins binding to the transcription start site of the lacZ gene. lacZ encodes an enzyme, β-galactosidase, which is responsible for the split of lactose into glucose and galactose. When the concentration of glucose in the cell is high, the expression of β-galactosidase is low, since there is no need for β-galactosidase activity. When the concentration of lactose is increased, synthesis of β-galactosidase starts. This was shown to be the result of a repressor protein binding to a region upstream of the transcription start site. When lactose concentration is low the repressor is bound at the transcription start site and prevents transcription. Aside from the lac-operon binding site, the repressor protein also contains a binding site for lactose located at a different position. Binding of lactose to the repressor protein will induce a conformational change that will affect the binding of the repressor to the operon. Lactose-bound suppressor cannot bind to the transcription start site and therefore transcription is initiated. *I.e.* binding of one molecule at one site affects a binding site located at another position in the protein. This is called an allosteric effect. It plays an important role in regulating proteins and it is found in many different enzymatic pathways.

If the two different binding sites are located on the same protein molecule, the allosteric effect (positive or negative) can be significant. On the
other hand, a protein consisting of two subunits with two sets of the two different binding sites positioned at different subunits, will give a more sensitive protein. This can be due to the increased affinity of the ligand to the second site when the first site is loaded. One well studied example is the binding of oxygen to hemoglobin. Binding of one oxygen molecule will increase the affinity for oxygen to another binding site located on another subunit. This will make sure that a fast loading of oxygen will take place in the lungs as well as a fast unloading in the tissues (70). Other examples of allostery are found in feedback mechanisms in metabolic pathways of nucleotide metabolism. One example is the enzyme aspartate transcarbamoylase, which is inhibited by its end product cytosine triphosphate (71). In these pathways, the end product regulates the activity of the processing enzymes to assure a proper supply of nucleotides in the cell.

Recently, a new family of lipid molecules, phosphoinositides, has been identified. Molecules of this family, such as phosphatidylinositol-4,5-bisphosphate, have been found to function as signaling molecules. Further, they have been identified as allosteric regulators of proteins, one example is the nuclear speckle targeted PIPKI regulated-poly(A) polymerase. It has been shown that phosphatidylinositol-4,5-bisphosphate interacts with the nuclear speckle targeted PIPKI regulated-poly(A) polymerase and interestingly, it stimulates the processivity of the polyadenylation reaction (72).

mRNA degradation

Eventually all mRNA in the cell will be degraded either as the result of a quality control step that has identified the mRNA as aberrant, or as a response to a signal that the mRNA is no longer needed for gene expression. Surveillance mechanisms will detect mRNA with errors originating from transcriptional and post-transcriptional processes e.g. mutations not originating from the DNA template, splicing, 3′-end processing or assembly errors of the mRNP. The checkpoints are located in the nucleus, at the nuclear pore complex upon transport and in the cytoplasm. In the nucleus a surveillance mechanism is carried out by the nuclear exosome, which degrade aberrant pre-mRNA, pre-transfer RNA, pre-ribosomal RNA and mRNA (reviewed in (73,74)). At the nuclear pore complex, intron-containing mRNA will be retained by myosine-like protein 1, although the degrading activity coupled to the retention is not known (47). Most of the activities involved in eukaryotic mRNA degradation are performed by exonucleases, although there are examples of endonucleolytic activities. In the cytoplasm, the mRNA will be degraded through either of two pathways where the first critical step is deadenylation i.e. shortening of the 3′-end located poly(A) tail.
Pathways of mRNA degradation

Although the underlying mechanisms of identifying and designating an mRNA for degradation are not fully understood and may vary depending on the mRNA and the situation, several of the pathways and the enzymes involved have been well characterized. mRNA decay can be studied by inhibiting transcription and thereafter follow the degradation of a certain mRNA over time by RT-PCR or northern blot, for example. To induce a short pulse of a reporter mRNA expressed during a short time and then follow degradation, a so called pulse-chaise experiments, is a way to avoid the problem of the steady state amount of mRNA that is present. It is also possible to introduce a reporter mRNA as done by injection used in *Xenopus* oocytes. Many components of the mRNA degradation machinery have been identified by knockouts and repressor mutants of yeast where genetics can easily be performed. The recent finding of RNAi as a biochemical tool to silence genes has given further insights to how mammalian mRNA degrading systems function. To understand the mechanisms of degrading enzymes it is important to investigate their biochemical properties. In this thesis I have focused on the biochemical properties of one of the enzymes involved in mRNA degradation, PARN, which degrades the poly(A) tail of eukaryotic mRNA.

Two major pathways of mRNA degradation have been described in yeast and they are basically the same in mammalian systems (reviewed in (5,75,76)). Since the eukaryotic mRNA bears the 5´-cap structure and 3´-poly(A), the hallmarks of the eukaryotic mRNA, and because almost all enzymes involved in eukaryotic mRNA degradation are exonucleases, these structures are the main targets for the enzymes involved in mRNA decay. They therefore play a crucial role in the fate of mRNA. Genetic studies in yeast have revealed the pathways and enzymes involved (77-81). The first and most commonly used pathway is the deadenylation-dependent decapping pathway, which is believed to be the major degradation pathway in mammalian systems (82). This pathway is initiated by degradation of the poly(A) tail, which is followed by either decapping of the mRNA by the decapping enzyme (Dcp1/Dcp2) and subsequent 5´ to 3´ degradation of the mRNA by the exoribonuclease 1 (Xrn1) or 3´ to 5´ degradation of the mRNA by the exosome. In the latter case the remaining cap structure is hydrolyzed by the scavenger decapping enzyme (DcpS).

In a second and what seems to be a less used pathway in yeast, the deadenylation-independent decapping pathway is initiated by decapping by Dcp1/Dcp2 and followed by the 5´ to 3´ degradation the mRNA by Xrn1. The two pathways are not mutually exclusive since separate knock-down of the different components of the two pathways (83) do not affect the transcriptome to a large extent. The actual contribution of each to overall mRNA degradation is not clear, and there might even be redundancy. Though knock
down of components of the two systems simultaneously is lethal in yeast (84).

Degradation of a minority of eukaryotic mRNA is initiated by an endonucleolytic cleavage, followed by the degradation performed by exonucleases. One important example is the specific cleavage of mRNA targeted by the RNA-induced silencing complex as a response in the RNAi pathway. This is in contrast with prokaryotic mRNA degradation where the major mRNA degradation pathway is believed to be initiated by an endonucleolytic cleavage by RNase E which will cleave off the stable 3′-end located stem loop. Interestingly, the target mRNA is polyadenylated by PAP 1 prior to degradation. It has been suggested that the newly synthesized poly(A) tail may serve not only as a signal for degradation, but also as an anchor for the degradasome, a multiprotein complex comprised of PNPase, RNase E and enolase (22).

