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Regulation of Mammalian Poly(A) Polymerase Activity

BY

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ACTA UNIVERSITATIS UPSALIENSIS
UPPSALA 2002

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Dissertation for the Degree of Doctor of Philosophy in Molecular Cell Biology presented at Uppsala University in 2002

ABSTRACT
Thuresson, A-C., 2002. Regulation of Mammalian Poly(A) Polymerase Activity. Acta Universitatis Upsalaensis. *Comprehensive Summaries of Uppala Dissertations from the Faculty of Science and Technology* 770. 42 pp. Uppsala. ISBN 91-554-5455-0.

Poly(A) polymerase (PAP) is the enzyme catalyzing the synthesis of the adenine tail to the 3'-end of mRNA. This A-tail is present on the majority of the primary RNA transcripts of protein-coding genes, and is important for mRNA stability, export to the cytoplasm and translation. Therefore, PAP is a key regulator of eukaryotic gene expression. This thesis describes the heterogeneity of PAP and the functional significance of multiple isoforms of PAP.
PAP exists in many different isoforms generated by three different mechanisms, gene duplication, alternative mRNA processing and post-translational modification. In HeLa cell extracts three different forms of PAP being 90, 100 and 106 kDa in size have been detected, where the 106 kDa isoform is a phosphorylated version of the 100 kDa species. It is shown that the N-terminal region of PAP contains a region required for catalysis, while the C-terminal end is important for the interaction with the cleavage and polyadenylation specificity factor (CPSF). Interestingly, it was found that also the extreme N-terminal end is important for the interaction with CPSF. This region is post-translationally modified by phosphorylation. Five alternatively spliced forms of PAP mRNAs are encoded by the PAPOLA gene while one unique species is encoded by the PAPOLG gene. The analysis showed that the exact structure of the alternatively spliced C-terminal end of PAP played an important role for catalytic efficiency. Thus, the C-terminal end contains a region important for modulating the catalytic efficiency of PAP.
Aminoglycoside antibiotics inhibit PAP activity, most likely by displacement of catalytically important divalent metal ions. Data shows that different aminoglycosides inhibit PAP activity by different mechanisms suggesting that the binding sites for the different aminoglycosides do not completely overlap. It is concluded that aminoglycosides interfere with enzymes important for housekeeping functions in mammalian cell, which may explain some of the toxic side effects caused by aminoglycoside antibiotics in clinical practice.

Keywords: poly(A) polymerase, PAP, polyadenylation, isoforms, phosphorylation, alternative splicing, CPSF interaction, aminoglycosides.

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ISSN 1104-232X
ISBN 91-554-5455-0

Printed in Sweden by Uppsala University, Tryck & Medier, Uppsala 2002

To

Maria, Erik and Anders

"I hope you don't mind, I hope you don't mind

That I put down in words

How wonderful life is while you're in the world"

Your song

- Elton John

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

I. A-C Thuresson, J. Åström, A. Åström, K-O.Grönvik and A. Virtanen. (1994) Multiple forms of poly(A) polymerase in human cells. *Proc. Natl. Acad. Sci. USA.* **91**, 979-983

II. H. Nordvarg, C:B: Kyriakopoulou, A-C. Thuresson, B. Larsson, H. Rodriguez-Martinez and A. Virtanen. (2002) Functional significance of multiple mammalian poly(A) polymerases: Modulation of a carboxyl terminally located regulatory region by alternative mRNA processing. (under revision)

III. A-C. Thuresson, U. Hellman, O. Noerregaard-Jensen and A. Virtanen. (2002) Identification of a non-consensus phosphorylation site in the N-terminal region of human poly(A) polymerase. (manuscript)

IV. A-C. Thuresson, L.A. Kirsebom and A. Virtanen. (2002) Inhibition of poly(A) polymerase by aminoglycosides. (submitted)

SUMMARY OF PAPERS IN THIS THESIS

- In paper I we identified a human poly(A) polymerase. Monoclonal antibodies raised against this recombinant hPAP identified three isoforms of PAP with the molecular weights of 90, 100 and 106 kDa. The 90 kDa form was shown to be exclusively nuclear while the 100 and 106 kDa forms were both nuclear and cytoplasmic. We also identified the 106 kDa PAP as a phosphorylated form of the 100 kDa PAP. In addition we identified the C-terminal of PAP as a region important for the interaction with CPSF, and the N-terminal to be important for catalysis.

- In paper II we identified five alternatively spliced forms of PAP mRNAs encoded by the PAPOLA gene and one unique species encoded by the PAPOLG gene. All the different isoforms of PAP were ubiquitously expressed both at the mRNA and protein level. We also identified a C-terminal regulatory region that modulates the catalytic properties of the different isoforms of PAPα.

- In paper III we identified a novel phosphorylation site located in the N-terminal of PAP. By this we could identify three amino acids probably important for the interaction between PAP and CPSF.

- In paper IV we showed that PAP can be inhibited by aminoglycosides and that the inhibition was most likely caused by displacement of catalytically important metal ions. The interaction was electrostatic. We also found that the binding site for the sisomicin family differed to that of the neomycin and kanamycin families.

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ABBREVIATIONS

A	adenosine
(A)	adenylate
ATP	adenosine tri-phosphate
BPV	bovine papilloma virus
cDNA	complementary DNA
CBC	cap binding complex
CF	cleavage factor
CP	creatine phosphate
CPSF	cleavage and polyadenylation specificity factor
CRM	cyclin recognition motif
CstF	cleavage and stimulatory factor
CTD	carboxyl terminal domain
DNA	deoxyribonucleic acid
DOS	deoxystreptamine
DSE	downstream sequence element
GTP	guanosine tri-phosphate
GVBD	germinal vesicle breakdown
kDa	kilo Dalton
MPF	mitosis promoting factor
mRNA	messenger RNA
NLS	nuclear localisation signal
PABPN1	nuclear poly(A) binding protein 1
PAP	poly(A) polymerase
PLE	poly(A)-limiting element
RNA	ribonucleic acid
RNAPII	RNA polymerase II
TF	transcription factor
UTR	untranslated region
Å	Ångström

THESIS

Regulation of Mammalian Poly(A) Polymerase Activity

Introduction

Mammalian cells consist of many different molecules that are essential for its survival. One of these molecules is the enzyme poly(A) polymerase (PAP), which is important for several different functions inside of the cell. It is therefore important to study the reaction mechanism for PAP, and how the activity of PAP is regulated. This enzyme forms the basis of my research presented in this thesis.

All the genetic information needed for the cell is stored in the cell nucleus as DNA. The DNA in eukaryotes is transcribed into RNA by three different RNA polymerases (RNAP I, II and III). Each RNAP gives rise to a unique class of RNA molecule required for different purposes in the cell. RNAP II transcribes protein-coding genes into pre-mRNA, which is processed in different ways to generate a mature mRNA before it is transported to the cytoplasm. In the cytoplasm the mRNA is translated into a polypeptide chain, that folds into a mature protein (Orphanides, 2002). Transcription, mRNA processing and nucleocytoplasmic transport machineries can be regarded as processes of a *RNA factory*. The processes of the RNA factory are not isolated reactions. In nature they are all highly co-ordinated. The efficiency of one of these processes can be positively or negatively regulated through an interaction with a factor of another processes within the RNA factory (Reviewed in Bentley, 1999; Hirose and Manley, 2000; Minvielle-Sebastia and Keller, 1999; Proudfoot, 2000; Proudfoot, 2002).

Polyadenylation

The pre-mRNA processing machinery is responsible for a series of reactions that includes 5´-end capping, splicing, editing and 3´-end processing. 3´-end processing or polyadenylation occurs as a two-step reaction (Moore and Sharp, 1985), where the first step is an endonucleolytic cleavage of the pre-mRNA at the poly(A) site, and the second is addition of a poly(A) tail to the upstream cleavage product (Barabino and Keller, 1999; Wahle and Ruegsegger, 1999; Zhao et al., 1999). The fragment downstream of the cleavage site is rapidly degraded. This A-tail is present on most eukaryotic mRNAs, with the exception of the histone mRNAs. At least seven different factors are required for these two reactions and the majority of them have been purified to homogeneity. The heart of the polyadenylation machinery, poly(A) polymerase (PAP), is a template-independent polymerase that selectively incorporates AMP to create the adenine-tail at the 3´-end of the mRNA. PAP is also required for efficient cleavage of the pre-mRNA.

PAP activity was originally detected more than 40 years ago in calf thymus extracts (Edmonds and Abrams, 1960; Edmonds and Abrams, 1962) and has later on been

isolated from many different organisms, including eukaryotes, prokaryotes and viruses. Since its discovery PAP has been subjected to extensive biochemical studies and *in vitro* systems for polyadenylation has been developed (Butler and Platt, 1988; Moore and Sharp, 1984). Using these systems it has been possible to identify and characterise a large number of factors needed for polyadenylation.

