Activation of the Yeast Exosome Complex

John LaCava

A thesis presented for the degree of

Doctor of Philosophy

The University of Edinburgh

2004

Declaration

I hereby declare that I alone have composed this thesis and that the work presented herein is my own except where stated otherwise.

John LaCava

September 2004

Acknowledgements

Tracing this all the way back to the beginning, I must pay enormous gratitude to my parents, John and Rosanne, for not giving in to the pressures of the teachers and administrators of Lincoln/Sudbury Regional High School, who wanted to see me medicated and shackled because I did not sit still in class; because I did not respond subordinately to arbitrary authority and, oh yes, because I asked questions which did not have answers that could be found in between the chapters of the teacher's edition text book. Although I did not always realise it in those years, looking back I can see that you were always there for me, and I love you for it. I could never forget to thank those who have shared friendship and brotherhood that could never be broken, could not have been more important, and which will never be replaced: Sean, Jesse, Rob, Dave, Abe, Josh, Jeff, Christen, Dan and the rest, you know who you are and I love you all enormously. Love and thanks to Mrs. Tina Ball for helping me out when, again, very few were willing and my parents simply could not. Your investment in me then has been instrumental in my arrival at this place and time.

It is obvious to me that, even with the emotional investment of everyone already mentioned, I could not have arrived here had I not received the attention and tutelage of Dr. Bruce Jackson. You only held me under your wing long enough to let me fly and gave me an opportunity to break the through all expectation. My ultimate thanks to you has yet to come. Thanks to Professor Raymond Rodriguez who gave me the opportunity to mature and to continue to cultivate my research experience during my undergraduate years at UC Davis. Love and thanks to Amanzio and to Yan and Joan Nascimbene who adopted me when I needed a family, you filled me with love and belonging.

I owe enormous thanks to many here in Edinburgh. To Dr. Jenni Connaughton for taking me into her home those first few weeks and treating so warmly, to David and Jaime and Cristina my first friends here and my only friends for quite a while. To Todd and Scott, my brothers in Tullibody, and to Cara, all of whom visited and showed their love, and kept me sane! Thanks to all of my colleagues for their help with advice and reagents, your sharing made this all the

more attainable. Special thanks to Dr. Philip Mitchell, your insights and humour were invaluable. Very special thanks to Dr. Rym Houalla. Rym, everything you touched turned to gold, including my heart, I could not have made it through the final months without your tireless unconditional support, for that and for so many things I will always love you.

And last, but certainly not least, my undying gratitude to my supervisor, Professor David Tollervey. From our first meeting in Davis I had great expectations and anticipation of my time here. Though at times it seemed like a rollercoaster ride, one I might never get off, ultimately I believe it was your approach, to focus on the positive, which helped me to turn this all around and finally have a thesis worth writing. Your positive reinforcement and encouragement when things were more promising served to spur me on to work even more passionately. Thank you for helping me realise my potential. Your demeanour was always one of a friend rather than a superior, something I have always valued greatly about your character, and one reason for which I suspect we will remain friends for years to come.

Thanks again to all; all of my love.

John

Abstract

The exosome complex was first discovered through analysis of the yeast ribosomal RNA processing pathway. This complex consists of 10 core components, all of which are known or predicted to be 3'-5' exoribonucleases. Six of these are homologous to the phosphorolytic bacterial exonuclease RNase PH and one is homologous to the hydrolytic bacterial exonuclease RNase R (an RNase II family member). The remaining three proteins show homology to each other, but not to characterised bacterial enzymes, and one, Rrp4p, shows hydrolytic exonuclease activity *in vitro*. The exosome has been implicated in the processing or degradation of numerous classes of RNA molecule including rRNA, snRNA, snoRNA, tRNA, pre-mRNA and mRNA. It exists as nuclear and cytoplasmic forms and is known to associate with an appropriately compartmentalised set of cofactors. These cofactors are thought to contribute to the robust and accurate activity of the exosome, perhaps conferring substrate specificity and kinetic stimulation.

This study concentrates on functional analysis of the *S. cerevisiae* exosome complex. It is likely that these exoribonucleases are in a complex in order to inhibit potential non-specific activities of the individual components. In this way potent but highly specific RNA degradation machinery is maintained. This model is supported by the lack of free components *in vivo* and the relatively low enzymatic activity *in vitro* of the purified exosome complex.

This study shows how the exosome can be stimulated into a highly effective and processive activity *in vitro* by incubation of purified exosome preparations with an activating complex that contains three proteins Mtr4p, Air2p, and Trf4p. Mtr4p is an essential nuclear RNA helicase previously reported to be required for nuclear exosome activity. Air2p is a RING-type zinc-finger protein. Trf4p is shown to be a poly(A) polymerase. The Mtr4p/Air2p/Trf4p polyadenylation complex (MATPAC) is responsible both for the polyadenylation of target RNAs prior to degradation and the facilitation of the processive exosome mediated degradation. Genetic analyses of *trf4Δ* and *air2Δ* mutations support these *in vitro* findings. In addition to the stimulation of exosome by the MATPAC complex, this study also presents evidence

for a distinct activation mechanism. This involves ADP and inorganic phosphate, strongly indicating that one or more of the phosphorolytic activities have been stimulated. These analyses yield important insights into the activation of RNA turnover by the exosome, and open many future lines of investigation.

Table of Contents

I Introduction

I.A Regulation of Gene Expression

Gene expression in eukaryotes has in recent times been described as a multistage process, whereby DNA in the nucleus is transcribed by RNA polymerase II, the resulting pre-mRNAs are spliced, modified and exported out of the nucleus, and the exported mRNAs are subsequently translated into proteins. The presence of the protein product is the fruit of the gene expression cascade.

In this simplified scheme are transcription and ribosome binding, and hence translation efficiency. It is now known that the cascade of gene expression is a vastly more complex and intricate process. The dawn of the post-genomics era brought the realisation that gene expression was not simply the result of mRNA produced from naked DNA made available en masse for bulk translation. Gene expression is carefully controlled both via epigenetic effects such as chromatin modification, affecting the availability of DNA for transcription, and post-transcriptional regulation at several steps, affecting the functional concentration of transcripts available for translation. It is post-transcriptional gene regulation that is relevant to the focus of this study.

Post-transcriptional gene regulation encompasses methods of control over gene expression which affect the lifespan or processing of the transcript rather than the rate of transcription itself. For example, nascent RNA transcripts that are aberrant or non-functional are targeted for degradation in order to prevent downstream abnormalities likely to arise from a potentially non- or mis-functional RNA or protein; this is referred to as RNA surveillance. Another example of post-transcriptional regulation is the specific elimination or prolongation of normal transcripts in order to affect the functional concentration of the transcript available for translation.

There are numerous species of stable non-coding RNA molecules that are as important as the protein coding mRNA. These include ribosomal (r)RNA, small nuclear (sn) and small nucleolar (sno)RNA, and transfer (t)RNA. These stable RNAs are also subject to regulation through the degradation of aberrant molecules or processing intermediates and the precise trimming of premature RNAs to mature forms.

In any case there is a necessity for efficient and specific RNA catabolism machinery; enter the exosome. The exosome is a multi-component enzymatic complex of 3'-5' exoribonucleases (Mitchell *et al.* 1997), responsible for the major pathway of pre-mRNA turnover in the nucleus (Bousquet-Antonelli *et al.* 2000), and the minor pathway for mRNA turnover in the cytoplasm (Anderson *et al.* 1998). The exosome is also responsible for the degradation and processing of numerous species of stable RNAs (Allmang[a] *et al.* 1999; Kadaba *et al.* 2004).

This study examines the structural, biochemical and functional nature of the exosome complex in order to better understand its role in RNA metabolism, to attempt to fit its as yet unknown form with its emerging functions and to place it within an evolutionary context.

I.B The Ribonucleases of *Saccharomyces cerevisiae*

Though the primary focus of this study is the exosome, a complex heavily implicated throughout a multitude of RNA catabolism reactions, it is not responsible for all RNA degradation and processing in the yeast. It is essential that this complex work in concert with the array of other nucleolytic activities in order to achieve the appropriate profile of post-transcriptional gene regulation. This section describes some of the other important ribonuclease activities. Table 1 lists the ribonucleases covered in this section, along with brief notes on the characterised activity and prominent substrates, and also indicates if a homologous protein exists in *E. coli*.

Table 1
Yeast Ribonucleases Discussed in Section I.B

Nuclease(s)	Activity	*E. coli* homolog
Xrn1p	5'-3' exo: cytoplasmic mRNA turnover	
Rat1p	5'-3' exo: nuclear mRNA turnover, sno & rRNA processing	
Rex1, 2 & 3p	3'-5' exo: rRNA and snRNA processing	RNase D
Pan2p + Pan3p	3'-5' exo: nuclear mRNA poly(A) trimming	RNase D (Pan2p)
Pop2p + Ccr4p	3'-5' exo: cytoplasmic mRNA deadenylation	RNase D (Pop2p) / Exo III (Ccr4p)
Rnt1p	Endo: sn, sno and rRNA processing	RNase III
RNase P	Endo: tRNA processing	RNase P (RNA component)
RNase MRP	Endo: 5.8S rRNA processing	RNase P (RNA component)

I.B.1.2 Phosphorolytic Mechanism

Enzymes such as RNase PH, PNPase and their homologues, which utilise inorganic phosphate and liberate nucleotide di-phosphates feature prominently in RNA degradation in bacteria and yeast. For convenience they shall be referred to here as exoribonucleases despite the technical distinction in the nomenclature.

Following the recent publication of the crystal structure of PNPase from *Streptomyces antibioticus* (Symmons *et al.* 2000), and crystals from the RNase PH of *Aquifex aeolicus* and *Bacillus subtilis* (Harlow *et al.* 2004; Ishii *et al.* 2003), the mechanism of the phosphorolysis is becoming much clearer. A number of conserved amino acid residues have been identified in the PH domain of RNase PH, the active site, and correlated the proposed active site of PNPase. These conserved residues map to a phosphate-binding region in the active site (Harlow *et al.* 2004; Ishii *et al.* 2003; Symmons *et al.* 2000). A less defined site in the *B. subtilis* structure is proposed to be a Mg^{2+} binding site (Harlow *et al.* 2004).

Both studies show RNase PH crystallises as a hexamer, or trimer of dimers more precisely (Harlow *et al.* 2004; Ishii *et al.* 2003)). Harlow *et al.* argue that the dimer is the active form. This is based on a model of proposed interaction of the nuclease with the tRNA secondary structure in the region of the protein that has structural similarity with the protein component of RNase P (βαβ motif (Stams *et al.* 1998)) and shows potential for phosphate binding (Harlow *et al.* 2004). An active RNase PH dimer is also consistent with observations made in *E. coli* that describe the ~25kDa protein purifying as a 45-50kDa fraction upon gel filtration chromatography (Kelly *et al.* 1992).

No explicit determination of mechanistic action is likely without a co-crystal structure that includes bound ssRNA. Only one Mg^{2+} binding site has been proposed for the active sites of both RNase PH and PNPase, residing between aspartic acid residues (Harlow *et al.* 2004; Symmons *et al.* 2000). The requirement for a divalent cation suggests a catalytic mechanism based on two metal ions, with the complication that the attacking nucleophile is a phosphate rather than a hydroxyl ion ((Beese *et al.* 1991; Steitz *et al.* 1993) and reviewed in (Coburn *et al.* 1999)).

The recent publication of the crystal structure of the catalytic domain of Pop2/Caf1p ((Thore *et al.* 2003) also see section I.B.3.2), has lent insights into the nature of the mechanism of RNase D family members, which share the DEDD motif also found in the 3'-5' exonuclease domain of DNA polymerases and members of DEDD superfamily (Zuo *et al.* 2001) including RNase T and oligoribonuclease (Deutscher *et al.* 2001). Therefore it is assumed these enzymes follow the same two metal ions catalytic mechanism (Steitz *et al.* 1993) previously described for the Klenow fragment of DNA polymerase I.

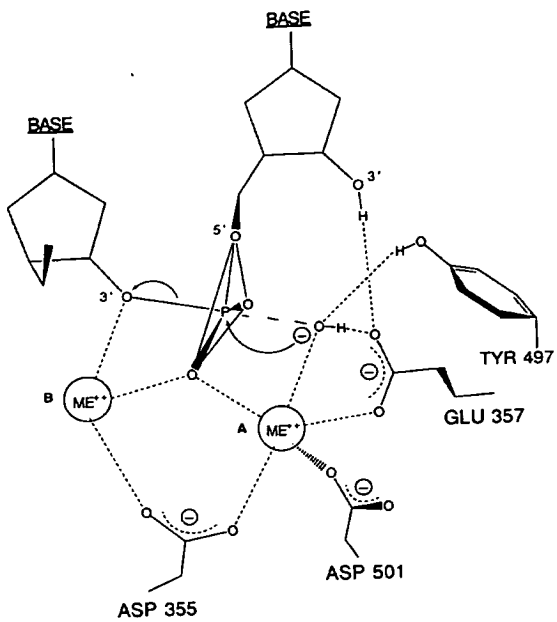


Figure 1
Mechanism of Klenow Fragment Activity
Figure taken from (Beese, 1991)

Two divalent cation exonucleolytic mechanism: the -OH (hydroxyl) anion trapped between the divalent cation at position A, GLU 357 and TYR 497, initiates nucleophilic attack on the phosphorous of the phosphodiester linkage.