**Exosome**

As previously mentioned, mRNA degradation takes place both in the nucleus and in the cytoplasm. In the nucleus, aberrant transcripts that i) contain mutations not encoded by the template DNA (85), ii) have failed to splice successfully, iii) with an abnormal 3′-end or iv) have been assembled incorrectly into an mRNP complex will be targeted by the nuclear exosome, a multi-protein complex consisting of at least 10 components (73). The exosome is located in close proximity to where active transcription is taking place (86). The exosome is therefore an important part of the nuclear surveillance machinery. The nuclear exosome was first identified to process ribosomal RNA, small nucleolar RNA and snRNA (reviewed in (87)). Although the mammalian core exosome components all contain RNase PH domains, it remains to be shown which of the subunits that are active in the exosome. The yeast exosome component Rrp44 and the nuclear exosome-associated auxiliary factor Rrp6 have been shown to harbor hydrolytic activity (74,88). Interestingly, the degradation by Rrp6 is poly(A) tail-dependent, resembling the prokaryotic degradation mechanism. Indeed a complex consisting of exosome component Trf4p in association with Air2p/Mtr4p PAP, has been purified (30).

The cytoplasmic version of the exosome is currently known to degrade deadenylated mRNA as part of normal degradation and additionally in response to a PTC-induced NMD and as well as AU-rich element (ARE) mediated mRNA decay (89). A coupling between the exosome and decapping has been demonstrated (82).
Deadenylation

In the majority of cases the first step of mRNA degradation is deadenylation i.e. removal of the 3’-end located poly(A) tail. In the deadenylation dependent pathway, the poly(A) tail in yeast is shortened down to 10 nucleotides (78) while in mammalians it is degraded to a length of 30-60 nucleotides (90).

Exonuclease activities with some poly(A)-specificity were observed in early preparations of different sources when purifying PAP (91). The activities were shown to be magnesium- or manganese-dependent with released product of 5’-adenosine monophosphate (92,93). Today three enzymatic deadenylation activities have been characterized. Two are shared between yeast and mammalians i.e. carbon catabolite repressor 4/carbon catabolite repressor 4-associated factor 1 (CCR4/Caf1) (Caf1 is also called Pop2) and the PAN2/PAN3 complexes. A yeast strain where both CCR4/Caf1 and PAN2/PAN3 have been deleted lacks deadenylation activity. It has been proposed that PAN2/PAN3 is involved in the trimming of the poly(A) tail in the nucleus since a deletion of the encoding gene induced longer poly(A) tails (94-97). PAN2 contains an RNase D domain which when deleted abolish hydrolytic activity (98). PAN2/PAN3 is a PABPI-dependent deadenylase i.e. PAN2/PAN3 activity is enhanced by the presence of PABPI. Immunostaining experiments of human PAN2/PAN3 showed a clear cytoplasmic distribution, indicating a role for PAN2/PAN3 in deadenylation of mRNA in the cytoplasm. This is in agreement with the finding that PAN2/PAN3 seems to be involved in the initial shortening of a β-globin mRNA in mouse NIH3T3 fibroblasts (99).

CCR4/Caf1 was first identified as a regulator of transcription (reviewed in (100)) and homologues are present in mammalian systems including human (101). CCR4/Caf1 has been shown to be the major deadenylase in yeast since the deletion of CCR4 as well as CAF1 in yeast drastically stabilize a number of transcripts through a decreased deadenylation (102). CCR4 plays also an important role in mRNA degradation in Drosophila (103). CCR4/Caf1 exists in a large 1 MDa complex together with the Not1-5 proteins. Recently two more proteins were found to be associated with the complex, Caf40 and Caf130 (104). The function of these factors are not fully understood but deletion of them affects deadenylation (103,105). CCR4 is active in vitro and magnesium-dependent. Mutation of a conserved Glu 556 to alanine abolish activity (101) and its activity is inhibited by PAB1 (105). Caf1 belongs to the RNase D family. Deletion of Caf1 in yeast causes a similar effect to that of a CCR4 deletion although not as drastic. Recombinant Caf1 is active (106), but mutations of amino acids in the catalytic site does not affect deadenylation in vivo (107,108). Deletions of regions which interact with CCR4 affect deadenylation in vivo. This fact together with the finding that Caf1 interacts with all the proteins in the CCR4/Caf1 complex...
has led to a proposed model where Caf1 plays an important role to link CCR4 to the other factors rather than being active in deadenylation.

In higher eukaryotes a third deadenylase, PARN, has been identified, (109,110). PARN is unique among the deadenylases because PARN interacts not only with the 3´-end located poly(A) tail but it also binds to the 5´-end located cap structure. The cap binding property of PARN has several important impacts on mRNA metabolism: i) The cap activates PARN and stimulates processivity and will lead to fast degradation of the mRNA (111,112) ii) an mRNA involved in PARN-mediated deadenylation will most likely not be translated since the cap is not available for the translational initiation complex. It is therefore of great importance to characterize the cap binding property of PARN to understand the mechanisms of PARN.

Very recently an involvement of microRNAs in deadenylation has been found (113-115). MicroRNAs are known to regulate gene expression though translational inhibition (reviewed in (116) and the finding of microRNAs regulating deadenylation, and thereby mRNA degradation, assigns a second post-transcriptional role for microRNAs. It remains to be shown whether PARN is involved in microRNA-mediated deadenylation.

**Decapping**

In contrast to deadenylation, whose effect can be reversed by polyadenylation to rescue the mRNA from degradation, decapping is an irreversible process and therefore plays a central role in both mRNA degradation pathways. Decapping will produce a 5´-end without a cap structure that can no longer be bound by eIF4E. As a result translation will be prevented, further emphasizing the importance of decapping on gene expression. In the deadenylation-dependent decapping pathway the cap is hydrolyzed, after deadenylation, by the Dcp1/Dcp2 complex. This will allow Xrn1 to access the remaining part of the mRNA and degrade the mRNA in a 5´ to 3´ direction. Dcp1/Dcp2 was first identified in yeast (117,118). Decapping activity has been recovered from HeLa cytoplasmic extracts (119). The activity was later cloned and recombinant Dcp1/Dcp2 was shown to be active in decapping (120). Hydrolysis by Dcp1/Dcp2, where Dcp2 is the catalytic subunit and Dcp1 the activator, will result in the release of 7-Me-GDP (121,122).