Polyadenylation is not an exclusively nuclear event even though the factors of the nuclear polyadenylation are the best characterised. In the cytoplasm, mRNA carrying a cytoplasmic polyadenylation signal (CPE) (Fox et al., 1989; McGrew et al., 1989; McGrew and Richter, 1990; Paris and Philippe, 1990; Sallés et al., 1992) can be subjected to cytoplasmic polyadenylation. Moreover, poly(A) metabolism is tightly coupled to translational control (reviewed by (Mendez R, 2001; Richter, 1999)). Translational control has mainly been studied in the early development of different higher eukaryotes. Cytoplasmic polyadenylation has also been found in somatic cells (Groisman et al., 2002; Wu et al., 1998).

Nuclear polyadenylation in mammalian cells

To accurately process pre-mRNA in eukaryotes the polyadenylation machinery require *cis*-acting signal sequences in the 3´ UTR and *trans*-acting factors to act on them (reviewed in (Wahle and Ruegsegger, 1999; Zhao et al., 1999). As previously mentioned, the cleavage reaction is the first step of the 3′-end processing (figure 1A). The assembly of the cleavage complex is absolutely dependent on recognition of the poly(A) signals by the cleavage factors. At the second step, the recognition of the poly(A) signal is necessary for the processivity of PAP (figure 1B). The resulting poly(A) tail at the 3´-end of the mRNA is important for mRNA stability (Beelman and Parker, 1995), efficient export of the mRNA from the nucleus to the cytoplasm (Huang and Carmichael, 1996), and also for efficient initiation of translation (Sachs et al., 1997; Sachs and Varani, 2000). Taking all of this into account the control of poly(A) tail synthesis must be a key regulatory step in eukaryotic gene expression.

Cis-acting elements of the polyadenylation machinery

Three *cis*-acting elements in mammalian cells, i.e. the AAUAAA motif, the poly(A) site and the downstream element, specify and direct the cleavage and polyadenylation reactions (figure 1). In addition to the conserved elements, there are also other sequences, not required for the polyadenylation, that positively or negatively modulate the efficiency of the 3´-end processing.

The highly conserved hexanucleotide sequence, AAUAAA (Montell et al., 1983; Proudfoot, 1986; Sheets et al., 1990; Wickens and Stepenson, 1984; Wilusz et al., 1989; Zarkower et al., 1986), is located 10 to 30 nucleotides upstream of the cleavage site and is essential for both cleavage and polyadenylation (reviewed in (Manley, 1988; Wahle, 1992; Wickens, 1990)). The only variant seen is AUUAAA. Any other mutation will have a severe effect on the reaction (Sheets et al., 1990; Wickens and Stepenson, 1984; Wilusz et al., 1989). The downstream element (DSE) (Chou, 1994; Gil and Proudfoot, 1987; McLauchlan et al., 1985) is located approximately 30 nucleotides downstream of the poly(A) site and is U- or GU-rich in sequence. It can exist as a single U or GU element (Chou, 1994; McDevitt et al., 1986) or as a combination of both (Gil and

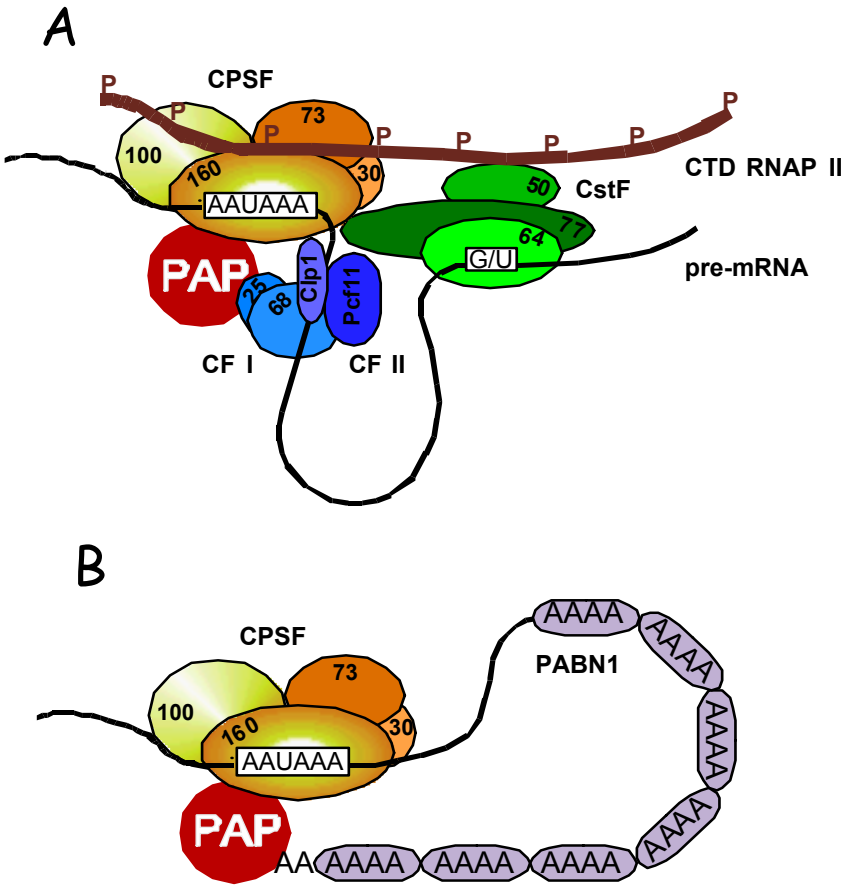

Figure 1. Model showing the cis-acting elements and trans-acting factors of the nuclear 3'-end processing of mammalian cells.
(A) The nuclear cleavage complex. **(B)** The nuclear polyadenylation complex.

Proudfoot, 1987). The distance between the AAUAAA and the DSE is what mainly determines the poly(A) site (Chen et al., 1995; Cherrington and Ganem, 1992; Cherrington et al., 1992; DeZazzo and Imperiale, 1989; DeZazzo et al., 1991; DeZazzo et al., 1992; Lutz and Alwine, 1994; Schek et al., 1992; Sittler et al., 1994; Valsamakis et al., 1991). The sequence at the poly(A) site is not highly conserved although the dinucleotide CA is the most common.

Other sequence elements known to affect the cleavage and polyadenylation exists both upstream and downstream of the three elements that directs the 3´-end processing. An enhancer element located upstream of the AAUAAA has been found primarily in viral poly(A) sites (Bhat, 1987; Brown et al., 1991; Carswell and Alwine, 1989; Cherrington and Ganem, 1992; Cherrington et al., 1992; DeZazzo and Imperiale, 1989; DeZazzo et al., 1991; DeZazzo et al., 1992; Lutz and Alwine, 1994; Prescott, 1994; Prescott and Falck-Pedersen, 1992; Schek et al., 1992; Silver Key, 1997; Sittler et al., 1994; Valsamakis et al., 1991). The poly(A)-limiting element (PLE) is another *cis*-acting sequence located 10-33 bp upstream of the translational stop codon. U2AF 65 binds to the PLE to inhibit the second step of poly(A) addition, resulting in mRNAs with poly(A) tails shorter than 20 nucleotides (Haidong, 1999; Vagner et al., 2000b). Secondary structure elements can also influence polyadenylation efficiency. A GU-rich region downstream of the murine IgM secretory poly(A) site forms a secondary structure that enhances the polyadenylation activity of the secretory poly(A) site (Phillips et al., 1999).

Trans-acting factors of the polyadenylation machinery

The cis-acting elements for polyadenylation are recognised by different trans-acting factors (figure 1). Six factors are essential to perform the reactions; polyadenylation specificity factor (CPSF), cleavage stimulatory factor (CstF), cleavage factor I and II (CF I_m and CF II_m), nuclear poly(A) binding protein (PABPN1) and of course poly(A) polymerase. A seventh factor, the carboxy terminal domain (CTD) of RNA polymerase II, is not essential but is known to have a stimulatory effect on the 3´-end processing.

The cleavage and polyadenylation specificity factor (CPSF) (Christofori and Keller, 1988; Gilmartin and Nevins, 1989; Takagaki et al., 1989) is a multi subunit factor required for both cleavage of the pre-mRNA and for the synthesis of the A-tail to the 3´-end of the mRNA. The mammalian CPSF protein complex consists of four subunits with molecular weights of 160, 100, 70 and 30 kDa. CPSF-160 binds specifically to the hexanucleotide sequence, AAUAAA, independently of any secondary structure (Bardwell et al., 1991; Bienroth et al., 1991; Keller et al., 1991). However, the other subunits of CPSF facilitate the recognition of the AAUAAA motif. The binding of CPSF by itself to the mRNA is weak, but is enhanced by the co-operative interaction with CstF, which is bound to the downstream sequence element (Gilmartin and Nevins, 1991; MacDonald et al., 1994; Weiss et al., 1991; Wilusz et al., 1990). The specific interaction between CPSF-160, CstF-77 and PAP forms a stable complex on the pre-mRNA, which is needed for efficient cleavage and polyadenylation. CPSF-30 has a zinc finger-like motif and can like, CPSF-160, be cross-linked to the

RNA, and has a preference for poly(U) (Barabino et al., 1997). The properties for CPSF-100 and -73 are at present not clarified, but CPSF-100 can be cross-linked to RNA in complex with the other subunits of CPSF. A possible role suggested for CPSF-100 is that it might play a role in RNA-binding (Edwalds-Gilbert and Milcarek, 1995).