There is second known mechanism of hydrolytic 3'-5' exoribonuclease activity that does not require divalent cations. These enzymes derive catalytic activity solely from the amino acid functional groups present in the active site and produce 3' phosphate and 5' hydroxyl products through a 2'-3'-cyclic intermediate. RNase A from bovine pancreas is a well-studied example of such a ribonuclease (Gerlt 1993). None of the exonucleases examined in this study fall into this category.

I.B.1 Nuclease, Phosphodiesterase, Hydrolase or Phosphorylase

Due to ambiguities in the nomenclature it is not always clear what similarities or difference some nucleases share. Endoribonucleases are those that make cleavages at internal sites in the RNA molecule. Conversely, exoribonucleases are those that cleavage starting from the extremities and proceeding inwards, either 5'-3 or 3'-5. Exoribonucleases achieve polymer cleavage through two distinct modes: phosphorolytic and hydrolytic. Phosphorolytic enzymes break down phosphodiester bonds using inorganic phosphate as the attacking group and releasing nucleotide diphosphates. This reaction involves little free energy change and is therefore readily reversible (Grunberg-Manago 1963; Kelly *et al.* 1992); hence phosphorolytic enzymes are also polymerases. Technically speaking phosphorylases are not true nucleases, which are defined as hydrolytic phosphodiesterases, catalysing the breakdown of the phosphodiester linkage between two neighbouring nucleotides and consuming water in the process. Hydrolysis is strongly energetically favourable and is not reversible.

I.B.1.1 Hydrolytic Mechanism

In a known hydrolytic mechanism the hydroxide anion from water is used as an agent of nucleophilic attack upon the phosphorous of the diester linkage. This reaction does not generally require additional cofactors other than two divalent cations. These cations are arranged to facilitate the generation of the hydroxide ion and to stabilise developing negative charges. These details were resolved through the solution of the structure of the 3'-5' exonuclease of the Klenow fragment of *E. coli* DNA polymerase I with ssDNA substrate and deoxynucleoside monophosphate (Beese *et al.* 1991). This structure is conserved in the 3'-5' exonuclease domains of many prokaryotic and eukaryotic DNA polymerases but a similar level of detailed structural information is not yet available for other exonucleases and the data suggests that there is not one canonical mechanism for these enzymes (reviewed in (Mitsis 2001)).

I.B.2 5'-3' exoribonucleases

5'-3' exoribonucleases feature prominently in RNA catabolism reactions in *S. cerevisiae*. Indeed the major pathway for cytoplasmic mRNA turnover in *S. cerevisiae* is carried out by the 5'-3' exoribonuclease Xrn1p ((Hsu *et al.* 1993; Larimer *et al.* 1992; Muhlrad *et al.* 1994) and reviewed in (Caponigro *et al.* 1996)). Rat1p and Xrn1p are homologous 5'-3' exonucleases found in the nucleus and cytoplasm respectively (Amberg *et al.* 1992; Johnson 1997; Larimer *et al.* 1990), which participate in many of the same processing pathways as the exosome. Rat1p participates in the generation of functional rRNAs, snoRNAs (Geerlings *et al.* 2000; Henry *et al.* 1994; Petfalski *et al.* 1998; Qu *et al.* 1999) and in a minor pathway of nuclear pre-mRNA turnover (Bousquet-Antonelli *et al.* 2000).

I.B.3 3'-5' exoribonucleases

There are several 3'-5' exoribonuclease activities in addition to the components of the exosome. The yeast *S. cerevisiae* was predicted to have seventeen 3'-5' exoribonucleases based on genome analysis of models built with *E. coli* enzymes (RNase II, PH and D) (Mian 1997; Moser *et al.* 1997). Add to that Rrp4p and its homologues Rrp40p and Csl4p, which have no *E. coli* homologues and were not previously predicted to be exoribonucleases, and the total number of potential exoribonucleases is twenty. Eleven of these nucleases, including the Rrp4p family, are found in the exosome complex (see table 2, pg. 15). Whilst all other exoribonucleases are individually dispensable, all components of the exosome are essential, with the exception of Rrp6p, which is temperature sensitive (ts)-lethal (Briggs *et al.* 1998).

I.B.3.1 REX proteins

Included among the other activities are the REX proteins (Rex1, 2 and 3p), which, like the exosome component Rrp6p, are RNase D homologues. These proteins are implicated in the 3' processing of 5S and 5.8S rRNA and U4 and U5 snRNAs. These proteins have some overlapping redundancy amongst themselves

and are synthetically lethal with Rrp6p, indicating their participation in an as yet undefined function (van Hoof[b] *et al.* 2000).

I.B.3.2 Poly(A) Nuclease and the CCR4-NOT Complex

An interesting story has unfolded in the identification of the yeast PAN enzyme, the poly(A) binding protein dependant 3'-5' exoribonuclease consisting of the Pan2p and Pan3p proteins (Boeck *et al.* 1996; Brown *et al.* 1996; Brown *et al.* 1998). The nuclease activity was originally attributed to the *PAN1* gene and linked with translation initiation (Sachs *et al.* 1992). This was later corrected and appropriately attributed to the *PAN2* gene product (Allmang[a] *et al.* 1999) and finally turned out to require both the Pan2p and Pan3p proteins (Brown *et al.* 1996; Brown *et al.* 1998) where Pan2p is assumed to be the catalytic unit based on its homology to RNase D (Moser *et al.* 1997). The apparent poly(A) exonuclease activity of the PAN enzyme is non-essential and appears to be involved in the nuclear trimming that results in the species-specific mRNA poly(A)-tail length of 50 – 90 nucleotides. The *pan2/pan3* deletion mutants do not significantly affect mRNA turnover rates but do affect poly(A) tail length and are linked to pre-mRNA 3'-end processing. PAN is not the major cytoplasmic poly(A) exonuclease responsible for poly(A) shortening to oligo(A) length that then results in rapid degradation. Nevertheless, PAN does stabilise a subset of cellular mRNAs approximately two-fold implicating it in post-transcriptional gene regulation (Brown *et al.* 1998).

The major cytoplasmic poly(A) exonuclease activity responsible for deadenylation of mRNA consists of at least two proteins, Pop2/Caf1p and Ccr4p, that also exist in a the CCR4-NOT complex (Chen *et al.* 2002; Collart 2003; Tucker *et al.* 2001; Tucker *et al.* 2002). The CCR4-NOT complex is also implicated in transcriptional regulation (Denis *et al.* 2001). It is unclear if these two functions are strictly linked but defects in some of the *NOT* genes can result in defects in deadenylation (Tucker *et al.* 2002) leading to speculation of a connection between transcription initiation and mRNA turnover. Both Pop2p and Ccr4p have exonucleolytic catalytic domains, Pop2p being an RNase D homolog (Daugeron *et al.* 2001) and Ccr4p being an Exo III homolog (Dlakic 2000). Pop2p was initially characterised as an active exonuclease (Daugeron *et al.* 2001) but it was later shown

that Ccr4p is the catalytic subunit *in vivo* (Tucker *et al.* 2002) in *S. cerevisiae*. The abolition of all detectable cellular deadenylase activity can be achieved in a *ccr4/pan2* double mutant deletion strain (Tucker *et al.* 2002).

I.B.4 Endoribonucleases

Endoribonucleases, while essential for cleavages and processing reactions on the stable RNAs, do not feature heavily in mRNA degradation in *S. cerevisiae*. This is not the case in higher eukaryotes, although the identities of the many endonucleases predicted to be involved in mRNA turnover remain elusive (reviewed in (Tourriere *et al.* 2002)). Notable is the *S. cerevisiae* endoribonuclease Rnt1p (Mead *et al.* 1983), an RNase III homolog required for the proper processing of snRNA, snoRNA and rRNA (Allmang *et al.* 1998; Chanfreau *et al.* 1997; Chanfreau *et al.* 1998; Elela *et al.* 1996; Qu *et al.* 1999). More recently Rnt1p has been implicated in pre-mRNA degradation of two intron containing yeast genes (Danin-Kreiselman *et al.* 2003).

Also notable are RNase P and RNase MRP, two related ribozymes consisting of RNA and accessory proteins (reviewed in (Xiao *et al.* 2002)). RNase P is involved in the 5' maturation of all pre-tRNAs (reviewed in (Hopper *et al.* 2003; Xiao *et al.* 2001)). RNase MRP is implicated in the maturation of the 5.8S rRNA, producing an endonucleolytic cleavage product which is subsequently shortened by the 5'-3' exoribonculease Rat1p ((Henry *et al.* 1994; Schmitt *et al.* 1993) and reviewed in (Fromont-Racine *et al.* 2003; Kressler *et al.* 1999)). The protein component of bacterial RNase P shares some sequence homology with RNase PH homologues (Stams *et al.* 1998; Symmons *et al.* 2000).

I.C The Exosome

Although this study was conducted in *S. cerevisiae* and concentrates specifically on the exosome of this organism, it is important to note that the exosome complex has been found in all eukaryotes examined and is comprised of proteins homologous to the yeast components; among them are *Trypanosoma brucei, Drosophila melanogaster, Arabidopsis thaliana* and humans (Allmang[b] *et al.* 1999;

Andrulis *et al.* 2002; Chekanova *et al.* 2002; Estevez *et al.* 2001). An exosome-like complex has been predicted in archaebacteria on the basis of sequence homology (Evguenieva-Hackenburg *et al.* 2003; Koonin *et al.* 2001).

No orthologous complex exists in eubacteria examined to date, but the exosome does contain many proteins which share homology with the 3'-5' exonucleases of *E. coli*. An exonuclease complex does exist in *E. coli* and is known as the degradosome (Carpousis 2002; Py *et al.* 1996). The degradosome complex, whose major components include the 3'-5' exoribonuclease PNPase, the endoribonuclease RNase E and the RNA helicase RhlB (Carpousis *et al.* 1994; Py *et al.* 1994; Py *et al.* 1996), is responsible for mRNA decay (reviewed in (Coburn *et al.* 1999)).

I.C.1 Exosome Composition

The exosome complex of *S. cerevisiae* is composed of ten core components, all of them shown or predicted to be 3'-5' exoribonucleases. Rrp4p, Rrp41p and Rrp44p have shown exonuclease activity *in vitro* (Mitchell *et al.* 1997). Of these ten core components six are RNase PH homologues: Rrp41-43p, Rrp45p, Rrp46p and Mtr3p (Allmang[b] *et al.* 1999). As such these enzymes contain the PH catalytic domain characterised for the homologous enzymes from bacteria. Of the remaining four components, three are hydrolytic enzymes, Rrp4p, Rrp40p and Csl4p. These proteins have significant homology only unto themselves and each contains S1 and KH RNA binding domains (Allmang[b] *et al.* 1999; Chekanova *et al.* 2002). Finally, Rrp44p is hydrolytic exoribonuclease with homology to RNase R from *E. coli* (Mitchell *et al.* 1997).

Protein	Activity	Motif / Homolog	Note
Rrp4p	Hydrolytic	S1/KH RBD	*in vitro*, distributive
Rrp6p	Hydrolytic	RNase D	*in vitro*, distributive
Rrp40	Hydrolytic	S1/KH RBD	sequence homolgy
Rrp41	Phosphorolytic	RNase PH	*in vitro*, processive
Rrp42	Phosphorolytic	RNase PH	sequence homolgy
Rrp43	Phosphorolytic	RNase PH	sequence homolgy
Rrp44	Hydrolytic	RNase R	*in vitro*, processive
Rrp45	Phosphorolytic	RNase PH	sequence homolgy
Rrp46	Phosphorolytic	RNase PH	sequence homolgy
Csl4p	Hydrolytic	S1/KH RBD	sequence homolgy
Mtr3p	Phosphorolytic	RNase PH	sequence homolgy

Table 2
Exosome Components

The core components of the exosome are stable in a $MgCl_2$ gradient up to 1.6M, with the exception of Rrp44p, which dissociates from the complex at 0.6M (Allmang[b] *et al.* 1999). This reveals the existence of a highly stable nine-component substructure containing six PH domains and three S1/KH domains.

Additionally, there are two population specific exosome components: Rrp6p, a non-essential nuclear specific 3'-5' exoribonuclease with homology to *E. coli* RNase D, shown to have exonuclease activity *in vitro* (Burkard *et al.* 2000) and found in stable association with the exosome up to 1.6M $MgCl_2$, is required for nuclear specific exosome functions in 3' processing (Allmang[a] *et al.* 1999; Bousquet-Antonelli *et al.* 2000; Briggs *et al.* 1998; Torchet *et al.* 2002); and Ski7p, a cytoplasm specific putative GTPase and found in stable association with the exosome is required for exosomal function in cytoplasmic mRNA turnover (Araki *et al.* 2001; Benard *et al.* 1999; Mitchell[a] *et al.* 2003; van Hoof[c] *et al.* 2000). Current estimates by the Tollervey lab based on staining intensity of gel separated protein bands from fractionated exosome, suggest that Rrp6p and Ski7p associated exosome complexes each make up about 15-20%, respectively, of the total cellular exosome pool (Allmang[b] *et al.* 1999; Mitchell[a] *et al.* 2003).