Dcp1/Dcp2 is an RNA binding protein complex that acts on capped RNA substrates, preferably 25 nucleotides or longer. Recent structural determinations together with modeling have revealed a Box B domain in Dcp2 that is predicted to be part of an RNA binding channel (123-125). Indeed mutations in this region decrease RNA binding drastically as well as the catalytic activity. Cap binding of Dcp1/Dcp2 seems to be weak but specific. A Tyr 220 is involved in stacking the base of an adenosine which was present in the crystallization and likely sits either where the inverted 7-Me-G is located or at
the first transcribed guanosine, since the mutation of Tyr 220 affects cap hydrolysis.

If deadenylation is followed by 3′ to 5′ degradation by the exosome, the remaining cap structure must be hydrolyzed. This is an activity performed by DcpS, which will yield 7-Me-GMP as the product (122). DcpS can hydrolyze free cap or capped oligonucleotides but not capped RNA. Interestingly, it seems that the activity of Dcs1p in yeast affects degradation events downstream of decapping i.e. 5′ to 3′ degradation. Deletion of Dcs1p in a yeast strain stabilizes mRNA, for example the TIF51A mRNA, and expression of Dcs1p could complement the phenotype but not an inactive dominant negative form of Dcs1p. This might reflect that either the substrate for Dcs1p or the hydrolyzed product i.e. 7-Me-GMP could act as a signal for downstream degradation events rather than the presence of the protein itself (126). The structure of DcpS has revealed a dimeric composition. In DcpS the 7-Me-guanosine is stacked between Trp 175 and Leu 206 (127). When bound to the 7-Me-GpppG substrate the enzyme exhibits an asymmetric structure. Both active sites are bound to the substrate but strikingly, one subunit has a closed conformation where the substrate interacts extensively with amino acids in the active site. The other subunit adopts an open conformation. In this situation the cap makes fewer contacts to the protein. It has been suggested that the closed form is the productive site, and that the conformational change that takes place between open and closed form is an important regulatory mechanism of DcpS.

A number of factors have been identified that regulates decapping. The Lsm1-7 proteins (reviewed in (128)) that become associated with deadenylated mRNA have been shown to interact with Dcp1p/Dcp2p. Deletion of the Lsm genes in yeast leads to stabilization of a MFA2pG mRNA and an accumulation of oligoadenylated and capped mRNA (129). Further analysis could show that the interaction between the Lsm proteins and Dcp1p/Dcp2p increases when the mRNA becomes deadenylated. This is also true in the interaction between the mRNA and the Dcp1p/Dcp2p complex (130). These data indicate that the Lsm1-7 proteins activate decapping although the mechanism is not yet fully understood. Another factor, a helicase DEAD box protein Dhh1 has been shown to activate Flag-tagged Dcp1p in yeast (131). This interaction is interesting since it also has been shown that the helicase DEAD box protein Dhh1p interacts with CCR4/Caf1 deadenylase (132). This is another example of coupled processes in mRNA decay. Dhh1p also functions as a translational repressor and induces the formation of processing bodies, suggesting a tight coupling of translation and mRNA degradation (133). Processing bodies are cytoplasmic foci where translationally dormant mRNAs are found in association with factors involved in mRNA decay such as the decapping machinery and 5′ to 3′ exonucleases (134). Of the deadenylases, CCR4 has been found in processing bodies (135) whereas PARN
together with exosome components have been found associated with ARE-containing mRNA. These complexes are found in granules and differ from processing bodies (136).

Further, a testis-specific cap binding protein VCX-A implicated in X-linked mental retardation has been shown to be a trans-acting factor that inhibits hDcp2 (137). A nuclear decapping enzyme, X29 is involved in the hydrolyses of 2,2,7 trimethylated capped U8 small nucleolar RNA (138) and notably shows another mode of cooperative metal-dependent cap binding (139).

An early observation in decapping assays performed with yeast extracts was the effect of the poly(A) tail on decapping. It was noted that an mRNA lacking a poly(A) tail was much more quickly decapped than one with a poly(A) tail (140). Was this due to the augment binding of the Lsm proteins to the deadenylated RNA? The addition of poly(A) to the reactions sequestered this effect, indicating that something binding to the poly(A) tail could cause the inhibition. A similar effect was observed in mammalian extracts (120). In an in vitro system the PABPI-inhibitory effect on Dcp2 could be reconstituted. UV cross-linking experiments could show that PABPI was able to associate with both the poly(A) tail and the cap simultaneously (141). It remains to be investigated at the molecular level how cap binding by PABPI is achieved and where a predicted cap binding site is located.

PARN

Introduction

Enzymatic activities with exoribonucleolytic properties were found early in tumor cells and in different mouse tissues. These activities degraded single stranded RNA and were shown to have some preference for poly(A) (92,93,142). The activity associated with poly(A) tail removal leaving the mRNA body intact was first shown in a partially purified preparation from HeLa cells (143). This activity specifically degraded poly(A) relative poly(C), poly(G) and poly(U). This activity was further characterized and it was found that the enzyme required a 3'-hydroxyl group for activity, the enzyme yielded a 5'-adenosine monophosphate product and a 3'-hydroxyl group was left on the last adenosine residue on the remaining poly(A) tail (144). It was also shown that the enzyme was magnesium dependent and required monovalent ions for activity. A deadenylase purified from calf thymus showed similar properties and the activity was inhibited by PABP1 (145). The human gene encoding the enzyme was later cloned and the 74 kDa recombinant protein expressed in E. coli showed poly(A)-specific ribonuclease activity (109,110). The enzyme was therefore named PARN for
poly(A)-specific ribonuclease. PARN is not present in yeast or *Drosophila* but found in *Xenopus* (146) and in higher eukaryotes.

PARN was found to be a member of the DEDD subfamily of the RNase D family of proteins based on sequence homology. The DEDD family is characterized by three exonuclease domains where three aspartates and one glutamate residue build up the active site (Fig. 3). Mutational analysis of D28, E30, D292 and D382 confirmed the importance of these amino acids for PARN activity (147). Furthermore, if the same four amino acids were substituted with cysteines, then the PARN mutants were no longer active in the presence of magnesium. However, the cysteine substituted PARN mutants were active in the presence of the softer manganese. This gave strong functional evidence for divalent metal ions being coordinated by the active site of PARN and directly involved in the catalytic activity of PARN (148). An estimated native molecular weight of 180-220 kDa was determined from gel filtration experiments with PARN purified from calf thymus, indicating an oligomeric composition of PARN. Indeed cross-linking experiments revealed an oligomeric statue of PARN (110). Furthermore, a co-crystal structure of PARN(1-430) bound to A₁₀ has been solved (149). Only the last three adenosines of A₁₀ were visible in the structure, but the binding of them to the active site could confirm the involvement of the well conserved DEDD exonuclease domain-containing amino acids. Notably no adenosine specific interactions were revealed. The structure showed a dimeric composition of PARN. Further, PARN was found to interact with the 5’- cap structure of mRNA, a property used during the initial purification of PARN where the affinity to 7-me-GTP-Sepharose could be used in the purification procedure (110).