The cleavage stimulatory factor (CstF) only participates in the cleavage reaction (Gilmartin and Nevins, 1989; Takagaki et al., 1989) and recognises specifically the GU/U-rich sequence downstream of the poly(A)-site. Three subunits with the molecular weights of 77, 64 and 50 kDa have been identified in HeLa cells (Gilmartin and Nevins, 1991; Takagaki et al., 1990). The three subunits are arranged in a linear fashion with CstF-77 being the bridging subunit (Takagaki and Manley, 1994). As mentioned earlier, CstF-77 strongly interacts with CPSF-160, probably to stabilise the RNA/CPSF complex (Murthy and Manley, 1995). The CstF-64 subunit binds to the downstream GU/U-rich sequence of the pre-mRNA (MacDonald et al., 1994) while CstF-50 can mediate protein-protein interactions (Takagaki and Manley, 1992). *In vitro* experiments revealed an interaction between CstF-50 and CTD of RNAP II (McCracken et al., 1997b). This interaction is interesting as it provides another proof for the coupling between pre-mRNA processing and transcription.

Cleavage factor I (CF I_m) is required for the cleavage reaction. It is a heterodimer consisting of a 25 kDa subunit and a 68 or 59 kDa subunit, where both CF I_m-25-68 and CF I_m-25-59 can be cross-linked to the pre-mRNA (Ruegsegger et al., 1996; Ruegsegger et al., 1998). A fourth subunit of 72 kDa co-purifies with the cleavage activity of CF I_m, but a possible role of that protein has not been clarified. The CF I_m-25 subunit was recently shown to interact with the C-terminal region of mouse PAP (Kim and Lee, 2001). Kinetic analysis suggests that CF I might be one of the first factors of the cleavage complex binding to the RNA and may in this way facilitate the recruitment of the other processing factors (Ruegsegger et al., 1998).

Cleavage factor II (CF II_m), when partially purified from HeLa cell nuclear extracts (de Vries et al., 2000), can be separated into two fractions, CF IIA_m and CF IIB_m, of which only CF IIA_m is essential for cleavage of the pre mRNA. CF IIB_m stimulates the cleavage activity but is not essential for the reaction. The CF IIA_m fraction contains more than 15 different polypeptides. Two of these polypeptides with the molecular weights of 47 and 200 kDa have been identified as the human homologues of yeast Clp I and Pcf 11, respectively. The 47 kDa polypeptide, called hClp I_m, interacts with and bridges CPSF and CF I_m within the cleavage complex. The other polypeptides that co-purified with Clp I_m and Pcf 11_m were CF I_m and several proteins known to participate in the splicing and transcription machinery.

The carboxy terminal domain (CTD) of RNA polymerase II (RNAP II) influence the different RNA processing reactions needed to synthesise a mature mRNA, thus connecting transcription to the pre-mRNA processing (Reviewed in Bentley, 1999; Hirose and Manley, 2000; Minvielle-Sebastia and Keller, 1999; Proudfoot, 2000; Proudfoot, 2002). The mammalian CTD contains 52 repeats of a seven-amino acid sequence element with the consensus YSPTSPS (Dahmus, 1996). The serines at positions 2 and 5 become phosphorylated when the length of the transcript is about 25

bases long (O'Brian, 1994), which would be at the switch from transcriptional initiation to elongation. This phosphorylation event probably releases the initiation complex from the promotor and recruits and activates the capping enzymes. TF IID brings CPSF to the CTD early during the initiation phase of transcription, and at some stage during the initial steps of transcription CstF is recruited to the complex (Dantonel et al., 1997; McCracken et al., 1997b). CPSF and CstF remain associated to the CTD along the elongation phase of transcription. It is believed that the binding of the two factors to the CTD promotes recruitment to the RNA substrate for polyadenylation and thereby enhances the cleavage reaction.

The nuclear poly(A) binding protein 1 (PABPN1) is a 33 kDa polypeptide that together with CPSF is required for the processivity of PAP activity (Bienroth et al., 1993; Wahle, 1991a; Wahle, 1995b). PABN1 is also involved in controlling the length of the A-tail. In mammals the tail is approximately 250 nucleotides long (Brawerman, 1981), compared to around 80 in yeast. The length determination is probably due to stoichiometric binding of the PABPN1 (Wahle, 1995b). Both linear filaments and oligomeric particles of PABPN1 have been detected (Keller et al., 2000). The linear filaments are proposed to be involved in controlling the length of the poly(A) tail, while the oligomeric structures are believed to be involved in the termination of the poly(A) synthesis (E. Wahle, personal communication).

Poly(A) polymerase is a single subunit enzyme that exists in multiple forms, which all have a molecular weight around 80 kDa. The different forms are generated by (i) alternative RNA processing of their C-terminal ends (splicing) (paper II, Zhao and Manley, 1996), (ii) post-translational modifications (phosphorylation) (paper I and III; Colgan et al., 1996; Colgan et al., 1998) but also by (iii) the presence of multiple genes (PAPOLA, PAPOLB and PAPOLG) (paper II; Kashiwabara et al., 2000; Kyriakopoulou et al., 2001; Lee et al., 2000; Perumal et al., 2001; Topalian et al., 2001).

3´-end processing

The 3´-end processing is a two-step reaction, where the first step is the cleavage of the pre-mRNA followed by the polyadenylation reaction.

The cleavage reaction

The cleavage complex (figure 1A) is assembled in a co-operative manner. The complex is formed rapidly *in vitro*. However, the actual cleavage reaction proceeds very slowly (Rüegsegger et al 1998). CPSF and CstF are handed over to the cleavage reaction by the CTD of RNAP II, where they have been associated to *the CTD platform* during the transcriptional elongation (Dantonel et al., 1997; Hirose and Manley, 1998; McCracken et al., 1997b). CPSF-160 and CstF-64 recognise and bind to the hexanucleotide sequence and the downstream GU/U rich element respectively. The binding of the two factors to the pre-mRNA is stabilised by the interaction between CPSF-160 and CstF-77 (Gilmartin and Nevins, 1989; Murthy and Manley, 1992; Murthy and Manley, 1995). CF I_m is thought to bind early in the cleavage complex assembly, and has even been suggested to be the first factor to contact the RNA (Ruegsegger et al., 1998), maybe preparing the RNA for the binding of the other factors required for the cleavage reaction. CF I_m can stabilise the CPSF-RNA interaction and vice versa, but

not the CstF interaction with the RNA (Ruegsegger et al., 1996). PAP is recruited to the cleavage complex by CPSF-160 (Murthy and Manley, 1995) as it has no specificity for the RNA substrate by itself. Even though most components of the cleavage reaction have been purified, no actual candidate has been identified as the endonuclease required for the actual cleavage of the phosphodiesterbond. A metal ion was suggested to be involved in the catalysis since the upstream fragment has a 3´-OH and the downstream fragment a 5´-phosphate (Wahle and Keller, 1996). The presence of the CTD of RNAP II stimulates the cleavage of the pre-mRNA *in vivo* (Hirose and Manley, 1997; Hirose and Manley, 1998; McCracken et al., 1997b). Both the unphosphorylated and the phosphorylated CTD can stimulate the cleavage, although the phosphorylated CTD was more active. *In vivo* studies show that the cleavage is knocked out when the CTD has been truncated (McCracken et al., 1997b). This stimulatory effect of the CTD i*n vitro* might be compensated by the addition of creatine phoshate (CP) (Hirose and Manley, 1998). In this report CP was presented as a necessary cofactor of the *in vitro* polyadenylation and hypothesised to mimic a phosphoprotein naturally recognised by the polyadenylation machinery.

Synthesis of the poly(A) tail

The polyadenylation complex (figure 1B) is, like the cleavage complex, assembled in a co-operative manner. How the transition from cleavage to polyadenylation is achieved is not known. CPSF, often regarded as the central player, probably remains associated to the AAUAAA sequence element during both steps. PAP has very low affinity to RNA and needs the tethering to CPSF to be active (Bienroth et al., 1993). The addition of the first adenines is very slow and distributive, but after the addition of approximately 10-15 adenines PABN1 binds to the complex and the synthesis becomes very fast and processive and proceeds until 200-300 adenines have been added (Wahle, 1995b). Termination of the processive reaction is caused by the disruption of the CPSF-PAP interaction and requires complete coverage of the poly(A) tail by PABN1. The length controlled by PABN1 is measured by stoichiometric binding (Wahle, 1995b).

The reaction mechanism for how the adenines are added to the growing tail is thought to act through a mechanism called two metal ion catalysis (figure 2). This type of reaction mechanism has been proposed for both protein enzymes, and also for a majority of the catalytic RNA molecules. The number of reactions catalysed by enzymes respectively ribozymes is much larger than the actual number of reaction mechanisms used (Steitz and Steitz, 1993).