The nuclear exosome has been shown to associate with an additional factor, Rrp47p, which participates in Rrp6p specific exosome functions relating to rRNA, snRNA and snoRNA processing, associates with the exosome is similar proportions to Rrp6p, but has a lower affinity for the complex in a $MgCl_2$ gradient (Mitchell[a] *et al.* 2003).

I.C.1.1 The Core Exosome and Rrp6p

In some cases the exonucleolytic effects of Rrp6p on nuclear RNA processing appear to be distinct but concerted with the core exosome. In the case of 5.8S rRNA synthesis, the core exosome acts prior to Rrp6p in the 3'-5' trimming of the 7S precursor to the mature 5.8S product. Rrp6p null alleles produce a characteristic 5.8S + 30 nucleotides 3'-extended precursor that is both smaller in size than the predominant 3'-extended precursors resulting from core exosome depletion, and which is not present in exosome depletions (Allmang[a] *et al.* 1999; Allmang[b] *et al.* 1999). The degradation of the rRNA 5' external transcribed spacer (5' ETS) is equally affected by depletion of core components or Rrp6p deletion (Allmang[b] *et al.* 1999).

With respect to snoRNA processing, such as U14, U18 and U24 for example, the exosome components are reported to have differential effects on the processing of the 3'-extended precursors. However, the data show the final trimming of the last three nucleotides to yield the mature product is an Rrp6p dependant process with no obvious effects upon depletion of the core exosome, and no additional phenotype in exosome depleted *rrp6*-delta strains. This is in contrast with the processing of snRNAs, such as U4 and U5, which appears to be similarly affected by exosome depletion or *rrp6*-delta mutations, and combinatorial effects are not synergistic. Deletion of Rrp6p also appears to be responsible for a 3' extended poly(A) phenotype in these RNAs ((Allmang[a] *et al.* 1999; van Hoof[a] *et al.* 2000), all reviewed in (Butler 2002)).

The core exosome and Rrp6p also play distinct roles in determining the fate of 3'-extended pre-mRNA. The core exosome is responsible for the trimming of the 3'-extended species to the mature length in an Rrp6p independent fashion, whereas the presence of Rrp6p determines the subsequent fate of the message in a carbon source dependant mechanism (Torchet *et al.* 2002). It is unclear whether Rrp6p functions in an exonucleolytic capacity or is required for a surveillance activity in this pathway or any pathway where Rrp6p and/or core exosome components show similar phenotypes.

I.C.2 Exosome Cofactors

Mtr4p, a putative ATP-dependant DExH-box RNA helicase, is essential and required for nuclear exosome function (Allmang[a] *et al.* 1999; de la Cruz *et al.* 1998; Liang *et al.* 1996; Torchet *et al.* 2002). Although Mtr4p can be detected only at substoichiometric levels by western blot in yeast exosome preparations (P. Mitchell, unpublished data), it readily co-purifies with the human exosome (Chen *et al.* 2001).

The DExH-box helicases are related to DEAD-box helicases homologous to the archetypal eIF4A, a translation initiation factor implicated in the facilitation of 40S ribosomal subunit binding to the 5' region of the mRNA. Its helicase activity is expected to play a role in unwinding secondary structures in the 5' untranslated region (5' UTR). Helicases are involved in various RNA metabolic reactions. Though these enzymes are termed "helicases" it is unlikely that they act in an identical fashion to the processive activity of the extensively studied DNA helicases. (reviewed in (de la Cruz *et al.* 1999; Lorsch[a] *et al.* 1998; Lorsch[b] *et al.* 1998)).

```
motifs.....I.....Ia.....Ib.....Ic.....II.....
DEAD_group.....TGTGKTxxF.....PTRELAxQ.....GG.....TPG.....VxDEAD....
DExH_group.....TGxGKTxxxL.....PxxAL.....T.....VxDExH....

motifs.....III.....IV.....V.....Va.....VI....
DEAD_group....SAT.....VIF.....P.....RGL.....HRxGRxGR.
DExH_group....SAT.....FxxS.....T.....VID.....QRxGRxGR.
```

Figure 2: Shaded letters represent identical residues, normal letters represent conservative substitutions, and x represents variable residues (Alignment from (Jankowsky *et al.* 2000)).

Mtr4p is homologous to the putative helicase Ski2p, which is not essential for viability but is required for cytoplasmic exosome function in 3'-5' mRNA turnover (Araki *et al.* 2001; van Hoof[c] *et al.* 2000). This protein can be isolated in a complex also including Ski3p and Ski8p (Brown *et al.* 2000), which were initially characterised for the superkiller phenotype of the mutant alleles, resulting from increased expression of the protein product of the M "killer" double stranded RNA virus (Rhee *et al.* 1989; Sommer *et al.* 1987; Toh *et al.* 1978).

The viral mRNAs are neither capped nor polyadenylated, hence efficient manifestation of the viral phenotype was detected in *ski* mutants that stabilise these RNAs (M "killer" virus molecular biology reviewed in (Wickner 1996)). This was initially proposed to be due to increased translation of viral mRNAs, but is now generally accepted to be a consequence of decreased degradation. The Ski-complex (Ski2,3 and 8p) and Ski7p are all essential for 3'-5' degradation of mRNA upon prior deadenylation and viral proteins lacking poly(A) tails make efficient targets for turnover. Mutants in these proteins are deficient for 3'-5' mRNA turnover and viral RNAs are stabilised.

The exosome component Csl4p was previously named Ski4p for this same reason. The *ski4-1* mutant allele contains a point mutation in the S1 RNA binding domain of Csl4p. This mutation affects the functions of the exosome in cytoplasmic mRNA turnover without apparent effect to its roles in nuclear RNA processing (Toh *et al.* 1978; van Hoof[c] *et al.* 2000). Similarly, Rrp41p was initially characterised as Ski6p. The *ski6-2* allele confers the superkiller phenotype, but also shows defects in rRNA processing (Benard *et al.* 1998). The 5'-3' exoribonuclease Xrn1p is also Ski1p, and it confers the superkiller phenotype through stabilisation of the uncapped viral mRNAs (Masison *et al.* 1995; Toh *et al.* 1978).

The Ski-complex has been shown to interact with the exosome via the N-terminal domain of the cytoplasmic specific co-factor Ski7p (Araki *et al.* 2001). The Ski complex is not essential under normal conditions but is essential and required for mRNA turnover in the absence of a functional 5'-3' pathway.

I.C.2.1 Exosome Structure

Current research supports the possibility that the exosome has structural similarity to the PNPase of *E. coli* (Aloy *et al.* 2002; Estevez *et al.* 2003; Raijmakers *et al.* 2002; Symmons *et al.* 2002). The PNPase is a homo-trimer of phosphorolytic 3'-5' exoribonucleases whose crystal structure was published in 2000 (Symmons *et al.* 2000). Each subunit has two PH domains and one S1 and KH binding domain. Although the complex has two PH domains, the first domain is highly divergent and not exonucleolytically active (1st-core domain, fig. 3). When compared to the stable nine-component core exosome it appears that the stoichiometry of PH domains and

S1/KH RNA binding domains is conserved between the two complexes. Hence the complexes appear to be linked through evolution. This symmetry is also conserved in the trimer of dimers formed in the RNase PH crystal structures described in section I.B.4.2.

I.C.3 RNase PH and PNPase

The following figure demonstrates an alignment of structural motifs across PNPase/RNase PH like proteins. The PH domain of RNase PH is aligned across the catalytically active 2nd-core domain of the PNPase.

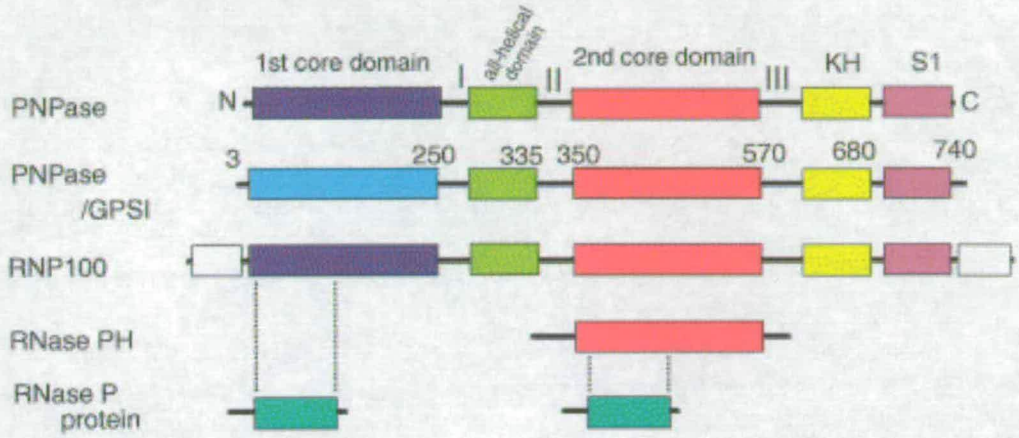


Figure 3: The crystal structure of the PNPase/GPSI enzyme from *S. antibioticus* (This figure taken from (Symmons *et al.* 2000)).

PNPase-like molecules carry S1 (Bycroft *et al.* 1997) and KH (Musco *et al.* 1996; Siomi *et al.* 1994) RNA binding domains (RNA binding domains reviewed in (Burd *et al.* 1994; Messias *et al.* 2004)), the all helical domain and duplicate RNase PH domains, although the amino-terminal (1st-core) PH domain is divergent and not associated with catalytic activity (Symmons *et al.* 2000). It is believed that the PNPase enzyme was derived from gene duplication of an ancient RNase PH like enzyme (Symmons *et al.* 2002). There is no direct evidence of how the substrate gains access to the active site. This could be through direct contact in a distributive mode on smaller substrates, or through RNA binding of the accessory motifs in a more processive fashion on larger ones. Neither mode of action is mutually exclusive. In the processive model involving direct RNA binding, RNA may be

wrapped around the molecule to gain access to the active site in the "lower face"/2nd-core domain (as described in (Symmons *et al.* 2000)), or pass through the central channel (Symmons *et al.* 2002). In this mode of processive action evidence suggests that the PNPase acts on one substrate at a time (Soreq *et al.* 1977), perhaps favouring the "central channel" model over the "wrap around" model, which might grant more leeway for multiple simultaneous degradation reactions.

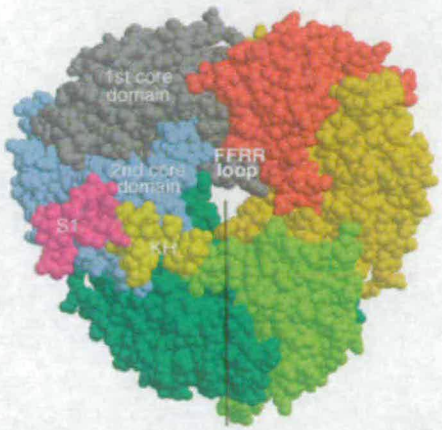


Figure 4: The PNPase (figure from (Symmons *et al.* 2000), on the crystal structure of the PNPase/GPSI enzyme from S. antibioticus)

In defining the essential composition PNPase catalytic site(s) cues can be drawn from the similarities in structural analysis that has been conducted on the RNase PH. This enzyme, despite a somewhat low sequence homology of just 17% to RNase PH, retains high structural super-imposablity across the enzymatic PH domain and a number of conserved amino acid residues known to be common amongst the PDX superfamily. The active site of RNase PH from *B. subtilis* consists of a phosphate-binding site Thr 125 and Arg 126 (corresponding to Thr 462 of PNPase), a conserved Gly 123 (Gly 460 PNPase), and the DX_4EDX_5D motif (PH: D175, E180, D181, D187; PNPase: D508, E513, D514, D520) (Harlow *et al.* 2004; Zuo *et al.* 2001).

A comprehensive mutational analysis carried out on *E. coli* PNPase characterises a number of essential residues required for specific aspects of PNPase catalysis (Jarrige *et al.* 2002). This study does not mutagenise Ser 461, corresponding to Thr462 of the *S. antibioticus* crystal structure (Thr479 from the

Protein Data Bank, http://www.rcsb.org/pdb/index.html, sequence; there is a 17AA discrepancy at the amino-terminus), which is predicted to be the P_i binding site of the catalytically active 2nd-core domain. However, the study does identify a mutation in Arg80 of E. coli PNPase (corresponding to Arg87 in the *S. Antibioticus* crystal) that affects PNPase catalysis differentially with regards to polymerisation, phosphorolysis and phosphate exchange. This arginine residue contacts Tyr404, also conserved in *E. coli,* in the predicted catalytic centre and also interacts with the tungstate binding Thr462. Mutations in Glu190, Gln349 and His403 (213, 372 and 462 respectively in *Symmons et al.* comparative alignment) significantly abolish enzymatic catalysis and His403 corresponds to His427 in the crystal structure and is known to be in direct contact with the tungstate. The other mutations are most likely disruptive to catalysis in the context of structural rearrangement, though only Gln349 appears to cause a loss in quaternary integrity (Jarrige *et al.* 2002; Symmons *et al.* 2000).