**Expression and purification of recombinant PARN (paper III)**

In order to assure the supply of good amounts of high quality recombinant PARN, we set up a protocol wherein we established conditions for high expression of soluble PARN expressed in *E.coli*. We found that expressing PARN from a pET33b vector in *E. coli*, grown in terrific broth media and induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside at OD ~1 yielded approximately 1 mg of soluble protein per liter culture (Fig. 1, paper III). Further, we established a purification protocol based on His-tag affinity puri-
fication, ion exchange chromatography and finally affinity chromatography using 7-Me-GTP-Sepharose (Fig. 2, 3 and 4, paper III). Due to the small but significant leakage of 7-Me-GTP from the sepharose this step was avoided for the following studies of the cap binding property of PARN. We found it important to optimize the ratio between the matrix and applied clarified lysate in order to saturate the beads with PARN to avoid unspecific binding of *E.coli* proteins. This purification protocol yields a highly active more than 95% pure PARN preparation which was used for mechanistic studies (Fig. 6, paper III).

**Cap binding induce allosteric activation of PARN (paper I)**

A unique property that distinguishes PARN from the other deadenylases is its ability to interact not only with 3’-located poly(A) tail but also with the 5’-located mRNA cap structure. Several observations indicate that a direct interaction of the cap structure and PARN takes place. Firstly, deadenylation activity of PARN is increased when a cap structure is present on the mRNA in an *in vitro* deadenylation assay (110-112). Secondly, PARN activity could be inhibited by the addition of a 7-Me-GpppG cap analogue *in trans* (Fig. 2, paper I). And finally, PARN binds to 7-Me-GTP-Sepharose, a ligand that represents the methylated inverted guanosine of the cap structure.

To further characterize the cap-stimulatory effect, the kinetic parameters $K_m$ and $V_{max}$ were determined for a capped, a non-methylated capped and a non-capped L3(A30) RNA substrate, using native PARN as the source of enzyme (Table I, paper I). Interestingly, the efficiency parameter $V_{max}/K_m$ for the different substrates relatively to each other were 1, ½, and 1/3 for capped, non-methylated capped and non-capped substrates respectively. When the distribution of substrate and product, during a time course experiment, was resolved by gel electrophoresis, it revealed a pattern of fast turn-over from full length capped polyadenylated RNA to completely deadenylated RNA without the presence of partially deadenylated products (Fig. 1 and 4, paper I). The degradation pattern using a non-capped RNA substrate gave a broader distribution of partially deadenylated products. This was indicative of a processive reaction of PARN acting preferentially on a capped substrate. To investigate processivity, the method of choice is *in vitro* deadenylation experiments using two different substrate concentrations in a time course experiment to determine the time point at which the first deadenylated product appears. If the reaction is highly processive, the time point where the first deadenylated product appears should be independent of substrate concentration, since the enzyme will be associated with the same substrate until it is fully deadenylated. Such experiments could show that both a capped and a non-capped substrate was deadenylated in a processive fashion by PARN since the time point when the first deadenylated product appeared was concentration independent (Fig. 4, paper I). Another signature of a proc-
ressive reaction is that both the substrate and the fully deadenylated product should be present sometime during the course of the reaction. When PARN was incubated with the capped substrate, the presence of both substrate and product could be visualized at some point but when PARN was incubated with a non-capped substrate, the presence of substrate and product could not be visualized at the same time (Fig. 1 and 4, paper I). If PARN degrades poly(A) in a processive way it should also be possible to capture an active deadenylating complex. Indeed an electrophoretic mobility shift assay (EMSA) could show that a cap-independent PARN-RNA complex forms (Fig. 5, paper I). In the PARN-RNA complex partially deadenylated RNA substrates could be detected (Fig. 6, paper I). This suggested, together with the efficiency parameters, that the cap structure not only activate PARN, but also enhances processivity. Therefore the cap functions as an allosteric activator of PARN.

Another interesting observation was that PARN activity could be inhibited by the addition of cap in trans to the reaction (Fig. 2, paper I). A non-methylated GpppG cap analogue inhibited approximately hundred fold less efficiently than 7-Me-GpppG, indicating that the methyl group is very important for PARN cap binding. It is worth mentioning that the end product of mRNA degraded by deadenylation and subsequent 3´ to 5´ degradation by the exosome, is the cap structure. This is further hydrolyzed by DepS to give 7-Me-GMP. Although speculative at the moment, PARN activity might be regulated by the end products of mRNA degradation just as it has been suggested that the substrates or the products of decapping regulates 5´ to 3´ degradation by Xrn1 (126).

Interestingly, when a non-methylated capped or a non-capped substrate was used a small but significant increase in activity was observed when low concentrations of 7-Me-GpppG were added in trans, before inhibition occurred at higher concentrations (Fig. 2, paper I). This activation was not observed when a 7-Me-GpppG capped substrate was used. It seemed that the cap ligand added in trans could mimic a cap present in cis on the substrate. A kinetic analysis could show that the cap inhibition pattern was non-competitive, meaning that the cap binds at a different site compared to where the hydrolysis of the substrate takes place (Fig. 3, paper I). Altogether we conclude that the cap interacts with PARN and that the cap stimulates PARN activity and enhances the processivity of PARN mediated deadenylation. Although the mechanism behind the stimulation was not fully revealed by these experiments, it was clear that cap binding induces an allosteric affect which could involve conformational changes that leads to an increased rate of hydrolysis and/or translocation of the substrate.

The cap binding property of PARN will not only influence the rate of degradation. It will also make sure that an mRNA involved in degradation by PARN will not be translated at the same time, since the cap is the major
structure recognized during initiation of translation through binding of eIF4E to the cap. Furthermore, deadenylation by PARN will release poly(A) tail-bound PABP1. That in turn will lead to a disruption of the interaction between eIF4G and PABP1 and prevent the circularization of the mRNA. This will affect ribosome recirculation and decrease translation efficiency further. PARN therefore regulates gene expression in two ways and the cap plays important roles in both.