Many of the polymerases belonging to the family of nucleotidyltransferases react through this two metal ion mechanism where three aspartates or glutamates are positioned in a triad-like configuration to co-ordinate two divalent metal ions (figure 2). The distance between the metal ions has in the crystal structure of different polymerases been measured to 3.9 Å. The metal ions will co-ordinate the ∝-phophorus of the incoming nucleotide and the 3´-OH of the primer. The 3´-OH, activated by metal ion A, makes a nucleophilic in-line attack on the ∝-phophorus of the incoming nucleotide. This result eventually in the breaking of the scissile bond and the nucleotide will be transferred to the primer. The negative charge that builts up on the leaving β-phosphate is stabilised by metal ion B, which also facilitates the leaving of the β- and γ-phosphates (Brautigam and Steitz,

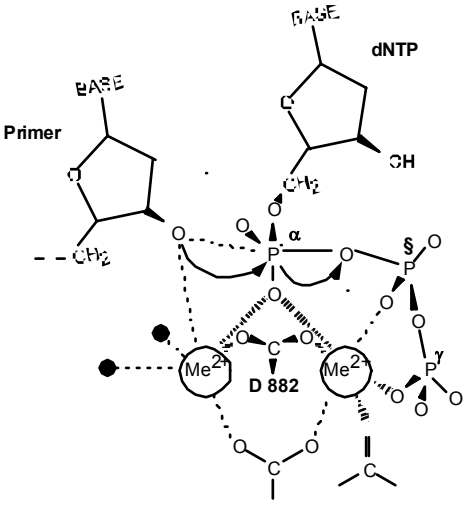

Figure 2. The active site o *E. coli* DNA polymerase I, showing the nucleotidyl transfer and the mechanism of two metal ion catalysis. The black dots (•) indicates water molecules.

1998; Steitz, 1999). PAP most likely uses this type of reaction, where the ∝-phophorus is used by the polymerase in a S_P-configuration. However, experiments done at this stage (Wittmann and Wahle, 1997) have not revealed the exact stereochemistry of the reaction mechanism for PAP.

Crosstalk between polyadenylation and other mRNA processing events

Capping and polyadenylation

The methylated cap structure at the 3´-end of the pre-mRNA is recognised by the cap binding complex (CBC), which is composed of the two proteins CBP20 and CBP80 (Izaurralde, 1995; Izaurralde, 1994; Izaurralde, 1992; Kataoka, 1995; Ohno, 1990). This interaction is important both for splicing (Colot, 1996; Izaurralde, 1994; Lewis, 1996) and export of the RNA from the nucleus to the cytoplasm (Görlich and E., 1996; Izaurralde, 1995). In addition, the presence of CBC appears to enhance the efficiency of the cleavage reaction and to stabilise the CPSF/CstF/RNA complex in HeLa cell nuclear extracts (Flaherty et al., 1997; Lewis and Izaurralde, 1997). This stimulation is, however, only visible with pre-mRNAs having the correct methylated cap structure.

Splicing and polyadenylation

During the last few years there has been an increasing amount of reports on how splicing and polyadenylation reactions affect one another (figure 3). The co-ordination of the two processes was first suggested with the exon definition model (Berget, 1995). In this model splicing factors binding to the 3´ splice site of an upstream intron promotes the binding of splicing factors to the splice site of the downstream intron and *vice versa*. When reaching the very last exon, the poly(A) site replaces the 5´ splice site, so that an interaction between the factors of splicing and polyadenylation defines the last exon. It is also evident that the presence of a 3´ splice site increases the rate of cleavage at a downstream poly(A) site and *vice versa* (Cooke, 1999; Niwa et al., 1990).

The proteins of the splicosomal U1snRNP have been reported to affect 3´-end processing in many different ways, both activating and deactivating. The N-terminal of U1A can interact with CPSF-160 to stabilise its interaction with the hexanucleotide sequence and thereby increase polyadenylation efficiency (Lutz et al., 1996). At higher concentrations of U1A this effect on the polyadenylation is lost and

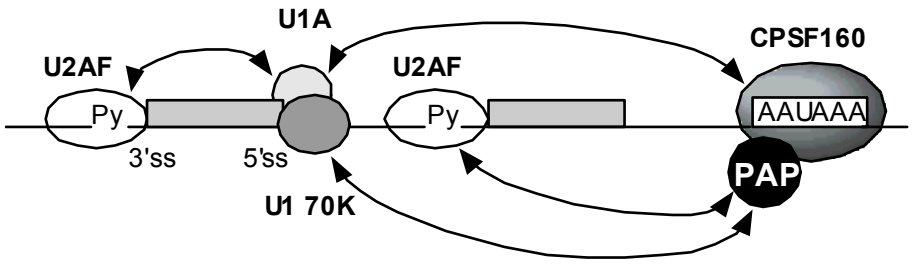

Figure 3. The efficiency of splicing and polyadenylation is regulated through specific interactions between the factors of the two reactions.

an inhibitory effect is observed. This might reflect the inhibitory effect observed when the C-terminal of free U1A protein interacts with PAP to regulate the polyadenylation of its own mRNA (Gunderson et al., 1994; Gunderson et al., 1997; Lutz et al., 1996). The U1 70K protein can interact with poly(A) polymerase to inhibit polyadenylation when bound to a 5´ splice site. U1 snRNP has been reported to inhibit polyadenylation in two mechanistically different ways depending on if it binds to a 5´ splice site upstream or downstream of the cleavage site. The U1 70K binding in bovine papilloma virus (BPV) is an example of how upstream binding directly inhibits PAP activity. Binding downstream of the cleavage site instead inhibits the cleavage reaction, although today the mechanism remains unknown (Vagner et al., 2000a).

U2AF 65 is considered to be involved in the definition of the terminal exon. The interaction observed between poly(A) polymerase and U2AF 65 stimulates the binding of U2AF 65 to the pyrimidine tract within an upstream intron to promote splicing [Vagner, 2000 #1151; EMBO 2002). However, there is also an observation where the binding of U2AF 65 to the pyrimidine tract stimulates 3´-end cleavage (Millevoi, 2002). There also seems to be a physical link to CF II_m, as U2AF 65 co-purifies with the partially purified CF IIA_m (de Vries et al., 2000). Taken together, all these different observations point out the tight coupling between splicing and 3´-end processing. So how does this work out? What comes first, *the chicken or the egg*? Is the cleavage complex formed as a first step to promote the binding of U2AF 65 to the pyrimide tract, so it then can stimulate the actual cleavage? Or do all the different factors gather at the last exon and poly(A) site to then co-operatively act to promote efficient splicing and polyadenylation? This still remains to be investigated.

Termination of transcription and polyadenylation

Termination of the RNAP II transcripts ((Proudfoot, 2002) and references therein) has by the studies made in both yeast and mammalians been suggested to be coupled to 3´-end processing. RNAP II transcription proceeds up to the site of termination before the transcript is cleaved at the poly(A) site. Transcriptional termination is dependent on

the presence of a functional poly(A) signal for termination to occur. The strength of the poly(A) site has direct effects on the efficiency of the termination. Pause sites located downstream of the cleavage site also affects the termination since they enhance the recognition of the poly(A) site. Splicing of the final intron also increases the efficiency of the 3´-end processing and thereby promotes termination.

Poly(A) polymerase (paper I, II, III and IV)

Structure and motifs of mammalian PAP

Several different motifs have been characterised for mammalian poly(A) polymerase (figure 4). The crystal structures of PAP reveal three domains (i) the N-terminal catalytic domain, (ii) the central domain and (iii) the C-terminal RNA-binding domain. PAP belongs to the superfamily of nucleotidyltransferases (Holm and Sander, 1995).

Nucleotidyltransferases is a family of enzymes catalysing the transfer of a nucleotide to an acceptor hydroxyl group. The sequence and structural folds of these enzymes show little similarity, but some common features like a highly conserved catalytic domain with three invariant aspartates (D) or glutamates (E) has been found. A helical turn motif has also been suggested for the nucleotidyltransferases. This motif is suggested to be involved in recognising the triphosphate moiety of the nucleotide (Aravind, 1999; Holm and Sander, 1995; Martin et al., 1999; Martin and Keller, 1996). The catalytic core of PAP consists of both a helical turn motif (aa 101-115) and three aspartates (D113, D115 and D167) positioned in a triad-like configuration. These aspartates co-ordinate the divalent metal ions crucial for catalysis (Bard et al., 2000; Martin and Keller, 1996; Martin et al., 2000). DNA polymerase β and kanamycin nucleotidyl transferase are found in the same family and the crystals structure of the catalytic domain of PAP shows a great structural homology to these two enzymes (Bard et al., 2000; Martin et

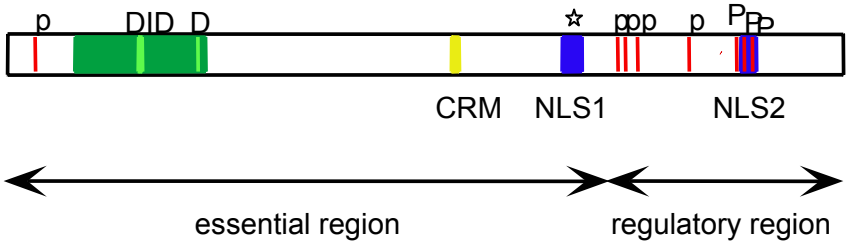

Figure 4. Schematic structure ofhuman poly(A) polymerase II. The green box corresponds to the catalytic domain, with the three invariant aspartates (D) and the helical turn motif. ☆ Indicates the primer binding site. Red bars corresponds to the cdk phosphorylation sites, where P denotes consensus cdk sites and p non-consensus cdk sites.

al., 2000). Superimposition with PAP and either one of the two shows an almost complete overlap of the catalytic site.