The exosome contains six RNase PH homologues, Rrp41-43, 45, 46 and Mtr3p. If these are arranged to accommodate a PNPase-like model, which is also in agreement with the observed hexameric RNase PH quaternary structure, they could provide a ring-like structural scaffolding:

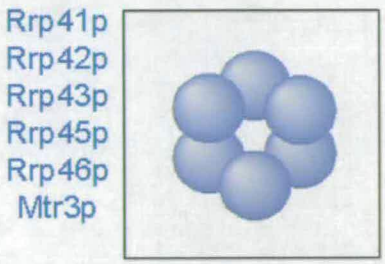


Figure 5
PH ring

I.C.4 The Rrp4p family, RNase R and RNase D

The exosome contains exonucleases from these three families that also represent the breadth of known hydrolytic 3'-5' exoribonucleases from *S. cerevisiae*.

Continuing to build our PNPase-like model, the Rrp4p family of enzymes, including Rrp4, Csl4 and Rrp40p, could be arranged along the upper face of the structural scaffolding:

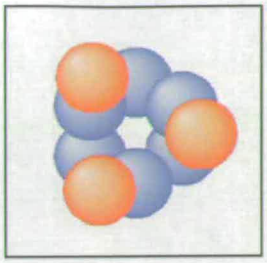


Figure 6
PNPase-like core

Rrp4p/Rrp40p/Csl4p

This arrangement would resemble the arrangement of S1/KH RNA binding domains found in PNPase.

In a departure from a strict protein for protein comparison to the PNPase, the exosome also has two additional exonuclease components, Rrp44p and Rrp6p, which belong to different exonuclease superfamilies RNase R and RNase D respectively.

RNase R homologues, part of the RNR superfamily (Zuo *et al.* 2001), are defined by the presence of an S1 RNA binding domain in the extreme C-terminus (Mian 1997), are maximally stimulated by magnesium and monovalent cations and act in a processive fashion releasing mono-nucleotide 5' phosphates. RNase D, as described earlier (see section I.B.4.1), is part of the DEDD superfamily that also includes proofreading DNA polymerases (reviewed in (Deutscher *et al.* 2001)). Rrp6p, however, is only found to be present in the nuclear exosome of *S. cerevisiae*.

Although these two additional components represent novel components not directly correlated to PNPase it should be noted that the degradosome complex, of which PNPase is a major constituent, also includes an additional nuclease activity in the form of RNase E (Carpousis *et al.* 1994). Furthermore, RNase II an enzyme closely related to RNase R in the RNR superfamily (Zuo *et al.* 2001) accounts for ~90% of total RNase activity in *E. coli* ((Deutscher *et al.* 1991) and review: (Deutscher *et al.* 2001)) and could have been incorporated into the exosome during the evolution of the complex.

Preparations of the yeast exosome have very little activity *in vitro*, suggesting that the complex largely represses the individual activities of components that can show robust activity as recombinantly expressed proteins. Thus the complexing of exosome components is likely to serve a regulatory function with relevance *in vivo*.

In the development of a sophisticated and consolidated system of regulation of cellular exoribonucleolytic activities, yeast may have incorporated the once autonomous RNase II/R-like activity of its prokaryotic ancestors into the exosome in the form of RNase R homolog Rrp44p. Identifying activating co-factors is a major driving force in current exosome research and the focus of this study. This is discussed in detail in sections I.F and I.G (and reviewed in (Mitchell *et al.* 2000)).

I.C.5 Bringing it Together

In a re-assuring return to the PNPase comparative model it should be noted that the ATP dependant DExH-box putative RNA helicase, Mtr4p, is required for exosome activity. This is potentially comparable to the association of the ATP-dependant DEAD-box helicase RhlB with the degradosome (Py *et al.* 1996). Schematically this culmination of elements known to be essential for exosome activity may be depicted as the following:

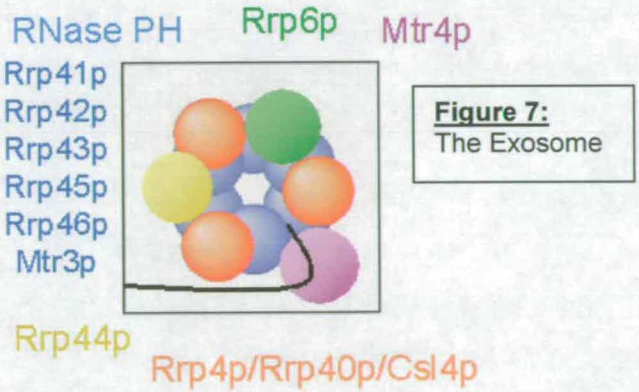


Figure 7:
The Exosome

This model also allows for many potential methods of modular exosome activity. All the PH domains of exosome components may be enzymatically capable. As such this allows for the potential of two distinct modes of phosphorolytic activation of the exosome. One may be a distributive action, whereby RNA substrates gain direct access to PH domains on the "lower" face of the above schematic, which is directly correlated with the 2nd-core domain on the "lower" face of the PNPase arrangement ((Symmons *et al.* 2000) fig. 4). The second mode of action may be processive, in which RNA gains access to internal or external PH

domains via interactions with the RNA binding domains of the Rrp4p family of enzymes arranged across the upper face of the PH ring-like scaffolding (see (Symmons *et al.* 2002) for speculation on PNPase/RNA interactions).

Additionally there remains potential for distributive Rrp4-like or Rrp6p hydrolytic activity and processive Rrp44p activity, all likely to be modulated through interaction with additional cofactors.

I.D Decipher the Structure

Understanding the structure of the exosome is central to determining how its activity is regulated. The exosome can degrade vast quantities of RNA, such as pre-rRNA processing intermediates or aberrant pre-mRNAs (Allmang *et al.* 2000; Allmang[a] *et al.* 1999; Bousquet-Antonelli *et al.* 2000), with such efficiency such that degradation intermediates cannot be detected in wild-type strains. In other contexts, it can be found performing the precise trimming of stable RNAs, resulting in mature 3' end formation (Allmang[a] *et al.* 1999; Torchet *et al.* 2002).

There are currently two models that attempt to address the dilemma regarding exosome structure and activation: proteasome and allosteric (reviewed in (Butler 2002; Mitchell *et al.* 2000; van Hoof *et al.* 1999). In one instance the exosome is likened to the proteasome, where by all of its catalytic active sites are proposed to be directed toward the centre of a barrel like complex, similar to the proteasome protein degradation complex as the name implies. In this way RNAs are directed toward the centre of the complex for inescapable destruction. This would generally be expected to also require the presence of an ATP dependant helicase or translocase to feed the RNA substrate into the mouth of the complex. Mtr4p and Ski2p, ATP dependant putative RNA helicases known to function together with the nuclear and cytoplasmic exosome, respectively, are the obvious candidates for this task.

In the allosteric model the exosome is likened more to a box with many doors. It may still be "globular" or barrel like in shape with active sited pointed toward the centre, but in this model the exosome is able to metabolise RNAs not by feeding them inward but by a conformational change which makes catalytic activity available at the surface of the complex. This model requires additional factors that can stimulate the exosome to adopt one conformation or another depending on the

required activity. This model would be compatible with the proposed PNPase homology.

I.E Exosome Activity

The recurring theme in any examination of the exosome is the breadth and efficiency of its activity. A list of its known functions include the following:

Nuclear

pre-rRNA processing (Allmang[a] *et al.* 1999; Mitchell[a] *et al.* 2003)

pre-rRNA spacer degradation (Allmang[a] *et al.* 1999; Mitchell[a] *et al.* 2003; Zanchin *et al.* 1999)

snRNA processing (Allmang[a] *et al.* 1999; Mitchell[a] *et al.* 2003; van Hoof[a] *et al.* 2000)

snoRNA processing (Allmang[a] *et al.* 1999; Mitchell[a] *et al.* 2003; van Hoof[a] *et al.* 2000)

SRP RNA processing (Grosshans *et al.* 2001)

pre-mRNA degradation (Bousquet-Antonelli *et al.* 2000; Torchet *et al.* 2002)

RNA surveillance (Kadaba *et al.* 2004; Kuai *et al.* 2004; Reed 2003; Torchet *et al.* 2002)

Cytoplasmic

mRNA degradation (Anderson *et al.* 1998; Chen *et al.* 2001; van Hoof[c] *et al.* 2000)

RNA surveillance (Mitchell[b] *et al.* 2003)

Despite the multi-enzyme composition of the exosome and its extensive list of functions, preparations of the yeast exosome show very little *in vitro* activity (Mitchell *et al.* 1996). This is likely due to the repressional constraints of the exosome quaternary structure and the loss of activating co-factors during the purification process (reviewed in (Mitchell *et al.* 2000). In the nucleus there is the protein factor Mtr4p, an essential putative ATP dependant RNA helicase, is required for nuclear exosome activity *in vivo* (Allmang[a] *et al.* 1999; de la Cruz *et al.* 1998). This factor, however, only weakly co-purifies with the yeast exosome, as determined

by Western blot (P. Mitchell, unpublished data). A similar homologous helicase, Ski2p, is required for cytoplasmic exosome activity, along with a complex (the Ski complex), which consists of Ski2p, Ski3p and Ski8p. Ski7p, a putative GTPase also co-purifies with the cytoplasmic exosome (Araki *et al.* 2001).

Hence, understanding the modulation of exosome activity appears to be dependant on identifying those factors that are able to mediate its interaction with the substrate and induce the essential conformational changes (reviewed in (Butler 2002)). This study reports the identification of other factors that appear to be linked to exosome function in the nucleus. Starting with a yeast two-hybrid analysis of Mtr4p, examination of TAP-purified Mtr4p and its ability to stimulate the exosome *in vitro,* we found Trf4p and Air2p as likely interacting partners that make up a complex that targets exosome activity. We have termed this complex the MATPAC for Mtr, Air and Trf PolyAdenylation Complex.

I.F The Role of Polyadenylation in RNA fate

It is now accepted that bacterial mRNAs are subject to polyadenylation and that this is directly related to turnover (Mohanty[a] *et al.* 1999; O'Hara *et al.* 1995). It has also been reported that other types of RNAs can be regulated through polyadenylation, such as RNA I the anti-sense inhibitor of replication of ColE1-type plasmids (Xu *et al.* 1993). More recently stable RNAs were shown to be subjected to polyadenylation, which may serve as a form of RNA surveillance for defective molecules (Gavin *et al.* 2002; Li[b] *et al.* 1998). In short, bacterial RNAs that are polyadenylated are also subjected to degradation.

The necessity for the elongation of RNAs prior to degradation may be simple matter of kinetics. The polyadenylation, or indeed polyuridinylation, of RNA 3' ends make them better substrates for PNPase degradation. This comes down to the enhanced binding of the enzyme to these sequences (Lisitsky *et al.* 1999). Additionally, PNPase is greatly inhibited by RNA secondary structure *in vitro*, however polyadenylation greatly enhances the ability of PNPase to degrade these structured RNAs processively. While ATP dependant RNA-helicase activity from RhlB in the degradosome complex with PNPase, for example, may be able to provide unwinding activity for some such structures, it has also been shown that on other

structures it is inefficient to this end and that polyadenylation and subsequent PNPase digestion is necessary and sufficient. Though it is likely that this increase in capability imparted by repeated rounds of polyadenylation and degradation may simply be the result of offering the enzyme multiple opportunities to bind and hydrolyse, it is still indicative of a level of enzymatic capability not achievable through complementation with traditional helicase activity ((Coburn *et al.* 1998; Xu *et al.* 1995) and reviewed in (Deutscher *et al.* 2001)). Perhaps unsurprisingly, bacterial mRNAs resultant from Rho-independent termination carry stable 3' stem-loop structures that protect them from 3' degradation. In addition to marking the target then, through increased PNPase binding efficiency, polyadenylation may be essential for the kinetics of degradation, allowing the enzyme to reach the heightened level of processivity needed to chew through such structures.

While this is the emerging picture in the prokaryotic world, there has been quite a different picture in the world of eukaryotes. It has long been accepted for that mRNA molecules are polyadenylated in animals (Darnell *et al.* 1971; Edmonds *et al.* 1969; Edmonds *et al.* 1971; Lee *et al.* 1971), plants (Fraser[a] 1975; Ragg *et al.* 1975) and yeast (Fraser[b] 1975; McLaughlin *et al.* 1973). However, in contrast to the prokaryotic system polyadenylation was found to enhance nuclear export, translational efficiency and message stability.