In this context it is also worth mentioning that regulation of translation occurs at the 5´-cap. The interaction between eIF4E and eIF4G is disrupted by eIF4E-binding protein 1 binding to eIF4E. This interaction is regulated by phosphorylation. Both eIF4E and eIF4E-binding protein 1 are phosphorylated, for example in a response to an extra cellular signal. It is not clear how phosphorylation of eIF4E affects translation but 80-85 % of eIF4E found in 40S initiation complex is phosphorylated at Ser 209 (150). It has also been shown that the affinity of eIF4E to the cap is decreased upon phosphorylation (151). A recent study has shown that cap binding by PARN is increased when cells are exposed to serum starvation and at the same time a decrease of the association of eIF4E to the cap is observed (152). In the same study it was shown that PARN is a phosphoprotein, and the increased association of PARN to the cap was accompanied by an increased phosphorylation level of PARN. It has also been shown that synthetically produced cap analogues with higher translational efficiency also leads to a stabilization of the mRNA, further emphasizing the interplay between translation and mRNA degradation (153). Therefore the cap is an indispensable structure that will influence what biological activity is associated with the mRNA.

Prokaryotes do not have 5´-end located cap structures on the mRNA and the major mRNA degrading activity is endonucleolytic cleavage by RNase E. It has been shown that the activity of RNase E is stimulated by the presence of a 5´-located monophosphate but not by a 5´-triphosphate (154-156). Even though the enzymatic entities are different it clearly shows that communication between the 5´-end of mRNA with the rest of the mRNA, and associated proteins, is a well conserved phenomenon between prokaryotes and eukaryotes.

**Regulation of PARN by cap binding complex (paper II)**

A number of proteins have been identified to regulate PARN-mediated deadenylation. A special type of fast mRNA degradation is dependent on ARE. Tristetraprolin binds to AREs and destabilizes ARE-containing mRNA (157). Destabilization is due to increased deadenylation by PARN whose activity is enhanced by Tristetraprolin. Another ARE-binding protein involved in ARE-mediated mRNA degradation that interacts with PARN is the KH-type splicing regulatory protein (158).
In contrast to the CPE element responsible for deadenylation/polyadenylation during *Xenopus* oocyte maturation, deadenylation occurring after fertilization is dependent on an embryonic deadenylation element present in the 3’-UTR. Deadenylation is regulated by an embryonic deadenylation element binding protein (159). The human homologue, CUG-BP, has been shown to interact with PARN. Extracts depleted with an antibody against CUG-BP reduces the level of PARN as well as the rate of deadenylation of TNFα and *c-Fos* mRNA containing a binding site for CUG-BP. mRNAs without CUG-BP binding sites were unaffected (160). The interaction was found to be RNA-independent since RNase treatment did not affect the interaction.

In order to further investigate the cap binding property of PARN and its biological importance, a study of CBC was initiated. When reconstituted CBC was added to an *in vitro* deadenylation reaction with PARN a clear inhibitory effect was detected (Fig. 1A and B, paper II). Since the cap stimulates the activity of PARN, the first obvious explanation to the inhibition was that the cap binding property of CBC prevented PARN from binding to the cap and thereby affects PARN activity. Therefore a non-capped L3(A30) RNA substrate was used in *in vitro* deadenylation reactions. PARN degradation of a non-capped L3(A30) RNA substrate was efficiently inhibited by CBC (Fig 1C, paper II). The data indicated that the cap was not a prerequisite and therefore the experiment was repeated with two PARN mutants where amino acids important for cap binding had been mutated to alanines (Table 1, paper IV). One PARN mutant contained a double mutation of Glu 455 and Trp 456 while the other mutant contained a single Trp 475 mutation. Both cap binding site mutants were inhibited by CBC, which is consistent with the result that PARN was inhibited by CBC in a cap-independent fashion (Fig. 2A and B, paper II).

A more careful analysis was therefore performed where the subunits of CBC *i.e.* CBP20 (which binds directly to the cap, see Fig. 5) and CBP80 were added separately to a PARN reaction (Fig. 3, paper II). Surprisingly, CBP80 inhibited PARN while CBP20 did not affect PARN. The 3 fold cap-stimulatory effect previously detected with native PARN was less pronounced when recombinant PARN was used, and therefore the effect of CBP20 binding to the cap was not detected. Control experiments with eIF4E were performed and no inhibition of PARN by eIF4E was observed (Fig. 1A, paper II). From these experiments we concluded that the CBC-mediated inhibition of PARN was due to a protein-protein interaction between CBP80 and PARN, in keeping with the observation that the inhibition was cap-independent.

In order to localize the region of PARN that interacted with CBP80, a deletion mutant corresponding to PARN(1-470) was used as the source of enzyme (Fig 2C, paper II). PARN(1-470) corresponds to a proteolytic fragment
of native PARN that was initially purified from calf thymus. Interestingly, PARN(1-470) could not be inhibited by CBP80, indicating that the region of interaction is located at the C-terminal part of PARN.

A steady state kinetic analysis was performed by incubating PARN with different CBC concentrations and varying the L3(A30) RNA substrate concentration, in order to determine the pattern of inhibition. When a non-capped substrate was used, a non-competitive inhibition pattern was obtained (Fig. 4B, paper II). Similar results were achieved with the cap binding site mutants, independent of the 5’-structure of the RNA substrate (Fig. 4C-F, paper II). This implied again that CBC interacts with PARN outside the active site of PARN. When PARN was incubated with a 7-Me-GpppG capped substrate an un-competitive inhibition pattern was observed (Fig 4A, paper II). The interpretation of an un-competitive mode of inhibition is that the inhibitor can only bind to the enzyme-substrate complex and that the inhibitory interacting site is only exposed upon enzyme-substrate complex formation. In the case of the CBC-mediated PARN inhibition it is not completely clear what the reason is behind the shift from non-competitive to un-competitive. It is clear though that CBC will bind to both the cap and to PARN when the cap is present on the RNA. This will somehow affect the inhibition pattern compared to when a non-capped substrate or cap binding site mutant of PARN is used.

CBC plays an important role in NMD and during the pioneer round of translation. Further, PARN has been found to associate with NMD factors such as Upf proteins. Knock down of PARN has also been seen to reduce NMD, indicating a role for PARN in NMD. In order to investigate the in vivo situation of PARN and CBC, co-immunoprecipitation experiments were performed. An antibody against PARN could bring down CBC from HeLa extracts and conversely an antibody against CBC could bring down PARN (Fig. 5, paper II). This suggests that PARN and CBC were associated in the cell. A provocative model for the biological relevance of the CBC-PARN interaction is that CBC brings PARN to an mRNA that undergoes a pioneer round of translation and that the CBC inhibitory property makes sure that PARN is inactive during the pioneer round of translation. In the case a PTC is recognized, NMD will be activated and in such case PARN is in close proximity to degrade the aberrant mRNA.