Several motifs have been mapped to the C-terminal domain of PAP. A cyclin recognition motif (CRM) was identified between amino acids 396-403. This stretch of amino acids is important for interaction with the G1- and G2-type cyclins of the cell cycle (Bond et al., 2000). Further down the sequence there are two nuclear localisation signals, NLS 1 and NLS 2, located at amino acids 489-508 and 643-659, respectively (Raabe et al., 1994). The studies in paper I suggest both the N- and C-terminal of PAP to contain domains important for PAP activity and CPSF interaction. Mutational analysis revealed amino acids 493 to 662 to be important for the CPSF dependent polyadenylation activity, but did not have any effect on the non-specific polyadenylation. Amino acids 1–662 revealed to be crucial for the catalytic activity of PAP. This was also verified by the studies where the mutation of the basic cluster overlapping with NLS 1 had severe effects on the CPSF dependent polyadenylation (Raabe et al., 1994). Later on, a more extensive mutational analysis proved the region between amino acids 488 to 503 to be the CPSF interacting motif and was suggested for primer-binding (Martin and Keller, 1996). Downstream of NLS1 there is a serine/threonine rich part in which seven cdk phosphorylation sites have been mapped (Colgan et al., 1996; Colgan et al., 1998). These C-terminal phosphorylation sites are involved in the regulation of poly(A) polymerase activity during the M-phase of the cell cycle. In paper III we also describe the mapping of a novel phosphorylation site located at the N-terminal. We believe that this N-terminal site of phosphorylation is involved in a protein-protein interaction with a factor affecting the polyadenylation reaction.

Specificity for ATP

ATP is specifically selected for the synthesis of the tail to the 3´-end (Wahle, 1991b). So why is adenine selected as the incoming nucleotide over guanine, the closest related naturally occurring nucleotide? Adenine and guanine differ in position 2 and 6 (figure 5A). The specificity for ATP comes from the different types of interactions made between PAP and the ATP molecule (figure 5B). A van der Waal binding is made between C2 of the adenine to Val154 and Val156 of the hydrophobic side chain, thus creating a steric hindrance against binding of GTP. In addition, the 6-amino group donates a hydrogen bond to Asp167. As guanine has a keto-group in the same position the directionality of the hydrogen bond would be unfavourable (Martin et al., 2000). A water-mediated contact has been observed between N1 of the adenine and the carbonyl oxygen of Val154.

PAP isoforms

Since isolation of the first clones of bovine PAP, it has been obvious that PAP exists in multiple forms (Raabe et al., 1991; Wahle et al., 1991). Some years later we identified multiple forms of PAP in HeLa cell extracts having the molecular weights of 90, 100 and 106 kDa (paper I). After that several different forms from various species have been identified, all generated by alternative splicing, phosphorylation or by the presence of multiple genes. Three different genes, PAPOLA, PAPOLB and PAPOLG were identified to encode for the human PAPs. Purification of the native isoforms of PAP (Kyriakopoulou et al., 2002) identified the 100 and 106 kDa PAPs (Paper I) as products of the PAPOLA gene. Most likely the 100 kDa PAP consists of all the different full length proteins encoded by the PAPOLA gene (see below), and the 106

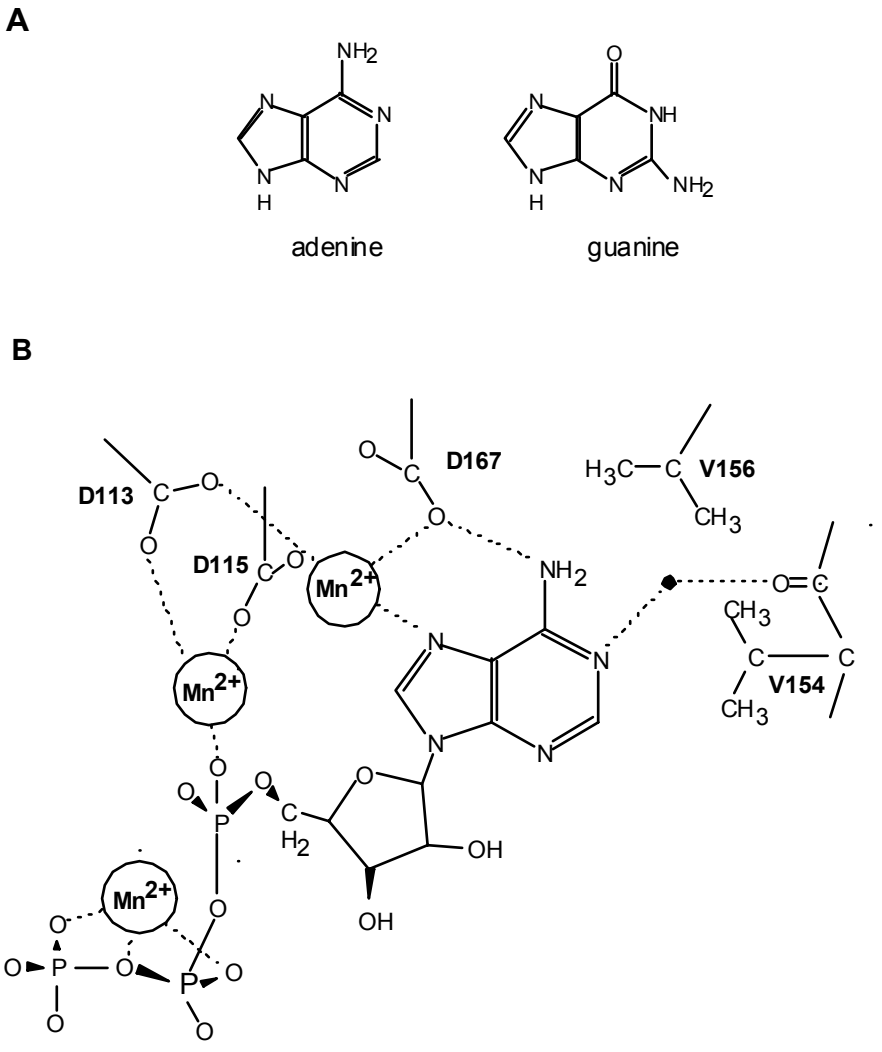

Figure 5. Base discrimination. **(A)** The structures of adenine and guanine. **(B)** Schematic drawing showing the active site of bovine PAP, and how the adenine is recognised. Dotted lines indicate contacts made between the amino acid residues of PAP, ATP molecule and Mn^{2+} ions. The black dot (●) indicate a water molecule. The van der Waal contacts between V154 and V156 to the C2 of the ATP is not shown.

kDa PAP is most likely a phosphorylated form of the 100 kDa PAP. The 90 kDa PAP was identified as a product of the PAPOLG gene (Kyriakopoulou et al., 2002).

The PAPOLA gene will give raise to five different full-length mRNAs, PAP I, II, IV, VIII and IX (figure 6). They all share the first 19 exons, but differ in their 3´-ends, due to alternative splicing of the last three exons 20, 21 and 22 (Paper II). These mRNAs, generated from the PAPOLA gene, can be divided into two classes, type I and II, based on the structure of exon 22. The PAP I-like mRNAs (PAP I and IX) use a proximal splice site in exon 22, which encodes three amino acids. The PAP II-like mRNAs (PAP II, IV and VIII) use a distal splice site in exon 22. The mRNAs using this splice site will encode for 32 amino acids of exon 22 (Paper II). Three truncated forms of PAP (PAP III, V and VI) have also been isolated from a mouse cDNA library (Zhao and Manley, 1996). However, these forms of PAP are inactive in polyadenylation.

The PAPOLB gene encodes for an intron-less testis specific poly(A) polymerase (PAPT) localised to the cytoplasm of spermatogenic cells (Kashiwabara et al., 2000; Lee et al., 2000). PAPT is 84% homologous to PAP II at the amino acid level, where the N-terminal 507 amino acids are much more conserved than the amino acids of the C-terminal. For instance, NLS2 found in PAPα is missing in PAPT and the amino acids of NLS1 differ to such an extent that it is probably not functional (Kashiwabara et al., 2000). The presence of testis specific PAP is interesting, especially as a testis specific 70 kDa variant of the CstF-64 subunit have been identified in meiotic spermatocytes and postmeiotic spermatids (Wallace et al., 1999).