I.F.1 Transcription, Cleavage and Polyadenylation

There are a number of steps involved in mRNA maturation that include the addition of a 5' methylguanosine cap, splicing, and polyadenylation (reviewed in (Kreivi *et al.* 2001; Proudfoot 2000)). Polyadenylation of nascent transcripts is directly linked with the process of cotranscriptional 3' cleavage and is also linked to transcriptional termination. As transcription proceeds, a site 3' of the open reading frame (ORF) is selected by the cleavage and polyadenylation machinery (CPF, cleavage and polyadenylation factor) and transcriptional termination is initiated through interactions with the carboxy-terminal domain (CTD) of RNA polymerase II. In some higher eukaryotes there is the conserved sequence of AAUAAA 20-30nt upstream of the polyadenylation site and a GU rich element (DSE, downstream element) downstream. The cleavage and polyadenylation signals in plants are far

more cryptic, and as with yeast there is no particular canonical sequence. In yeast, however, three general elements can be identified. These are an efficiency element, a positioning element and the poly(A) site. These sequences occur over about 100nt of pre-mRNA sequence. The RNA is endonucleolytically cleaved at the poly(A) site. It has been suggested that Yth1p could be the protein enzyme responsible in yeast (Zarudnaya *et al.* 2002) but this activity has recently been attributed to CPSF-73, the mammalian homolog of the yeast Ysh1p (Ryan *et al.* 2004). The cleaved mRNA is subsequently polyadenylated by Pap1p to an mRNA-specific length. The precise mechanism of this remains elusive. It has been attributed to the initial default production of long tails that are then trimmed by the Pab1p dependant poly(A) nuclease (PAN) in yeast (reviewed in (Proudfoot[b] *et al.* 2002; Wahle[b] *et al.* 1999; Zorio *et al.* 2004) and see section I.B.2.2). Another protein, Nab2p, has also been implicated as essential for proper nuclear poly(A) length control and also subsequent nuclear export (Hector *et al.* 2002; Marfatia *et al.* 2003). Polyadenylated mRNAs are spliced and translocated to the cytoplasm for translation (reviewed in (Reed 2003)).

I.F.2 mRNA Life in the Cytoplasm

Once messages reach the cytoplasm they are subject to translation in a process that is dependant on the 5' cap structure and whose efficiency is synergistically enhanced by the presence of the poly(A)-tail associated with the poly(A)-binding protein (PABP, Pab1p in yeast). In the "closed loop" model for translation the initiation factor eIF4E binds the cap, whereas PABP binds the poly(A) tail. Both proteins interact with eIF4G, which functions in recruitment of the 40S ribosomal subunit.

In contrast to translation, mRNA turnover is dependant on poly(A) shortening and therefore loss of PABP binding. Shortening to approximately 10nt results in subsequent decapping and rapid 5'-3' degradation by Xrn1p (see section I.B.1), or alternatively, further 3'-5' degradation by the exosome (see section I.G). However, cytoplasmic deadenylation, which is carried out by the CCR4-NOT complex (see section I.B.2.2), is a process inhibited by the presence of PABP *in vitro* (in contrast to the activity of the nuclear PAN (Tucker *et al.* 2002)). Thus mRNA lifespan *in*

vivo may be dependant upon PABP dissociation and message specific poly(A) length (the roles of CCR4-NOT in mRNA metabolism are reviewed in (Denis *et al.* 2003) and the roles of PABP in translation and mRNA decay are reviewed in (Kuhn *et al.* 2004)).

I.F.3 Mixed Signals

So far we have discussed the role of polyadenylation in RNA degradation in prokaryotes, and also to the concept of polyadenylation as a means of targeting and protecting eukaryotic mRNAs. As it turns out, polyadenylation does not only serve to protect eukaryotic messages.

It has been shown that polyadenylated species of stable RNAs accumulate in yeast mutants of the exosome component Rrp6p (Allmang[a] *et al.* 1999; Kuai *et al.* 2004; van Hoof[a] *et al.* 2000), or when 3' end processing is accomplished through non-standard means (Duvel *et al.* 2003). While the function of polyadenylation of the target RNAs described in these studies has not been explicitly determined, polyadenylation has been shown to be directly linked to the RNA surveillance-based turnover of defective tRNA (Kadaba *et al.* 2004).

mRNA that has been retained in the nucleus due to export defects or improper 3' end recognition has been shown to become hyper-adenylated (Hector *et al.* 2002; Hilleren[b] *et al.* 2001; Jensen *et al.* 2001), and the retention and degradation of these transcripts is linked to the exosome (Hilleren[a] *et al.* 2001). This suggests a hyper-adenylation as mechanism for tagging mRNA for turnover when defective or not competent for export.

I.G The Aim of This Project

This work was carried out with the intention of stimulating the *in vitro* activity of the exosome. The objective: to increase our understanding of the nature of RNA metabolism. The exosome lies at the very heart of RNA processing and degradation pathways. To identify factors that are able to stimulate enzymatic activity or differential processivity and specificity of the exosome is to peer ever closer into the mechanisms of its action at the centre of RNA metabolism.

An *in vitro* analysis is advantageous because it allows the identification of specific activating elements in the absence of background noise and cross-protein redundancy. These analyses also pave the way for an informed genetic approach to confirm *in vivo* to what extent the activity of the exosome relies upon the stimulating co-factors identified *in vitro*.

Two approaches have been used. One reconstitution method that combined affinity-purified exosome with affinity purified candidate protein co-factors, and another biochemical approach that sought to probe potential enzymatic similarities between the PNPase and the exosome.

This study will present the identification of three additional exosome co-factors and two new complexes implicated in the function of the exosome in nuclear RNA turnover. It will also be demonstrated that the exosome can be biochemically unlocked into different modes of stimulated exonuclease activity.

II Materials and Methods

II.A Materials

Restriction enzymes and other modifying enzymes were obtained from New England Biolabs (NEB), Roche, Promega, Sigma and Stratagene. Standard laboratory reagents were purchased mainly from Sigma; other suppliers were Gibco BRL, Difco, Bio-Rad, and Fluka. Reagents obtained from specific sources are indicated.

II.B Culture media

E. coli cultures were grown in Luria-Bertani medium (LB) supplemented with ampicillin to 50μg/ml where needed for selective pressure in the propagation of plasmids.

Standard S. cerevisiae growth and handling techniques were employed (Sherman 1986). *S. cerevisiae* strains were grown either in rich (YPD) or in minimal media (SD).

To make agar plates, bacto-agar was to 2% (w/v) prior to autoclaving at 15lb/sq.inch for 20 minutes in a liquid cycle.

Bioreactor cultures of *S. Cerevisiae* were carried out using standard YPD medium.

II.C Bacterial Strains

E. coli strain XL1-Blue was used for propagation of all plasmids. This strain is recombination deficient (*rec*A) and foreign DNA is protected from host restriction and recombination (*hsd*R *end*A).

II.D Yeast Strains

The yeast strains used in this study are shown in table 1.

Table 1: Yeast strains

Strains *S. Cerevisiae*	Genotype	Reference
P51	MATa *ade2-1 his3-11 leu2-3 trp1-1 ura3-52 rrp4p-Δ + [pRS415/RRP4]*	(Mitchell *et al.* 1997)
SC1157	MATa *ade2 arg4 leu2-3, 112 trp1-289 ura3-52 MTR4::TAP*	(Gavin *et al.* 2002)
SC1415	MATa *ade2 arg4 leu2-3, 112 trp1-289 ura3-52 CSL4::TAP*	(Gavin *et al.* 2002)
SC1449	MATa *ade2 arg4 leu2-3, 112 trp1-289 ura3-52 DIS3::TAP*	(Gavin *et al.* 2002)
SNU66-TAP	MATα *hisΔ200 leu2-3, 112 ura3-1 trp1Δ1 ade2-1 can1-100 SNU66::TAP::TRP*	Unpublished, Jean Beggs
SC1652	MATa *ade2 arg4 leu2-3, 112 trp1-289 ura3-52 RRP45::TAP*	(Gavin *et al.* 2002)
YSC1178-7499673	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 air2::TAP*	(Ghaemmaghami S *et al.* 2003)
YSC1178-7502709	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trf4::TAP*	(Ghaemmaghami S *et al.* 2003)
YSC1178-7502549	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trf5::TAP*	(Ghaemmaghami S *et al.* 2003)

II.E Radiolabelled Compounds

All radionuclides were purchased from Amersham (UK): {γ-^{32}P} ATP (6000Ci/mmol) and {α-^{32}P} ATP (3000Ci/mmol). {α-^{32}P} ADP was produced in a

reaction using {α-^{32}P} ATP and hexokinase (Sigma H 5000). Reaction conditions as follows: 20mM Tris pH 7.6, 5mM MgCl2, 100mM glucose, 10μCi {α-^{32}P} ATP and 2mg/ml Hexokinase; 50μl final volume, incubated for 10min at 30°C. Analysis by TLC shows no residual ATP after the full incubation time.

II.F Plasmids

The plasmids used in this study are listed in table 2.

Table 2: Plasmids

Plasmid	Reference/ Remarks
pBS +/- (Bluescript)	Stratagene
pSP64-supSI	(Krupp *et al.* 1985)

II.G Antibodies

The antibodies used in this study are listed in table 3.

Table 3: Antibodies

Antibody	Description	Supplier
Peroxidase-anti-Peroxidase (PAP)	rabbit IgG	Sigma, UK
anti-mouse IgG	anti-mouse IgG conjugated to horseradish peroxidase, secondary	Amersham, UK
anti-rabbit IgG	anti-rabbit IgG conjugated to horseradish peroxidase, secondary	Amersham, UK
anti-Mtr4p	rabbit IgG, primary	(de la Cruz *et al.* 1998)
anti-Nop1p	mouse IgG, primary, monoclonal	(Wu *et al.* 1998)

II.H Bacterial techniques

Standard bacteriological techniques were carried out according to Sambrook *et al.*, 2001. This includes the preparation of electro-competent cells and electrotransformation.

II.I Recombinant DNA techniques

Standard recombinant DNA techniques were carried out according to Sambrook *et al.*, 2001. This includes agarose gel electrophoresis of DNA, ethidium bromide staining, PCR, phenol/chloroform extraction and alcohol precipitation.

II.I.1 Restriction enzyme digests

Restriction digests were performed in total volumes of 20-50µl, using variable quantities of DNA and appropriate amounts of enzyme in the supplied buffer [1:10] and BSA (bovine serum albumin) [1:100] if required. The buffer used was the one most suitable for the particular digest, according to the manufacturer's instructions. Digests were incubated at optimal temperature for 1-2 hours and then analysed by agarose gel electrophoresis with appropriate molecular weight markers.

II.I.2 Plasmid preparation

Small and medium scale preparations of DNA were performed using the Qiagen plasmid preparation kit and following manufacturer's instructions for mini/midi preparations.

II.I.3 DNA sequencing

All sequencing reactions were performed on double stranded plasmid DNA using the Amersham DYEnamic ET Terminator Cycle Sequencing Kit and following

the manufacturer's instructions. Sequencing reactions were subsequently analysed at the ABI PRISM facility in ICAPB, Edinburgh University.

II.I.4 Polymerase Chain Reaction

PCR was used to amplify regions of DNA plasmids and regions of the yeast genome for cloning, to produce recombinant constructs and to generate expression cassettes for *in vitro* transcription/translation. All applications followed the general protocol described below or modifications upon it.

PCR reactions in a volume of 50µl contained 1.5mM magnesium chloride, 0.2mM of each dNTP, 0.25µM of two oligonucleotide primers, 1x of the supplied reaction buffer, 2.5u of thermostable polymerase and template DNA. The reactions were subjected to temperature cycling in a Hybaid Thermal Reactor (Biometra) with hotlid function. A typical temperature profile was:

95°C for 5min, 1 cycle

95°C for 30sec, 45°C for 30sec, 72°C for 2min, 30 cycles

72°C for 5min, 1 cycle

II.J DNA techniques

Genomic DNA from *S. cerevisiae* was generously provided by Dr. Martin Kos and was carried out as described previously (Hoffman 1993).

II.K RNA techniques

II.K.1 RNA extraction

S. cerevisiae total RNA samples from strain BMA38 (Baudin *et al.* 1993) generously provided by Dr. Rym Houalla and were carried out as described previously (Tollervey *et al.* 1987).

II.K.2 RNA gel electrophoresis

Polyacrylamide gel electrophoresis was performed as described previously (Sambrook *et al.* 1989). Low molecular weight RNAs (less than 100nt) were separated on 12% polyacrylamide, 50% w/v urea gels (1x TBE: 0.9M Tris-borate pH 8.3, 20mM EDTA). High molecular weight RNAs were separated on 6% polyacrylamide, 50% w/v urea gels. Preparative gels for purifying in vitro transcripts were mini-gels and measured 8 (h) x 8 (w) x 1.5mm (d). Analytical gels for studying *in vitro* assays measured between 170 x 200 x 0.5mm for "short gels (20 lanes)", 375 x 200 x 0.5mm for "long gels (20 lanes)", and 175 x 340 x 0.5mm for "wide gels (40 lanes)". Analytical gels for northern blotting were 170 x 200 x 1.5mm. Prior to loading RNA samples were mixed at least 1:1 with formamide gel loading buffer: 95% formamide, 5mM EDTA, 0.025% (w/v) for both xylene cyanol and bromophenol blue; and heated to 65°C for 10min.