**Identification of the cap binding site of PARN (paper IV)**

Cap binding has been studied in several cap binding proteins *e.g.* eIF4E, CBC20 and vaccinia virus VP39 (40,49,161). The cap binding property, shared between these proteins, has been characterized by both biochemical and structural methods. It has been shown that the main interaction for cap binding is achieved through a π-π stacking between the inverted guanosine residue and aromatic amino acid residues in the proteins. In the cases of
CBP20 and VP39, the cap is bound by tyrosines while in eIF4E two tryptophans are involved in cap binding.

Since cap binding involves aromatic amino acids, it has been possible to investigate and quantify cap binding by fluorescence spectroscopy (162,163). This technique utilizes the biophysical properties of aromatic amino acids to fluoresce and the quench that occurs upon binding to a cap analogue. Therefore we investigated whether the cap binding property of PARN could be analyzed by fluorescence spectroscopy. When PARN is excited with 280 nm light a peak at 342 nm is emitted (Fig. 2A, paper IV). The wavelength of free tryptophan is 340 nm suggesting that the peak originates from the fluorescence of tryptophans. Furthermore, upon addition of cap analogue to PARN, the 342 nm peak is drastically decreased in intensity, indicating that the cap quenches the emitted light from a bound tryptophan (Fig. 2A, paper IV). It is also possible that the observed quench could originate from a structural change in the micro environment of the emitting tryptophan.

To identify possible cap binding tryptophans within the PARN sequence, a mutational analysis was performed where all 6 tryptophans in PARN were mutated to alanines. In an initial screen the affinities of the mutant PARN polypeptides to 7-Me-GTP-Sepharose were investigated. In this screen one mutant polypeptide, PARN(W475A), showed a significant drop in its affinity to 7-Me-GTP-Sepharose (Fig. 4).

![Figure 4](image-url)  
**Figure 4.** Cap binding properties of PARN and PARN mutant polypeptides assayed by affinity to 7-Me-GTP Sepharose. 0.25 mg of indicated polypeptide was applied to the 7-Me-GTP-Sepharose and fractions were collected and separated by 7.5 % SDS-PAGE followed by silver staining. Above the lanes are the KCl concentrations used in the elution buffers indicated.

To further characterize and quantify the cap binding properties of PARN and mutants thereof we used fluorescence spectroscopy measurements to determine the equilibrium binding constant, $K_d$, of the PARN cap binding. PARN
interacts with both the cap and 7-Me-GTP with a $K_d$ in the low micromolar range (Fig. 2 and Table 1, paper IV). As predicted, PARN(W475A) did not interact with the cap and it was not possible to determine a $K_d$-value.

Two more mutants, PARN(E455A) and PARN(W456A) showed a slight decrease in affinity to the cap. Recently, the structure of a region from mouse PARN, mPARN(430-516) was determined by NMR (Fig. 5). The NMR structure revealed that this part of PARN was folded into a classical RRM (reviewed in (164)). In CBP20 the two tyrosines involved in cap binding, Tyr 20 and Tyr 43 are sticking out from the surface built up by two $\alpha$-helices supporting four $\beta$-strands (Fig. 5). When the identified amino acids important for cap binding in PARN were located within the structure of the RRM, they were surprisingly not present on the surface of the pleated $\beta$-sheet but rather outside. Strikingly, there is a His 442 (His 449 in human PARN) present at the corresponding position to where Tyr 43 in CBP20 is located (Fig. 5). In the NMR structure of the RRM of mPARN, a Tyr 505, corresponding to Tyr 512 in human PARN, is present and it is positioned where the inverted 7-Me-guanosine is situated when bound to Tyr 43 in CBP20. Assuming the NMR is correct, there is no space for a guanosine residue to bind to His 442. It is possible that a conformational change may take place upon cap binding that will shift the position of the Tyr 505. Against this suggestion and favoring cap binding to Trp 468 (corresponding to Trp 475 in human PARN) outside the $\beta$-pleated sheet, is the observation that a mutation of His 449 to an alanine does not affect cap binding. PARN(H449A) binds to 7-Me-GTP-Sepharose as PARN (Fig. 4) with an unaffected PARN-cap $K_d$-value of 1 $\mu$M, as determined by fluorescence spectroscopy.

To further characterize this domain of PARN, a sequence corresponding to PARN(443-560) was cloned, expressed and purified and its cap binding properties examined. PARN(443-560) bound to the 7-Me-GTP-Sepharose and interacted with the cap with a $K_d$-value 7 fold higher than PARN, as determined from fluorescence spectroscopy (Fig. 2B and C and Table 1, paper IV and Fig. 4). The two tryptophans, Trp 456 and Trp 475, were substituted with alanines and the resulting PARN mutant polypeptide was assayed for cap binding. As predicted, cap binding of PARN(443-560,W456,475A) was severely affected as revealed by its affinity to 7-Me-GTP-Sepharose and as determined by fluorescence spectroscopy (Fig. 2C and Table 1, paper IV and Fig. 4).

To rule out the possibility that the loss of cap binding was not due to a major conformational change in the PARN(443-560) structure upon introducing the mutations, far-UV CD spectra of PARN(443-560) and PARN(443-560,W456,475A) was performed (Fig. 2F, paper IV). No major change in the recorded spectra was observed, indicating that the introduced mutations did not severely affect the folding of PARN(443-560).
Since the cap stimulates native PARN activity, we investigated the deadenylation properties of recombinant PARN and the cap binding deficient mutant, using capped and non-capped L_3(A_{30}) RNA substrate. Recombinant PARN expressed in *E.coli* shows a less pronounced cap-dependent stimulatory effect compared to native PARN. Nevertheless, a small but significant difference in catalytic efficiency was detected between a capped and a non-capped substrate. A capped substrate was degraded faster than a non-capped. PARN(E455,W456,475A) was active in deadenylation but interestingly, the small cap-stimulatory effect was not observed when using this mutant (Fig. 3, paper IV). The reason as to why recombinant PARN is less sensitive to cap stimulation is not yet known but can be due to a number of reasons *e.g.*, post-translational modifications or correct folding/oligomerization.