The PAPOLG gene encodes one unique mRNA species (Kyriakopoulou et al., 2001; Perumal et al., 2001; Topalian et al., 2001). There is no evidence for alternative splicing of the PAPOLG gene (Paper II). The distance between the 5´ splice site of exon 21 and the 3´splice site of exon 22 is almost

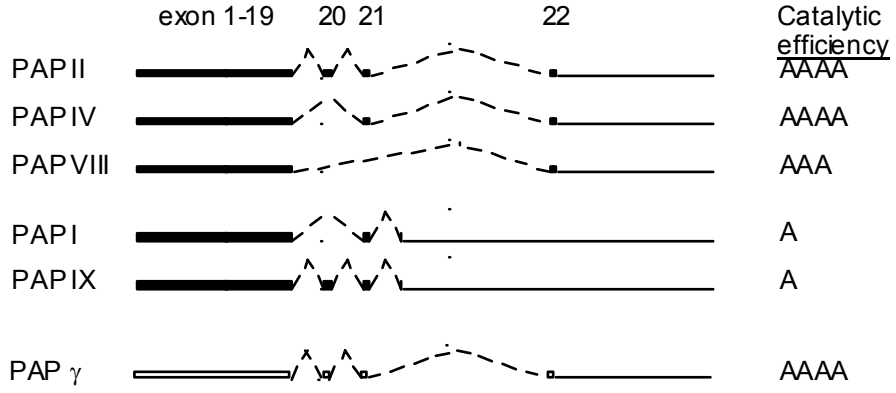

Figure 6. Schematic drawing of the full length mRNAs generated from the PAPOLA and PAPOLG genes. In the list to the right, the numbers of As symbolises the catalytic efficiencies of the different PAP isoforms.

identical to the same region of the PAPOLA gene. This indicates that the PAPOLG gene could generate alternative spliced mRNAs. However, this does not seem to be the case. At the amino acid level PAP γ has an overall identity of approximately 60% compared to PAP II, where the amino acids N-terminally located of NLS1 are more conserved than the amino acids of the C-terminal. Another striking observation is the existence of a putative third NLS (Kyriakopoulou et al., 2001), not present in the PAPs encoded by PAPOLA or PAPOLB gene. Purification of the native isoforms of poly(A) polymerase from HeLa cells extracts, revealed PAPγ as the previously identified 90 kDa form of paper I (Kyriakopoulou et al., 2002).

Expression profile and sub-cellular localisation of PAP isoforms

The expression profiles of the gene products of the PAPOLA and PAPOLG genes (the 90, 100 and 106 kDa isoforms) show that these different PAPs are all present to the same extent in a variety of bovine tissues (Paper II). Based on EST and RT-PCR analysis it is clear that PAP II of the PAPOLA gene is the most abundant mRNA. The gene products of the PAPOLA gene, named PAPα, can be detected in both the nuclear and cytoplasmic fractions of HeLa cells (Paper I). The gene product of the PAPAOLG gene, PAPγ, is unique in that it is exclusively nuclear (Kyriakopoulou et al., 2001). Immunostainings of HeLa cells with antibodies directed against exon 20, 21, 22 or PAPγ (figure 6) confirmed that PAPα localised both in the nucleus and the cytoplasm (paper II) while PAPγ was exclusively present in the nucleus (paper II) (Kyriakopoulou et al., 2001). Immunostainings in the nucleus did not reveal any differences in the localisation between PAPγ and the different isoforms of PAPα. However, all the different PAPs co-localised with nuclear polyadenylation, splicing and transcription factors. In the co-localisation with the basal polyadenylation factor CstF64 or hyperphosphorylated RNAP II a punctuated pattern was detected, which has previously been observed in co-localisation studies between both newly synthesised RNA and CstF64 respectively hyperphosphorylated RNAP II (Schul et al., 1998; Schul et al., 1996; Zeng et al., 1997). In the studies with splicing factors, co-localisation was observed in coiled bodies. These results are in good agreement with all the different studies showing the coupling between different events of the pre-mRNA processing.

Regulation of PAP activity (papers I, II and III)

Protein-protein interactions and phosphorylation are two examples of how the activity of an enzyme can be regulated. Phosphorylation is used to regulate both protein-protein and nucleic acid-protein interactions (Idriss, 1994; Tacke and Y. Chen, 1997; Xiao, 1997). The CTD of RNAP II, for example becomes phosphorylated at the transition from transcriptional initiation to elongation. This in turn regulates the interaction and recruitment of a subset of different proteins.

Regulation of PAP activity through its C-terminal (paper II)

The five different isoforms of PAPα differ in polyadenylation activity (Paper II). These differences can be seen in both the specific CPSF-dependent activity assay and the non-specific polyadenylation activity assay in the presence of Mn(II) ions. The

PAP II-like PAPs are significantly more active than the PAP I-like PAPs. From the determined kinetic parameters (visualised in the right panel in figure 6) in paper II, it is obvious that the exact structure of the C-terminal end plays an important role for the catalytic efficiency and the K_M-parameter. The determined kinetic parameters of the two classes compared to those determined for PAP lacking exon 20 to 22, suggest that the inclusion or exclusion of these exons will regulate the catalytic efficiency of the different PAP isoforms. Inclusion of exon 20 and 21 appear to have an inactivating effect on PAP, which can be overcome by the inclusion of exon 22. PAPγ has a catalytic efficiency in the same range as the PAP II-like PAPs and do also behave in the same way as those in the specific CPSF-dependent activity assay.

It has previously been reported that PAP I, II and γ are equally active polymerases (Raabe et al., 1991; Topalian et al., 2001; Zhao and Manley, 1996). As mentioned above, our results show that PAP I is far less active than PAP II and γ. At the moment we do not have any explanation as to why our results differ from what was previously reported. It is worthwhile to mention that our study is the first to present the determination of the kinetic parameters of different PAP isoforms. Thus, the earlier comparisons were not made at saturating substrate concentrations, which could be the reason for the differences.

Several of the factors of the splicing machinery interact with the C-terminal of PAP to inhibit polyadenylation or to regulate splicing. The U1A protein of the U1 snRNP regulates polyadenylation of its own mRNA by binding two U1A molecules to a regulatory stem-loop element in the U1A pre-mRNA. The resulting complex interacts with the U1A interacting motif found in exon 22 to prevent polyadenylation (Gunderson et al., 1994; Gunderson et al., 1997). As the PAP I-like PAPs do not have this motif they will not be able to be regulated by the U1A protein. Three U1A binding motifs have also been found upstream of the secretory site of the IgM heavy chain (μ) pre-mRNA. Binding of U1A in conjugation with the cleavage and polyadenylation complex inhibits poly(A) addition at the μ secretory poly(A) site (Phillips et al., 2001).

U1 70K, another protein of the U1 snRNP, have been implicated to interact with the C-terminal of PAP. The interaction motif for U1 70K in PAP is similar in sequence to the one found for U1A (Gunderson et al., 1998). In bovine papilloma virus (BPV) this interaction acts to shut off the late gene expression of the viral genes. Upon binding of the U1 snRNP to the splice site sequence located upstream of the late polyadenylation signal, the U1 70K will interact with PAP to inhibit polyadenylation of the 3´-end cleaved RNAs produced from the late BPV genes, which are rapidly degraded. The inhibition of polyadenylation while permitting cleavage can therefore be used as a way to regulate the expression of certain genes.

The specific interaction between the splicing factor U2AF 65 to the C-terminal of PAP can act to stimulate splicing. The direct interaction increases the binding of U2AF 65 to the pyrimidine tract of the 3´ splice site adjacent to the 3´-end formation signal (Vagner et al., 2000b).

The extreme C-terminal region of PAP II has also been reported to interact with CFI_m-(Kim and Lee, 2001). The interaction was mapped to the last 60 amino acids and would therefore include exon 21 and 22.This in turn is another example of where the structure of the C-terminal region of PAP might have a regulatory role for PAP activity. There is, however, no report today

on the functional significance of this protein-protein interaction.

All of the above mentioned interactions regulate PAP activity through an interaction with its C-terminal end. As the exon composition of the PAPαs differ in the C-terminal, some of them will not be able to be regulated through the proteins mentioned here.

Regulation of poly(A) polymerase activity through the N-terminal (paper III)

Earlier reports have by mutational analysis shown that the extreme N-terminal in yeast poly(A) polymerase is responsible for a specific protein-protein interaction. A deletion of the first 18 amino acids in yeast poly(A) polymerase knocks out the specific polyadenylation activity, but has no effect on the non-specific activity (Zhelkovsky et al., 1995). Surprisingly, deletion of the corresponding amino acids in bovine poly(A) polymerase, amino acids 1-31, effects both the specific and non-specific polyadenylation activity (Martin and Keller, 1996). A mutation of three amino acids in the N-terminal of hPAP (figure 7) has effect on the specific polyadenylation activity, but does not affect the catalytic efficiency. This mutation, T23AS24/27A, gives a phenotype synthesising shorter A-tails compared to the wild type PAP (Paper III). In conclusion, our data (paper III) together with the data of bovine PAP (Martin and Keller, 1996)) and the N-terminal deletion of yeast PAP (Zhelkovsky et al., 1995) suggest this domain to be important for the interaction with PAP. A possible explanation for the phenotype of the T23AS24/27A mutant can be that this mutation destabilises the interaction between PAP and CPSF.