For autoradiography or phosphorimaging, 0.5mm gels were fixed post-electrophoresis in an aqueous solution of 20% methanol/10% acetic acid for ~20 minutes, transferred to two sheets of Whatman paper and dried under heat and vacuum for 2.5hrs at 80 degrees C.

II.K.3 *In Vitro* Transcription

The following steps were carried out: ~10μg plasmid DNA was linearised by restriction digest and gel purified on 2% agarose. The DNA was recovered using the Qiagen gel extraction kit in 50μl solution EB. ~2μg were then used in a 50μl *in vitro* transcription reaction which included 1X manufacturer supplied reaction buffer, 5mM rNTPs, 0.8u/μl RNasin (Promega) and 50u of T3 or 40u of SP6 RNA polymerase. The reaction was incubated at 37°C for 1hr, an additional 25u of T3 or 20u of SP6 RNA polymerase was added and incubated 1 more hour. For preparing transcripts for 5' labelling 5u of calf intestine alkaline phosphatase (CIP) was added directly to the *in vitro* transcription after the 2hr incubation and incubated an additional 1-hour at 37°C. This mixture was ethanol precipitated in the presence of 0.3M sodium acetate pH5.2 and 10μg of glycogen.

Following precipitation the pellet was re-dissolved in 10µl of diethyl pyrocarbonate (DEPC) treated ddH_2O. Samples were gel purified on a 12% acrylamide gel for pBluescript (T3, 37nt) transcripts or a 6% acrylamide gel for pSP64-SupSI (SP6, 110nt) transcripts. In both systems the transcript migrated in close proximity to the xylene cyanol loading dye.

The gel-purified transcripts were observed by UV shadowing and the appropriated areas of gel excised. The gel slices were crushed inside a 1.5ml microfuge tube and the RNA was recovered by elution in buffer under the following conditions: 200µl of 10mM Tris-HCl pH7.6, 0.1% (w/v) SDS, 1mM EDTA pH8.0, incubated at 30°C with shaking for 2hrs. The solution was collected and an additional 200µl added for 2 more hours. The solutions were combined and ethanol precipitated in the presence of 150mM NaCl and 10µg glycogen. The resulting pellet was re-suspended in 20µl DEPC treated ddH_2O.

II.K.4 5' Labelling

Transcripts that had previously been subjected to treatment with CIP were 5' labeled with polynucleotide kinase (PNK). 5' labelling reactions were conducted using 2µl of RNA in the presence of 1X manufacturer supplied reaction buffer, 5mM DTT, 5µCi $\{\gamma\text{-}^{32}P\}$ ATP and 1µl T4 PNK (NEB) in a total volume of 15µl. The reaction was incubated at 37°C 30min. Following incubation the reaction was ethanol precipitated in the presence of 3M ammonium acetate and 10µg of glycogen. The resulting pellet was re-suspended in 8µl RNA loading buffer and run on the appropriate percentage urea-acrylamide gel. The labeled RNA was detected by auto-radiography using Kodak XAR film. The gel was wrapped in plastic cling film and the film lay on top in a hyper-cassette for ~½hr exposure. Another marker, a phosphorescent ruler, was used to give orientation of the film post-development. After development of the film it was laid back on top of the gel aligned to the marker to ensure correct orientation of the labeled RNA signal. A razor scalpel was used to cut through the film into the gel, excising the area where the RNA was located.

The gel slices were crushed inside a 1.5ml microfuge tube and the RNA was recovered by elution in buffer under the following conditions: 200µl of 10mM Tris-

HCl pH7.6, 0.1% (w/v) SDS, 1mM EDTA pH8.0, incubated at 30°C with shaking for 2hrs. The solution was collected and an additional 200µl added for 2 more hours. The solutions were combined and ethanol precipitated in the presence of 150mM NaCl and 10µg glycogen. The resulting pellet was re-suspended in 20µl DEPC treated ddH_2O.

Approximately 0.5 - 0.3µg of substrate RNA were recovered from gel purified *in vitro* transcription reactions, based on the intensity UV shadowing. Of this, 2µl were used for 5' labelling, and subsequently precipitated and resuspended in 20µl. 0.25ml of labelled RNA were routinely used for *in vitro* assays. For example: 0.5µg/20µl X 2.0µl/20µl X 0.25µl/25µl = 0.000025µg/µl = 25pg/µl. Hence, *in vitro* assays are estimated to contain substrate RNA at between 15 – 25 pg/µl. This does not take into account RNA losses incurred during the gel elution steps after UV shadowing or 5' labelling, which can be as low as 50% efficient and generally not better than 70%. Considering that two such elutions are used the actual concentration of 5' labeled RNA used in the in vitro assays could be as low as 25% of the stated figure.

To obtain 5' labelled poly(A)+ RNA, 5µl of 5'-labelled substrate was incubated in the presence 8µl of high stringency affinity purified Trf4p (see II.M.2) for ½hr at 30 degrees C, in the following conditions: 20mM Tris pH7.6, 5mM $MgCl_2$, 50mM KCl, 2mM DTT, 5mM ATP, 100µg/ml BSA, 0.8u/µl RNasin in a total volume of 50µl. The RNA was subsequently phenol/chloroform extracted and ethanol precipitated in the presence of sodium acetate and glycogen and re-suspended in DEPC H_2O.

II.K.5 Production of {α-^{32}P} Labelled ADP

Transfer of the gamma-phosphate of {α-^{32}P} labelled ATP to glucose in the presence of hexokinase to yield ADP and glucose-6-phosphate produced alpha-labelled ADP. The following conditions were applied in a 50µl reaction: 20mM Tris pH7.6, 5mM $MgCl_2$, 100mM D-glucose, 60nM {α-^{32}P} ATP (3pmol/50µl) and ~3u of hexokinase were incubated at 30°C for 10 min. Analysis by thin-layer

chromatography (see II.M.1.1 for method) indicated that the input ATP had been quantitatively converted to ADP. These reaction solutions were frozen and used in assays to detect the incorporation by the exosome of alpha-labelled ADP into a primer RNA (II.M.4.2).

The hexokinase enzyme was obtained from Sigma, product H-5000. The enzyme stock solution was prepared at 50mg/ml in 20mM Tris pH7.6, 5mM $MgCl_2$ and 10% glycerol (v/v) and stored at –20°C. One unit will phosphorylate 1.0μmol of D-glucose / minute at pH7.6 at 25°C.

II.K.6 Secondary Structure Prediction

The secondary structures *in vitro* transcripts and fragments were modeled using the MFOLD program for RNA secondary structure prediction. Included parameters were: 86mM Na^+, 5mM $MgCl_2$ and 30 degrees Celsius. 86mM represents the combined concentration of monovalent K^+ and Na^+ ions in a reaction prepared with enzyme in 150mM NaCl (TMN150, see section II.M); does not include Na^+ derived from other sources such as sodium phosphate, nor does it account for a decrease in Na^+ when lower stringency enzyme preparations were used. The program returns the same structure when Na^+ is specified up to 1M. (http://bioweb.pasteur.fr/seqanal/interfaces/mfold.html, (Zuker *et al.* 1999)). The sequence of the 37nt transcript is taken to be: 5' GGGAACAAAA GUUGCAUGCC UGCAGGUCGA CUCUAGC 3', from the +1 transcriptional start site of the T3 promoter to the 3' XbaI cleavage site. Secondary structure predications of truncated products were predicted in the same fashion.

II.L Protein and immunological techniques

Standard protein purification, handling, staining and analysis techniques preformed as described by Rosenberg, 1996.

II.L.1 Preparation of Yeast protein extracts

Yeast cells were grown in 2 litres of YPD to an optical density of approximately 2.0-3.0 at 600nm. Cells were harvested by centrifugation (Beckman Avanti J-25 series, JLA-10.5 rotor) in aliquots of ~500ml. Each resulting pellet was washed with 50ml sterile ddH_20. The resuspension was transferred into a 50ml-Falcon tube and pelletted by centrifugation (4krpm Beckman GS-6R). This pellet was then subjected to lysis with glass beads (Ø 0.400-600mm) either in the 50ml Falcon tube using a vortex mixer or using the Bio-spec bead beater.

Depending on the protein to be purified the extraction buffer was either TMN 150, 100 or 75: 20mM Tris-HCl pH 7.6, 5mM magnesium chloride, 0.1% Nonidet P40 and either 150mM, for the former, 100mM or 75mM NaCl for the latter (method adapted from Mitchell *et al.* 1996). Small scale extraction were kept cold by incubation on ice and generally did not contain protease inhibitors, save for small scale extractions carried out using the bead beater, which were cooled using the ice jacket and included protease inhibitor cocktail (Roche, The Complete-mini EDTA free). Large scale extractions carried out on bio-reactor scale yeast pellets were always carried out using the Bio-spec bead beater, with ice jacket, in the 4°C cold room using a protease inhibitor cocktail (Roche, The Complete EDTA free), Pepstatin at 1µg/ml and EGTA at 2mM.

For small-scale extractions of pellets up to ~5ml in volume the cell pellet was resuspended at a ratio of 1:1:1, pellet to lysis buffer to glass beads. The cells were lysed by vortexing 5min in 30sec intervals, remaining on ice in between rounds of vortexing. The lysate was centrifuged for 5 minutes at 4krpm (Beckman GS-6R) to pellet the glass beads and any unbroken cells. The supernatant was saved and the remaining pellet re-extracted in ½ the original volume of buffer for 2min in 30sec intervals on ice. After centrifugation the appropriate supernatants were combined. These crude extracts were next subjected to ultracentrifugation at 30,000*g* for 20min at 4°C using a Beckman JA25.5 rotor. This clears the solution of macromolecules of ~40S and larger using any tube with a K Factor of 418. The cleared extract was poured off into a clean 50ml-Falcon tube and subjected to affinity chromatography.

Small-scale pellets 5ml or greater were extracted using the BioSpec bead beater; carried out according to the manufacturers instructions. Large-scale reactions

were performed in the 350ml chamber with 150ml of glass beads and pellet resuspended in ~200ml of lysis buffer (filled until no air in vessel). This mixture was subjected to homogenisation for 3min. After lysis the extract was transferred to 50ml Falcon tubes and centrifuged for 10min at 4krpm. The supernatants were collected and subjected to ultracentrifugation at 30,000*g* for 20min at 4°C using a Beckman JA25.5 rotor and 50ml polycarbonate tubes (Beckman part 361693). The cleared extract was poured off into a clean 50ml-Falcon tube and subjected to affinity chromatography.

II.L.2 Immuno-precipitation/Affinity purification

ZZ and TAP – tagged yeast proteins were precipitated using IgG Sepharose 6 FF (Amersham). For up to 50ml of protein extract 200µl of the stock suspension were equilibrated in column (Bio-Rad 10ml Poly-Prep Column) with 2 x 5ml lysis buffer. After equilibration the protein extract was allowed to pass through the resin by gravity flow. Once all the extract has passed over the column it was washed ten times with 10ml lysis buffer, including two 5ml washes with agitation. When high stringency was required a given preparation received at least two 5ml washes of TMN500 with agitation (As TMN but with 500mM NaCl), and was equilibrated back to low salt with additional washes of lysis buffer thereafter. After washing, the buffer was allowed to drain the top of the resin bed. 200µl of lysis buffer and 2µl of recombinant TEV protease (Promega) were added and incubated at 4°C overnight. The eluate was drained into a primary fraction using a 10ml syringe to aid liquid flow. 200µl lysis buffer were added back to the resin and a secondary fraction was collected. Finally 200µl of lysis buffer with 3% glacial acetic acid was added to the drained resin and incubated for 5min at room temperature. This was collected and used as a control for TEV cleavage. The proteins were analysed by Western blot and Coomassie staining either directly or after precipitation with acetone, TCA/acetone (internet resource, see references (HCPF)) or methanol/chloroform (Rosenberg 1996).

Similarly, when the behaviour of protein complexes was assayed at several stringencies, such as the binding of Mtr4p by Trf4p, Trf5p and Air2p, batch binding

was used under the above-described conditions. Equilibrated resin was added to the cleared protein lysate and incubated at 4°C with rotation for 2 hours. The resin was collected by centrifugation at low speed (1krpm in Beckman GS-6R), washed once with 3ml of TMN75 and pelletted. Finally resuspended in a small volume (~1.5ml) and divided evenly over 4 columns (Bio-Rad Micro Bio-Spin) for 5 washes of TMN75, 150, 300 and 500 respectively; likewise, in separate experiments samples were divided across 3 columns for washes with TMN75, 150 and 500 or 2 columns for washes with TMN100 and 500 respectively. Primary and secondary eluted fractions were combined and methanol/chloroform precipitated.

When several hundred ml of extract had to be processed the cleared lysate was passed over 2-10ml IgG sepharose in 1cm (ID) x 20cm (h) glass column (Bio-Rad Econo-Column) with flow adapter (Bio-Rad 738-0014) under peristaltic pump action with a flow rate of 1.8ml/min. Washing was done at a minimum of 2ml/min and a maximum of 3ml/min. Elution was achieved in the same fashion as on the small scale. The buffer was drained to the top of the resin bed 2 – 5 ml of lysis buffer was added with 20 – 50µl TEV protease. The resin was agitated in the presence of the cleavage solution and allowed to incubate overnight at 4°C prior to elution.