![Figure 5. Structures of RRMs from mPARN, hCBP20 and hPABP.](image)

*Figure 5.* Structures of RRMs from mPARN, hCBP20 and hPABP. In the RRM of PARN (upper left panel), Trp 449 and Trp 468 (corresponding to Trp 456 and Trp 475 in human PARN respectively) are highlighted. In the upper right panel His 442 and Tyr 505 (corresponding to His 449 and Tyr 512 in human PARN respectively) are highlighted. In the lower left panel is the crystal structure of CBP20 bound to cap (blue) shown. Tyr 20 and Tyr 43 are highlighted in pink. In the lower right panel is the crystal structure of hPABP bound to A_{11} (blue).
Since the structure of PARN(430-516) showed that this domain is folded into a RRM, we investigated the RNA binding properties of this domain. EMSA was performed using oligo(A) of different lengths and indeed a complex was detected when PARN or PARN(443-560) was incubated with A_{20} (Fig. 5A and C, paper IV). Competition experiments with cold A_{5}, A_{10} and A_{20} showed, as expected, that A_{5} did not compete, A_{10} competed inefficiently, whereas A_{20} efficiently competed the PARN-A_{20} complex as well as the PARN(443-560)-A_{20} complex (Fig. 4A and B, paper IV). Furthermore, since PARN shows high specificity for poly(A), we addressed the issue whether there is specificity for adenosines in the RRM. EMSA was performed where the PARN-A_{20} complex or the PARN(443-560)-A_{20} complex were competed with cold poly(A), poly(G) and poly(C). Poly(A) competed both PARN-A_{20} and PARN(443-560)-A_{20} complexes efficiently while poly(G) competed to some extent and poly(C) to a very little degree (Fig. 4A and B, paper IV). These data show that the RRM harbor, at least some, of the poly(A)-specificity associated with PARN.

The finding that the amino acids important for cap binding i.e. Trp 456 and Trp 475, are not located on the surface of the β-pleated sheet raised the question whether the cap can bind simultaneously as poly(A) binds PARN and if cap binding affects RNA binding or vice versa. To address this question EMSA were performed with the cap binding deficient mutants PARN(E455,W456,475A) and the fragment PARN(443-560,W456,475A). Both mutants were able to form a complex with A_{20} (Fig. 5B and D, paper IV). Further, addition of cap analogue in trans up to a concentration of 500 times the K_{d} for the PARN-cap interaction did not affect PARN-A_{20} or PARN(443-560)-A_{20} complex formation (Fig. 7, paper IV).

A more elaborate study based on the filter binding assay was performed in order to determine the K_{d}-value of the PARN-RNA interaction. PARN binds A_{20} with a K_{d} in the low nanomolar range and a similar K_{d}-value was obtained for PARN(443-560) as well as for the cap binding deficient mutant PARN(443-560, W456,475A) (Table 2, paper IV). To further investigate the RNA length requirement of RNA binding to the RRM, we performed filter binding assays with oligo(A) of increasing length. Interestingly, a fast drop in K_{d} was observed when the oligo(A) was 10 adenosines or longer (Table 2, paper IV). In the crystal structure of the RRM of PABP1 it can be seen that about 8 adenosines fit to the surface of the pleated β-sheet. In the case of PARN it seems that 10 adenosines are required for low nanomolar K_{d}. Furthermore, the addition of cap at a concentration of 50 times K_{d} of the PARN-cap interaction did not affect the RNA binding, as visualized by similar K_{d}-values. This data together with the results of the EMSA suggested that cap binding does not affect RNA binding.

Even though the K_{d}-values are not affected by the presence of cap it could still be possible that the association and dissociation rate constants (k_{a} and k_{d}
respectively) were affected but to the extent that the $K_d$-value would still be the same (since $K_d = k_d/k_a$). Therefore the decay of the PARN-A$_{20}$ and PARN(443-560)-A$_{20}$ complex were followed and the $k_{d,r}$-values, were determined (Table 2, paper IV). Interestingly, the $k_{d,r}$-value of the PARN-A$_{20}$ complex is 4-5 times higher than that of the PARN(443-560)-A$_{20}$ complex. This implies that the kinetics of the RNA binding of PARN is different from the RNA binding to the RRM. In PARN, a R3H domain is present, apart from the nuclease domain, and predicted to bind single stranded nucleic acids. The R3H domain might contribute to the RNA binding in PARN and affect the binding kinetics. Finally, none of the $k_{d,r}$-values were affected by the presence of cap, further supporting a model where cap binding does not affect RNA binding (Table 2, paper IV). Altogether the data indicates that the cap and the RNA binding site of the RRM are structurally and functionally separate. However, PARN is an oligomer and it could be possible that one subunit of PARN binds the cap while another subunit binds RNA.

**Crystal structure of PARN in complex with cap (paper V)**

To gain more insight into how PARN cap binding is mediated at the molecular level, a crystal structure of mPARN(1-515) in complex with 7-Me-GpppG has been solved. The resolution of the structure is 3 Å. A modeled structure, based on the crystal structure of human PARN(1-430) and the crystal structure of mPARN(1-515) bound to cap, contains the nuclease domain, the R3H domain and the C-terminally located RRM (Fig. 1, paper V). The RRM is positioned in close contact with the nuclease domain (Fig. 1, paper V and Fig. 6).

![Figure 6. Schematic representation of a modeled dimeric PARN. A 7-MeGpppG cap analogue is bound to one subunit of PARN in the overlapping cap binding and active site. In the other subunit three adenosines are bound.](image)

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The binding of the cap analogue is mediated by both the nuclease domain and the RRM. The inverted 7-Me-guanosine residue of the cap is stacked by Trp 468 (corresponding to Trp 475 in human PARN), which belongs to the RRM (Fig. 2, paper V). The stacking interaction is almost planar and with a distance of 3.5 Å between Trp 468 and the inverted guanosine residue (Fig. 7). The drastic effect achieved when Trp 475 in human PARN is mutated to an alanine (Table 2, paper V), is easily explained; the important stacking interaction will be removed.

The three phosphate groups of the cap are associated with a number of amino acids through hydrogen bonds. The first transcribed guanosine is recognized by Asn 281 (corresponding to Asn 288 in human PARN). The $K_d$-value of the cap interaction of PARN(N288A) is drastically affected, reflected by a $K_d$-value increased at least 1000 fold (Table 2, paper V).

**Figure 7.** The cap binding site of PARN. Trp 468 and Asn 281 involved in cap binding are shown from the crystal structure of PARN in complex with 7-Me-GpppG.