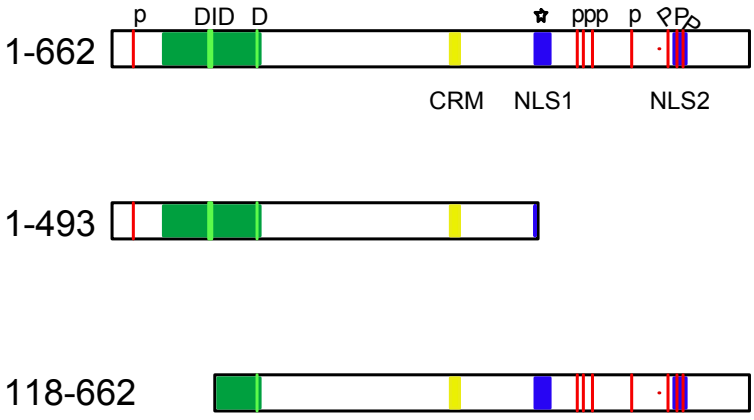

Figure 7. Map of PAP(1-662) (PAP12 in paper I) and the deletion mutants PAP(1-536) respectively PAP(1-50). The vertical lines of the C-terminal indicate the seven cdk sites. TSPIS indicate the novel phospho-rylation site located at aa 23-27 (paper III).

Regulation of poly(A) polymerase through phosphorylation *(papers I and III)*

Poly(A) polymerase is known to regulate its activity through phosphorylation of its serine/threonine-rich C-terminal (figure 4). During the M-phase of the cell cycle the mitosis promoting factor (MPF), composed of cyclin B and cdc2, inactivates PAP by phosphorylation of the seven cdk-sites found between NLS1 and NLS2 (Colgan et al., 1996; Colgan et al., 1998). The kinase of MPF, cdc2, has the optimal phosphorylation site T/SPXK/R. However, the requirement for a basic residue at position +3 can quite often be relaxed (Nigg, 1991). In human PAP three of the cdk-sites are of the consensus type and four of the non-consensus type (Colgan et al., 1998). To completely inactivate poly(A) polymerase all seven sites need to be phosphorylated. The combination of consensus and non-consensus phosphorylation sites is not a unique feature of PAP. It has also been found in lamins (Peter, 1990), non-muscle caldesmon (Yamashiro, 1995) and $p60^{src}$ (Morgan, 1989; Shenoy, 1989). In PAP it was shown that the consensus cdk-sites become phosphorylated at much lower levels of MPF than the non-consensus cdk-sites, and that the phosphorylation of these non-consensus sites followed the activity of MPF during mitosis (Colgan et al., 1998). As the consensus sites are constitutively phosphorylated during mitosis, this could likely be a way for the cell to fine-tune PAP activity during mitosis. The changes in the pattern of phosphorylation of PAP have also been detected in maturing *Xenopus oocytes* (Ballantyne et al., 1995; Colgan et al., 1998), where the pattern of phosphorylation becomes altered after germinal vesicle breakdown (GVBD). PAP is essentially unphosphorylated until GVBD. At the first signs of GVBD, PAP becomes phosphorylated mainly at its consensus sites. It is not until late in the metaphase arrest of meiosis II that PAP becomes fully phosphorylated, which is at the time point where the concentration of MPF is high.

Phosphorylation of the native poly(A) polymerase was already detected in 1994 in HeLa cell nuclear extracts (paper I), and recent kinetic analysis of the homogeneously purified native isoforms of poly(A) polymerase revealed that phosphorylation affects both the specific and non-specific polyadenylation (Kyriakopoulou et al., 2002) (Wittmann and Wahle, 1997).

A phosphorylation site has also been detected in the N-terminal of PAP (figure 4). This site is of the non-consensus cdk type, and can be phosphorylated by cdc2 protein kinase (paper III). This is extremely interesting as this site of phosphorylation overlaps with the previously suggested interaction between CPSF and the N-terminal of PAP (paper III) (Martin and Keller, 1996; Zhelkovsky et al., 1995). One might expect that this phophogroup might enhance the interaction between PAP and CPSF, as CPSF brings PAP into the cleavage complex. Alternatively, it might be that this phosphogroup works to disturb the interaction between the two in the elongation step. As mentioned before, termination of the processive polyadenylation reaction is caused by the disruption of the CPSF-PAP complex (Wahle, 1995a). However, we have not been able to correlate this site of phosphorylation with a function. One hypothesis is that a majority of the 32 N-terminal amino acids are required for the N-terminal interaction between PAP and CPSF.

Poly(A) polymerase as a therapeutic target (paper IV)

Aminoglycosides (figure 8) are a group of small naturally occurring molecules, which today are among the most commonly used antibiotics against bacterial infections (Davies, 1994; Davies, 1997)

Aminoglycoside antibiotics bind to the decoding region A-site of the prokaryotic 16S rRNA to induce codon misreading and inhibit translocation (Brodersen et al., 2000; Davies, 1965; Edelmann, 1977; Fourmy et al., 1996; Moazed and Noller, 1987; Woodcock et al., 1991; Yoshizawa et al., 1998). The binding mechanisms of the aminoglycosides have been extensively studied as it turned out that they also have the potential of inhibiting catalytic RNA (Mikkelsen et al., 1999; Rogers et al., 1996; Stage et al., 1995; von Ahsen et al., 1991; von Ahsen et al., 1992). Inhibition occurs most likely through displacement of catalytically or structurally important divalent metal ions (reviewed in (Schroeder et al., 2000)). This was also recently shown in a crystal structure of yeast $tRNA^{phe}$ in complex with neomycin B, where the binding site of the aminoglycoside overlaps with the binding site of the divalent metal ions (Mikkelsen et al., 2001). There are also biochemical data suggesting that binding of neomycin B competitively displaces the metal ions bound to RNA (Clouet-d'Orval, 1995; Hoch et al., 1998).

The binding of the aminoglycosides to the active site of different RNA molecules is the effect of electrostatic interactions (Stage et al., 1995; Tor et al., 1998; Werstuck et al., 1996; Zapp et al., 1993). At physiological pH the main part of the amino groups of the aminoglycosides are protonated (Botto, 1983; Szilagyi et al., 1993), making them positively charged and thereby attracted by the electronegative binding pockets of the RNA (Hermann and Westhof, 1998). As mentioned before, PAP belongs to the DNA polβ-like nucleotidyltransferase superfamily (Aravind, 1999; Holm and Sander, 1995; Martin and Keller, 1996). Within this family we can also find DNA pol I, DNA pol-β, HIV-I reverse transcriptase and T7 RNA polymerase. The active sites of these enzymes are similar in structure and share the same reaction mechanism, the two-metal ion catalysis (figure 2) (Joyce and Steitz, 1995). In 1998 Herman and Westhof (Hermann and Westhof, 1998) suggested that the negatively charged pockets of the ribozymes also to be present in the active sites of these nucleotidyltransferases. They also showed that the protonated ammonium groups of the aminoglycoside antibiotics that displace the metal ions of the active site of the hammerhead ribozyme are located at distances of around 4 and 8 Å apart. Such distances between magnesium ions are commonly found in the active sites of RNA molecules or protein enzymes like the nucleotidyltransferases (Steitz and Steitz, 1993). In bovine PAP the metal ions are 3.6-3.7 Å apart (Martin et al., 2000). This hypothesis was quite recently supported by the experiments showing that aminoglycosides also have the potential of inhibiting Klenow polymerase (pol), poly(A) specific ribonuclease (PARN) (Ren et al., 2002) and PAP (paper IV). These enzymes that are all dependent on metal ions for their activity. Experiments revealed that the aminoglycoside inhibition is dependent on electrostatic interactions and that the aminoglycosides compete with the metal ions for binding, which is the same mechanism as proposed for the ribozymes (paper IV) (Ren et al., 2002).

When we by kinetic analysis investigated the mechanism for how the aminoglycoside antibiotics inhibit the reaction of polyadenylation, we found that the

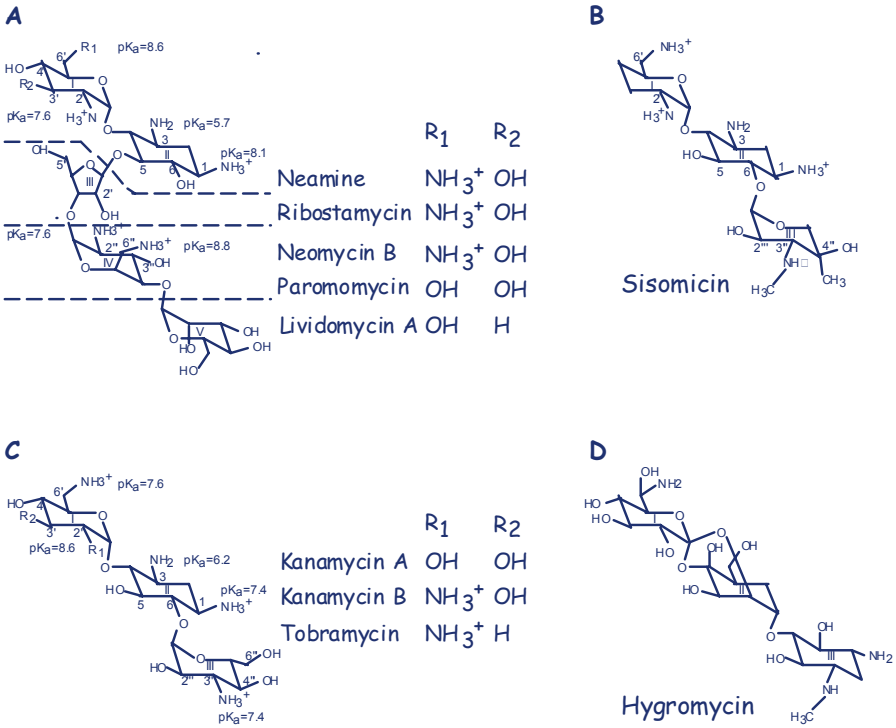

Figure 8. Structural drawings of aminoglycosides showing (**A)** the neomycin family, **(B)** the kanamycin family, **(C)** the sisomicin family and **(D)** hygromycin.

members of the neomycin and kanamycin family were acting as mixed non-competitive inhibitors for both the oligo A_{15}RNA substrate and ATP. This type of inhibition affects both the K_M and V_{max} and indicates that the binding site of these aminoglycosides partially overlaps with the binding sites of the two substrates. To our surprise sisomicin and 5-epi-sisomicin were acting in a competitive manner for the RNA substrate, but in a mixed non-competitive manner for the ATP. This implies that the binding site for the sisomicins overlaps with the RNA primer, but only partially with the ATP. Taken together, the data of aminoglycoside inhibition of PAP suggests that the binding site of sisomicin and 5-epi-sisomicin differ to the binding site of the other aminoglycosides tested.