II.L.3 SDS-Polyacrylamide electrophoresis

Proteins were separated on polyacrylamide gels containing SDS as described by (Laemmli 1970). Adding 2X or 10X protein loading buffer denatured the samples.

2X: 125mM Tris-HCl pH6.8, 200mM DTT, 4% SDS, 40% glycerol, and 0.001% bromophenol blue.

10X: 10g Sucrose, 2.5ml 2-Mercaptoethanol, 2.5 ml 10% (w/v) SDS, 2.5ml 2M Tris-HCl pH6.8, 0.01% (w/v) Bromophenol blue, top up to 10ml w/ ddH_2O.

II.L.4 Western blotting

Following separation of proteins by SDS-PAGE the proteins were transferred to a nitro-cellulose or PVDF membrane (Amersham) in a Semi-Dry blot apparatus (Bio-Rad).

The gel was assembled on the blotting membrane between 2 sheets of Whatman paper on each side, soaked in transfer buffer (25mM Tris base, 40mM glycine, 0.05% SDS, 20 % methanol) and taking care to remove air bubbles from between the layers. Transfer was performed at 150mA for 2 hours. After the transfer the membrane was blocked in 3.3% low-fat dried milk (w/v) in PBS for 1-2 hour shaking at room temperature or overnight at 4°C.

Depending on the protein to be detected the blot was treated with different immunological reagents. For detection of Protein A (ZZ) or TAP–tagged proteins the membrane was incubated with a 1:5000 dilution of peroxidase anti-peroxidase antibody (PAP, Sigma) for 1-2 hour at RT. Then the blot was washed twice for 15 minutes with phosphate buffered saline with 0.1% Tween 20 (PBST; 137mM sodium chloride, 3mM potassium chloride, 10mM di-sodium phosphate, 2mM potassium di-phosphate, 0.1% Tween 20), and one time with PBS (as PBST but without Tween 20) while detection reagents were prepared. For detection of Mtr4p, Rrp4p and Nop1p, the membrane was incubated with anti-Mtr4p, anti-Rrp4p or anti-Nop1p with a 1:5000 dilution for 1-2 hours at RT, or overnight at 4°C. The blot was washed twice for 15 minutes with PBST and incubated with the HRP-conjugated-secondary antibody for 1-2 hours with a 1:10,000 dilution then washed again twice for 15 minutes with PBST and finally once with PBS while detection reagents are prepared. The proteins were detected using an enhanced chemiluminescence (ECL) kit (Amersham), following the manufacturers instructions.

II.M In Vitro Assays

In vitro assays were carried out on low or high stringency affinity-purified proteins (II.L.2). All protein preparations used in the assays described below were prepared from ~2L cell culture grown to ~2-3u OD_{600} by the method described previously in section II.L.1. Standard procedure during time point collection was to collect the zero time point immediately after the mixing of the sample upon the addition of enzyme. The only deviation from this procedure is described in section II.M.4.2. Samples for mock RNase and polymerase assays were prepared from strain P51, as were "WT" total protein extracts.

II.M.1 ATPase assay

ATPase activity assays were carried out on affinity purified Mtr4p at high and low stringency (after 150 and 500mM NaCl washes respectively) and were conducted under the following conditions: 20mM Tris pH7.6, 5mM $MgCl_2$, 50mM KCl, 2mM DTT, 1mM ATP, 100μg/ml BSA, +/- Phosphatase inhibitors (Calbiochem, Phosphatase Inhibitor Cocktail Set II 524625; containing: imidazole, fluoride, molybdate, orthovanadate and tartrate), 2.5μCi {α-^{32}P} ATP. 10ul of the Mtr4p IP were used in each assay in the presence of 0.28μg/μl or 0.03μg/μl total RNA (prepared from *S. cerevisiae* BMA38). 20μl reactions were assayed at 37°C over a time course of 2hrs. 4μl samples were collected and mixed with an equal volume of stop buffer (5mM EDTA pH8.0, .1% (w/v) SDS, 0.01% (w/v) xylene cyanol) and stored at -20°C (method adapted from (Schmitt *et al.* 1999)).

Calf intestine alkaline phosphatase (CIP) was used as a control for the production of ADP, AMP and P_i from ATP. CIP was obtained from NEB and used at 0.005u/μl concentration. *S. cerevisiae* Dbp4p was used as a control for DEAD-box RNA helicase related ATPase activity. Dbp4p was provided by Dr. Martin Kos and was produced recombinantly in *E. coli* as a glutathione s-transferase fusion protein, and subsequently purified by glutathione-resin affinity chromatography. Affinity-purified Snu66p was used as a negative control and was prepared as described in section II.L.2.

II.M.1.1 Thin-layer Chromatography (TLC)

The results of these assays was analysed by TLC on PEI-cellulose (Macherey-Nagel, Polygram CEL 300 PEI/UV_{254} 801 063). TLC plates were pre-run in ddH_2O and dried; the top 5cm was cut off. 1-2μl was spotted on to the TLC plate, ~0.5 inches from the bottom, and allowed to dry. The plated was then set to run in 750mM KH_2PO_4 ~pH4.0 until the moving phase reached within ~0.5 inches of the top of the plate (~15cm). The plates were dried and subjected to autoradiography or phosphorimaging.

II.M.2 RNase Assay

RNA degradation assays were carried out on two substrates. One, a 37nt transcript produced by *in vitro* transcription of XbaI linearised pBS +/- with T3 RNA polymerase. The second, 110nt, was produced by *in vitro* transcription of a $Taq^{\alpha}I$ linearised construct containing a DraI-PstI fragment of the *S. pombe* pre-$tRNA^{Ser}$ (*supSI*) gene, subcloned into vector pSP64 cut with HindIII-PstI (the HindIII site was made blunt), provided by Iain Willis (Krupp *et al.* 1985) (referred to as pSP64-SupSI in plasmids table). This transcript contained a 28nt long 5' flank and ended with the guanosine preceding the CCA of the complete mature tRNA.

Purified labeled transcripts were subjected to digestion by affinity purified exosome complex (500mM NaCl wash stringency, see II.L.2) under the following conditions: 20mM Tris-HCl pH7.6, 50mM KCl, 5mM MgCl2, 2mM DTT, +/- 1mM ATP, 100μg/ml BSA, 0.8u/μl RNasin and 4μl of exosome preparation per 25μl reaction (method adapted from Mitchell *et al.* 1997).

RNAse assays were also carried out on 3' extended polyadenylated species of the above-described substrates. The method as described in the next section.

Additionally, RNase assays were carried out in the absence of added ATP and in the presence of inorganic phosphate at 20mM. Phosphate was derived from a 0.1M stock solution of sodium phosphate prepared as follows: combine 0.13ml of a 0.2M solution of sodium di-hydrogen phosphate, 0.87ml of di-sodium hydrogen phosphate and 1.0ml of double distilled sterile water. The resultant stock solution is 0.1M sodium phosphate buffer, pH 7.6.

These enzymatic reactions were terminated by combining the collected time point sample with an equal or greater volume of formamide RNA gel loading buffer (see II.K.2).

II.M.3 Polymerase Assays

RNA extension assays were carried out on the two RNA substrates described in the previous section. Through addition of the affinity purified exosome complex to RNA substrate in the presence of ATP and affinity purified TAP-tagged proteins (Mtr4p, Air2p and Trf4p) coupled polymerase-RNase assay was preformed.

Polymerase activity was initially characterised for affinity purified Mtr4p samples obtained at 150mM NaCl stringency under the following conditions: 20mM Tris-HCl pH7.6, 50mM KCl, 5mM MgCl2, 2mM DTT, +/-1mM ATP (or ADP and/or 5mM P_i) 100µg/ml BSA, 0.8u/µl RNasin and 2µl of Mtr4p per 25µl reaction. Similarly polymerase assays were carried out on high stringency (500mM NaCl) affinity purified samples of Air2p, Mtr4p, Trf4p and Trf5p under the same conditions, and also substituting UTP, GTP and CTP, respectively, for ATP.

These enzymatic reactions were terminated by combining the collected time point sample with an equal or greater volume of formamide RNA gel loading buffer (see II.K.2).

II.M.4 Coupled polymerase-RNase assays

These assays were designed to produce conditions favorable to elongation of *in vitro* transcripts, but were also subject to the degradative activity of the exosome.

II.M.4.1 Standard-type

Coupled polymerase-RNase assays were made under the previously described conditions for the standard RNase assay using ATP and combinations of exosome and affinity purified proteins used in the polymerase assays. The ratio of 4µl exosome preparation to 2µl Mtr4-TAP (150mM) was used as the standard reaction and these conditions maintained such that no more than 6µl of exosome and any combination of other affinity purified proteins was used in a 25µl reaction. When more than 6µl of protein preparation were used in a given reaction the total volume was scaled up accordingly such that the glycerol content never rose above 2.4% (v/v) and NaCl not above 36mM.

II.M.4.2 PNPase-like

This polymerization assay was extrapolated from the previously described method (Beers 1957). The conditions were the same as the traditional polymerase assay described above, but carried out on purified exosome only and without ATP.

4µl of exosome preparation were assayed in the presence of ADP at up to 5mM. $MgCl_2$ was supplemented at 1mM per millimole of ADP over 1. This assay was also carried out in the combined presence of {α-^{32}P} ADP (II.L.7) and/or 20mM P_i. When this assay was conducted in the presence of {α-^{32}P} ADP, unlabelled *in vitro* transcripts were used and a glucose hexokinase reaction containing the {α-^{32}P} ADP was used in place of DEPC treated water and at the same volume. ADP was added from a 100mM stock solution made from a di-sodium salt powder (84% ADP, Roche 127-507) dissolved in DEPC treated water and stored at –20 degrees C.

For some assays the zero time point was collected prior to the addition of enzyme. The standard reaction volume was 25µl and called for the addition of 4µl of exosome to 21µl of reaction mix (a 20% increase in volume). 7µl (28% of 25µl) were the standard sampling volume, thus 5.88µl were collected for the zero time point to compensate for the volumetric difference. 2.88µl of exosome were added to the remaining sample in order to dilute the solutes to the standard concentration and yield the same enzyme concentration as would otherwise have been.

II.M.4.3 RNA Precipitation Assay

To determine if the 5'-labelled RNA was precipitating from reactions under the described PNPase-like coupled RNase assay conditions the samples were allowed to incubate for one hour at 30 degrees C, in the presence or absence of enzyme during collection of the zero time point, and then centrifuged for 5 minutes at 13000 rpm in a bench top centrifuge at room temperature. The supernatant was transferred to a clean microfuge tube and radio decay counts were monitored by Geiger counter to determine qualitatively where the counts fractionated. When a significant portion of the counts remained in the original tube after transfer of the aqueous solution, it was taken to indicate precipitation of the input RNA had occurred.

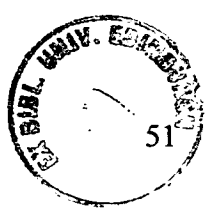
II.N Phosphorimager and ImageQuant Quantification

When quantitative analysis of radiolabelled species was required Molecular Dynamics storage phosphor imaging plates were exposed to TLC plates or dried

acrylamide gels. The storage phosphor plates were scanned and imaged using the STORM scanning system (Amersham) and data was collected using the ImageQuant v5.2 software and exported to Microsoft Excel. Calculations and graphs were done in MS Excel.

III Results and Discussion

Exosome Cofactors Mtr4p and the MATPAC



III.A Mtr4p, an Essential Cofactor

With the goal of stimulating exosome activity *in vitro*, the essential protein and known exosome cofactor Mtr4p was selected for initial reconstitution studies. Genetic depletion of Mtr4p closely phenocopies the effects of depletion of the core exosome for a variety of different substrates, including pre-rRNA, pre-snoRNAs, pre-snRNAs and pre-mRNAs (Allmang *et al.* 2000; Allmang[a] *et al.* 1999; Bousquet-Antonelli *et al.* 2000; Torchet *et al.* 2002; van Hoof[a] *et al.* 2000). As the repertoire of affected RNA species suggests, Mtr4p is localised to the nucleoplasm and nucleolus (de la Cruz *et al.* 1998; Huh *et al.* 2003; Liang *et al.* 1996). In yeast, a small fraction of the Mtr4p population was co-precipitated with the core exosome component Rrp4p (unpublished observation, Philip Mitchell). In contrast, hMtr4p was readily identified by proteomic analysis of purified preparations of the human exosome (Chen *et al.* 2001). These observations suggested that Mtr4p can interact with the core exosome and may participate directly in the recruitment of the exosome to target RNAs.

The literature describes Mtr4p as a putative ATP-dependant RNA helicase based on sequence homology (Ski2p family, DExH-box related to DEAD-box), but it had not been characterised for RNA dependence or ATPase activity *in vitro*. Thus *in vitro* experiments were carried out to test these qualities of the affinity-purified protein prior to *in vitro* reconstitution with affinity-purified exosome.