The most striking property of how PARN cap binding is achieved in PARN, revealed by the co-crystal structure, is the localization of the cap in the active site (Fig. 1, paper V and Fig. 6). A finding that is supported by the fact that several of the amino acids important for cap binding are also found to be involved in the hydrolysis of poly(A) by PARN (Fig. 3, paper V). For example, the mutation of Asn 288 to an alanine reduces PARN activity by 50% and leads to a complete loss of cap binding. Taken together these are very strong indications that determinants for the active site and the cap binding site are the same and that the sites to some extent must overlap. These data together represent a very important step towards understanding the cap-dependent mechanisms of PARN. For example, the inhibition of PARN by cap analogues can be explained by partially overlapping sites. The first obvious explanation to the cap *in trans* inhibitory effects is that cap binding will prevent the substrate from binding. It is important to mention though that the pattern of the inhibition was found to be non-competitive and that
might reflect the fact that PARN is an oligomer and that there are several active and cap binding sites present. It is also very likely that there are conformational changes involved upon cap binding, suggested from the cap \textit{in cis} stimulatory effect as well as the \textit{in trans} stimulatory effect at low concentrations of cap when a non-capped substrate is being hydrolyzed. Therefore the inhibition does not follow a clear competitive pattern that would have been expected if only one subunit and one overlapping active site and cap binding site was present in PARN. Taken together, and in spite of both detailed structural and functional studies, the exact underlying molecular mechanism behind the cap-dependent allosteric effect on PARN still remains unsolved. The story about PARN is likely to continue.

Concluding remarks

In this thesis I have investigated the cap binding property of PARN. It has been demonstrated that the cap has several affects on PARN. The cap stimulates PARN activity and enhances the processivity of PARN mediated hydrolysis. Therefore we conclude that PARN is an allosteric enzyme regulated by the cap. Further, CBC was found to interact with PARN and inhibit PARN activity. The inhibitory property of CBC might be important for NMD. To further characterize cap binding of PARN, I have indentified the cap binding site at a molecular level and found that Trp 475 is crucial for cap binding. A crystal structure of PARN bound to cap could show that Trp 475 binds the 7-Me-guanosine residue though a stacking interaction. Strikingly, the cap is recognized by the RRM together with the nuclease domain and amino acids involved in cap binding are also important for hydrolysis. Therefore, PARN is a unique allosteric enzyme where the regulation is mediated by the cap. That is, the two hallmarks of the mRNA molecule, the cap and the poly(A) tail, are recognized by sites in PARN that are structurally different but based on the same molecular determinants and the site that binds the cap allosterically activates the hydrolysis of poly(A) bound in the active site of the other subunit of PARN.
References

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En organism består av ett stort antal celler. Cellen byggs upp av bland annat proteiner. I generna finns information lagrad om hur proteinerna ska se ut. Det som styr när och hur mycket som skall finnas av ett visst protein regleras av transkriptionen av genen, en process där informationen överförs från genen till mRNA. mRNA fungerar som mall för hur proteinet ska se ut. Genprodukten, det vill säga proteinet, translateras (översätts) sedan från mRNA.

Hur stort uttrycket av en gen i en cell blir beror dels på hur aktivt genen transkriberas, dels på hur stor mängd av mRNA som finns tillgänglig och som kan translateras till protein. Ett viktigt steg i regleringen av genuttrycket är därför kontrollen av nedbrytningen av mRNA. I eukaryota celler initieras nedbrytningen av mRNA av att den i ena änden (3´-änden) placerade poly(A)-svansen bryts ned. En av poly(A)-svansens roller är att skydda mRNA från nedbrytning. När väl ett mRNA skall brytas ned sker detta genom att exonukleaser, enzymer som bryter ned från ändan av en molekyl, attackerar mRNA från 3´-änden och hydrolyserar (bryter ned) poly(A)-svansen.

I den här avhandlingen presenterar jag en studie av ett av de enzymer som bryter ned poly(A)-svansen, nämligen poly(A)-specifikt ribonukleas (PARN). PARN är ett processivt enzym som känner igen poly(A) och bryter ned det. Att PARN är processivt innebär att PARN binder till en mRNA molekyl för att sedan bryta ned hela poly(A) svansen innan PARN binder till nästa mRNA molekyl.

Vidare är PARN en oligomer, det vill säga PARN består av flera likadana subenheter, vilket har bekräftats av tidigare biokemiska och strukturella studier. En viktig egenskap hos PARN som gör enzymet unikt är att PARN inte bara interagerar med den 3´-placerade poly(A) svansen, utan PARN binder också till den i den andra änden (5´-änden) placerade cap-strukturen på ett mRNA. Cap-strukturen består av en guanosin (en av byggstenarna i DNA och RNA) som är metylerad och som via tre fosfatgrupper sitter ihop med den första guanosinen i det transkriberade mRNA:t. En av cap-strukturens uppgifter är att skydda mRNA:t från nedbrytning. I en av studierna presenterade i den här avhandlingen visas att PARN interagerar direkt med cappen och att den interaktionen gör så att PARN aktiveras. Dessutom ökar prosessiviteten hos PARN om det finns en cap närvarande på mRNA:t då PARN bryter ned dess poly(A)-svans. Detta sker antagligen genom att bindningen
inducerar en konformationsförändring (en strukturell förändring) i PARN som gör att hydrolysen blir mer effektiv. Vidare så har jag undersökt hur PARN binder cappen på molekylär nivå och jag har identifierat det cap-bindande sätet i PARN med hjälp av mutationsanalyser. Cap-bindnigen studerades med hjälp av fluorosens spektroskopi och vi kunde visa att tryptofan 475 i PARN var absolut nödvändig för cap-bindningen. En viktig observation som kunde göras var att vid tillsats av fri cap (ej bundet till mRNA) till en PARN reaktion inhihberade PARN.

Ett annat protein som binder till cappen på mRNA är det nukleära cap-bindande komplexet (CBC). CBC är involverat i transporten av mRNA från cellens kärna till cytoplasman. Vi fann att aktiviteten hos PARN inhiberades av CBC och att inhibitionen berodde på en protein-protein interaktion mellan PARN och CBC.

För att ytterligare studera den cap-bindande egenskapen hos PARN, togs en kristallstruktur fram av PARN bundet till cappen. Strukturen visade att tryptofan 475 band till cappen via en så kallad stackningsinteraktion. En mycket intressant observation som kunde göras från kristallstrukturen var att cappen bands till aminosyror som finns i det aktiva sätet. Det vill säga det ställe på enzymet där katalysen äger rum. Alltså är det cap-bindande sätet överlappande med det aktiva sätet och det förklarar varför cappen inhiberar PARN. De strukturella data som tagits fram är ett viktigt led i förståelsen av PARNs mekanism, samt hur cappen aktiverar PARN. Tillsammans bidrar dessa studier till en ökad förståelse för hur mRNA metabolismen i cellen fungerar och därigenom hur genuttrycket regleras.
A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology”.)