The kinetic data also revealed the importance of the structure of the aminoglycoside for efficient inhibition. Neamine and ribostamycin (figure 8) are poor inhibitors of the polyadenylation reaction. For efficient inhibition they need to be substituted with the aminosugars of ring III and IV. This is in agreement for what has been suggested for the aminoglycoside interaction with the RNA backbone of the ribosome (Cashman et al., 2001; Hermann and Westhof, 1999; Ma et al., 2002). The obtained data also revealed the importance of the number of protonated ammonium groups for inhibition activity.

The antibiotic effect of the aminoglycosides was discovered more than 50 years ago and is still one of the most widely used compounds of antibiotics. Even though the amount of bacteria acquiring resistance against aminoglycoside antibiotics is increasing (Davies, 1994), and also the increasing amount of evidence of toxic side effects like hearing loss and kidney dysfunction (Forge and Schacht, 2000; Hoffmann et al., 2002), it is still the main compound of the antibiotics. One of the main reasons for this is, of course, the low price of the aminoglycoside antibiotics, but also because there is nothing better to exchange it with. Taking all of this into account, it is important to understand the basis for how, for instance aminoglycosides interact with their targets within the cell of both eukaryotes and prokaryotes to be able to design new rational drugs. This has become even more important especially as the work on PAP, pol and PARN (paper IV) (Ren et al., 2002) proves that aminoglycosides interact with enzymes important for the housekeeping functions of the mammalian cell by the displacement of metal ions. This might also explain some of the toxic side effects of the aminoglycoside antibiotics.

This is of course only a short chapter in the *tale* about polyadenylation and the poly(A) polymerase; the heart of the 3´-end processing. I am sure you will hear more of this exciting story in the future.

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ACKNOWLEDGEMENTS

There are so many incredible persons I had the privilege of meeting during my years at the departments of Cell & Molecular Biology, Medical Genetics & Microbiology. Not mentioned here does not mean that you are forgotten, it is just the limit of space….I would like to thank everybody at these departments, in particular:

★ **Anders Virtanen**. For 12 great years! Thank you for being a great boss during our first years together, and being the best of and most optimistic supervisor during the following years, even your time schedule is optimistic. It was all your belief in me, your support and encouragement that made me take the step to become a graduate student. It was worth it!

❀ **Leif Kirsebom**, always knocking on Anders door, in the middle of a discussion, but I don't mind. Thanks for all the comments and tips you gave me, as my mentor at the basic course of pedagogic. You taught me a lot. Also, it's always fun travelling with you!

❀ **Ulf Petterson** and **Kurt Nordström**, for creating such a nice atmosphere at the MM departments, and also I would like to thank you Ulf, for giving me the opportunity to start my Ph D studies being a technician.

The **poly(A) group**. Many constellations of it have passed through the years, and I wouldn't have liked to miss one of them!

★ The poly(Å) guys: **Anders Åstöm**, an incredible person, who I had the privilege of knowing for a few years. **Jonas Åström,** for the great fun start at the lab and for cold moment with the *thymus-group-prep*. in the cold room!

★ My dear friends, the poly(A) girls: **Helena Nordvarg**, for all the small notes cheering me up, jokes, fun and laughter and for being around. **Christina Kyriakopoulou**, for different types of discussions, support and always giving me time whenever I needed it, even if you were busy. Did you ever catch the last bus? Lab work is easier with the two of you being around!

★ The poly(A) boys: **Yan-Guo Ren**, to be short, I really do like you! Now I also know the meaning of titrating the window! **Javier "**2µl" **Martinez**, I 'll never forget the dialysis-tubing joke! or you counting sunny days of November. The great *new comers* of the group: **Per** "Ulf Lundell" **Nilsson** and **Niklas** "I hurt my eye in the forest again" **Henriksson**. I've enjoyed having you around, sharing the daily work, computer help, cleaning of the lab., but now you have to bother someone else with your questions. Good luck for the future! **Nikos Balatsos**, just two years with us! You got the record this far! What did we do?

★ **Cathy Philips**, we had our fights and arguments, but now a very good friend. Hope to see you again somewhere, as we won't meet at conferences any more.

❀ **Solan**, for everything! **Ulrika, Pernilla, Patricia, Anna-Chey, Monica, Janne, Fredrik, Magnus** for all nice moments over a cup of cooffe, especially during

the time I had to spend at the other side of BMC. **Mattias B**, for all kinds of help, and ok, you got ahead of me! **Karin H**, my *sparring thesis writer*. **Coby**, for splitting things in Madison. **Klas U**, Sorry for the gel! **Susana C**, for all the chats over the lab. bench. **Helene B, Mirjana**, for all the nice chats. **Eva F**, for your kindness and service. **Christer F** and **Joakim B**, for fixing my ideas and pipettes. **Solveig**, my fiddle mate. **Björn**, for hours with computer help. **Santanu, Klas F, Gerhart, Karin C, Dermaid**, and All you people of **Micro**, for creating the nice atmosphere of the department, which meant a lot for me!

❀ **Rigmor, Margit, Birgitta** and **Mervi**, for your incredible service and putting special things aside for me!

❀ **Sigrid P, Christina F, Jeanette B** and **Ehlisabeth S**, for always, with a smile, helping me filling in forms and taking care of all the things around so that I never had to care.

❀ **Cattis Hemström-Nilsson** and **Eileen Bridge**, sharing beer and chips at the Friday meetings, trying to explain *the world of adenovirus* to me. **Anders Aspegren**, **Kai-Uwe Riedel and Hans Johansson** for being the best collaborators in the gene expression group.

❀ *Tanterna* (sorry) **BM C, Elsy, Maria, BM J** and **Lena** for guiding me around and teaching me all the *good thing to know* during the MM-era. **Gunnar W** and **Peter L** for laughter and sharing the early mornings with me. **Agge**, my sparring partner. **Maud Forsberg**, for dinners, laughs and *Fredags maraccas*. **Fredrik S, Staffan S, Lotta I, Hilde E, Helena S, Maggan, Danne** for the sharing the days at MM.

❀ **Margareta Ingelman** and **Hans Eklund**, for the nice collaboration we had for some years. Margareta, for all chats we had by the fermentor.

❀ **Fredrik Österberg**, for help with the picture at the front page, and the aminoglycoside dockings.

❀ **Søren Andersen**, for being friendly and help with sample preparations.

❀ **Ghil Jona**, for the great time we had at the FEBS-EMBO course. Time away from the family didn't feel so bad in your company. Thanks for TFIIH!

★ **Gary**, for reading my thesis and correcting my swinglish.

★ **Ronny**, for healing my body.

❀ **Maggan & Örjan**, for being my oldest and long-time friends.

❀ **Kaarina & Per, Ulla & Anders**, for all the dinners, long walks, *gympa*, Friday beer & drinks in the garden…

♫ **Hans, Cajsa** and **Malin** for letting me enjoy the music in your company.

❀ **Britta & Åke**, för alla de gånger ni ställt upp som barnvakter i sista minuten och all annan hjälp runtomkring.

❤ **Patric & Ulrika,** for being a part of my family, help in the past with the kids, dinners, wedding and party fixing. Still one to go though! Also for sharing **Arvid** & **Oskar** with me! Love you all!

❤ **Mormor**, för att du finns och för alla riktiga sommarlov hos dig som liten. Tycker så mycket om dig!

❤ **Mamma & Pappa**, det är svårt att uttrycka sig vad ni har betytt för mig.

Det enorma stöd, kärlek, hjälp med barnen på alla möjliga sätt och för att ni alltid har varit mina vänner, har betytt mycket för att jag kom så här långt! Jag älskar er!

Sist men inte minst, de viktigaste av alla!

- ♥ **Maria & Erik**, mina solstrålar. Ni är det bästa som hänt mig! Att få älska och älskas av er, är något av det finaste som finns.
- ♥ **Anders**, för alla tokiga ideer som vi hittar på och genomför, för att du finns och är den du är. *Att älskas utav dig, är det vackraste för mig*

.