III.A.1 Mtr4p: RNA stimulated ATPase activity

Yeast strain SC1157, expressing *MTR4*-TAP, was prepared as described in section II.L, at 150mM salt. These preparations were tested for their ability to convert {α-^{32}P} ATP to ADP *in vitro* and analysed by thin-layer chromatography (TLC, section II.M.1.1). 20µl of Mtr4p preparation, representing 10% of a typical TEV cleavage reaction from 2L of culture grown to between OD2-3, generally gave Coomassie stainable bands with approximately 200-250ng/band upon SDS-PAGE. With a predicted molecular weight of 122kDa, that is approximately $1.64 - 2 \times 10^{-12}$ moles (~1.64 – 2 picomoles) of Mtr4p in 20µl. Hence each reaction conducted with

2µl of standard Mtr4p preparation can be expected to have on the order of 0.16 – 0.2 picomoles of Mtr4p. This approximation works out to ~6.4 – 8nM in a 25µl reaction volume. An example of an SDS-PAGE analysis is shown in figure 1. Wild-type Mtr4p has a predicted molecular weight of 122kDa. The product of TEV cleavage lacks the protein-A tag, but still carries the calmodulin binding peptide at the C-terminal (+ ~5kDa).

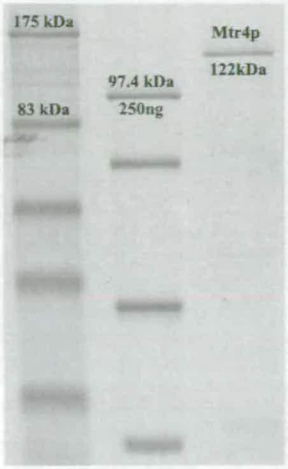


Figure 1: Affinity Purified Mtr4p 10% of the TEV cleaved eluate separated on a 5%/10% discontinuous polyacrylamide gel: (Left-Right) molecular weight marker, molecular weight marker standardised for 250ng/band, Mtr4-TAP

Figure 2 shows the results of an ATPase assay (methods II.M.1). Calf intestine alkaline phosphatase (CIP) and the DEAD-box RNA helicase Dbp4p were included as controls. CIP is expected to produce ADP, AMP and P_i. Its inclusion here allows a point of reference for differentiating the species. Both Dbp4p and Mtr4p are expected to hydrolyse ATP to ADP and P_i. The results indicate that the conditions are conducive to ATPase activity for this class of enzyme, as shown by the Dbp4p sample. However, the Mtr4p samples appear to also be producing AMP. This is not expected and may be indicative of a phosphatase having been carried through in the purification procedure. Even with the appearance of the unexpected AMP, an increase in ATP hydrolysis is observed in the Mtr4p sample treated with total RNA. Analysis of the phosphorimager data indicates a 21% increase ADP present after 120 min.

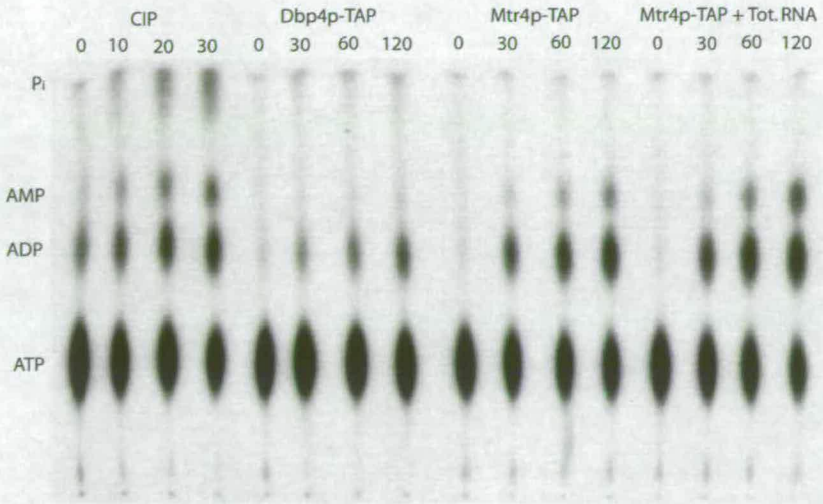


Figure 2: ATPase Assay. TLC on PEI-cellulose. Time course shown in minutes: (Left-Right) CIP positive control; Dbp4p positive control; Mtr4p; Mtr4p + total RNA extract

In order to address whether the generation of AMP was intrinsic of Mtr4p or some other factor in the preparation, the experiment was conducted a second time using samples purified under higher stringency conditions. This time a wash buffer containing 500mM salt (2 x 5ml with agitation, see section II.L.2) was used for part of the purification procedure. Phosphatase inhibitors were also added to the reaction mixture. Figures 2 and 3 show the results of ATPase assays carried out on these samples. An affinity purified sample of Snu66p (Stevens *et al.* 1999) from strain SNU66-TAP was prepared under the same conditions and was included as a negative control. This protein is not expected to posses ATPase activity.

These results confirm the detected ATPase activity of Mtr4p against the negative control, Snu66p. Quantification of the phosphorimager data shows no appreciable accumulation of ADP in the negative control across all Snu66p samples. There is also no appreciable accumulation of AMP across the Mtr4p control and +phosphatase inhibitor samples, although a small accumulation is observed in the presence of total RNA after 120 minutes. This indicates that the generation of AMP was dependant upon a factor that was mostly lost upon washing with 500mM NaCl.

The quantified data indicates a 15% drop in ADP accumulation between the Mtr4p control and +phosphatase inhibitor samples across all time points. Stimulation of ATPase activity in the presence of total RNA was observed between the Mtr4p sample and the +total RNA (both w/ phosphatase inhibitors). Based on the accumulation of ADP as a percentage of the combined signal detected for ADP

and ATP, the data indicates a 2.5X increase in the amount of ADP accumulated in the sample treated with RNA compared to without, across all time points (fig. 2C).

The Mtr4p vs. Mtr4p + RNA ATPase assays were repeated to confirm the level of stimulation conferred by the addition of total RNA to the high salt TAP-purified Mtr4p sample in the presence of phosphatase inhibitors. Amongst these assays was included a +RNA sample at tenfold lower concentration (data not shown). The data from the experiment carried out as previously showed a 2.2X increase in the accumulation of ADP, in keeping with the previous observation. The experiment conducted with 10% the amount of total RNA continued to show an increase in ADP accumulation vs. the non-RNA containing sample, albeit less pronounced by comparison to the previous result.

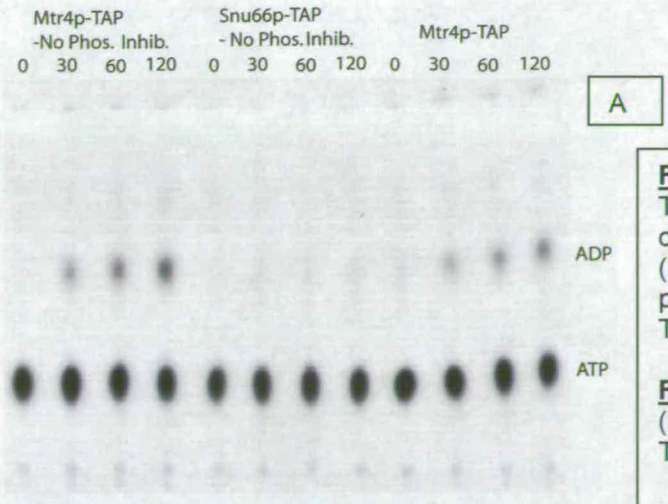


Figure 3A: ATPase Assay
Thin-layer chromatography on PEI-cellulose, time in minutes:
(Left-Right) Mtr4p-TAP control (-phosphatase inhibitors), Snu66p-TAP negative control, Mtrp4p-TAP

Figure 3B: ATPase Assay cont.
(Left-Right) Snu66p-TAP, Mtr4p-TAP + RNA, Snu66p-TAP + RNA

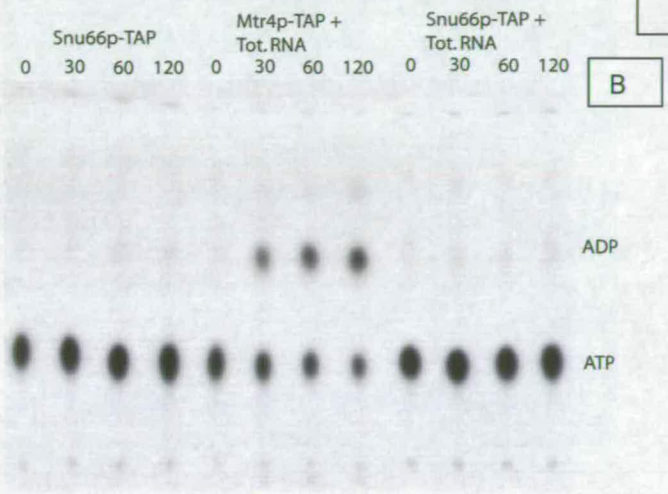
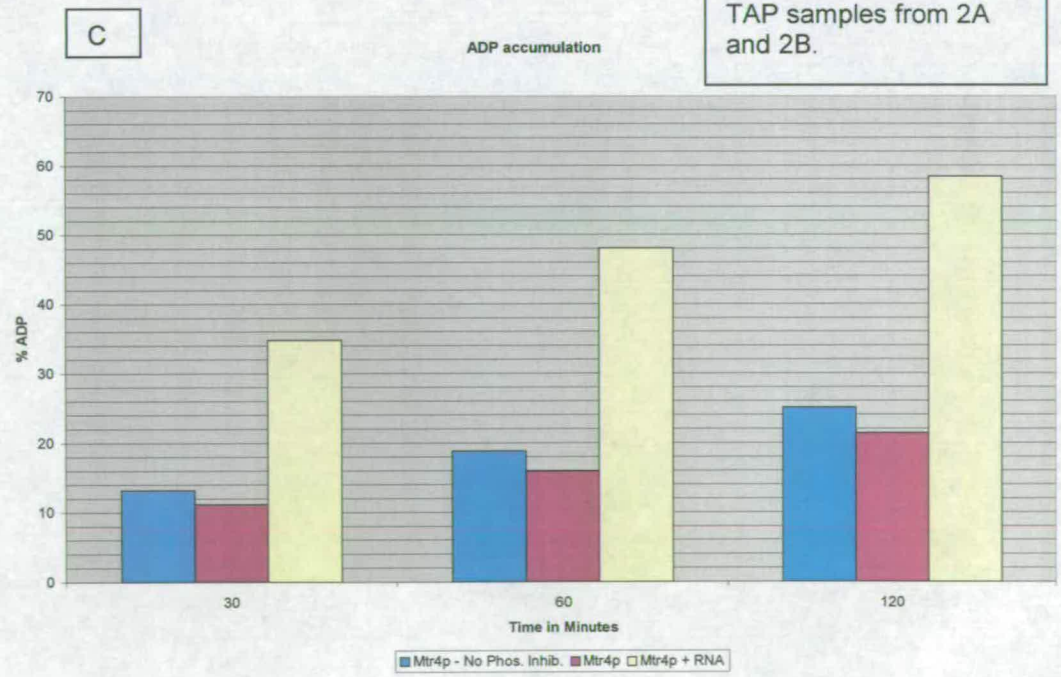


Figure 3C:
Quantification of Mtr4p-TAP samples from 2A and 2B.



III.B Proteomics

The driving force behind this work has been the identification of co-factors that are capable of stimulating the activity of the exosome, with the intention of screening such candidates *in vitro*. Mtr4p was an obvious first candidate as it is known to co-immunoprecipitate with the exosome and shows an exosome-like phenotype upon depletion. Attempts were made to identify other factors that interact with Mtr4p on the assumption that such proteins may also be required for interaction with or stimulation of the exosome *in vivo* and *in vitro*.

III.B.1 Two-hybrid screen

Simultaneous with the *vitro* analysis of Mtr4p described in the previous section, a two-hybrid analysis was performed to identify proteins that interact with the exosome cofactor using the entire *MTR4* open reading frame (ORF) as bait, in a fusion with the *GAL4* DNA binding domain in plasmid pB27 (Hybrigenics SA, Paris) (Fromont-Racine *et al.* 2003). Preparation of the samples for analysis was carried out by Liz Thompson of Jean Beggs' lab. This screen identified 22 clones containing regions of the Trf5p ORF and 5 clones with the Trf4p ORF. No other significant hits were found.

Trf4p and Trf5p are homologous proteins with significant homology to the beta nucleotidyltransferase superfamily (Aravind *et al.* 1999), which includes poly(A) polymerase and the tRNA CCA-adding enzyme. For Trf5p, the minimal region common to all of the clones interacting with Mtr4p is amino acids 53 to 199, which lies immediately N-terminal to the nucleotidyltransferase domain. Each fragment of Trf4p recovered contains a large region of the ORF, precluding more accurate mapping, however, given the high homology between Trf4p and Trf5p (57% identity, 70% similarity over 559 amino acids) it seems very likely that they share a homologous Mtr4p-binding site. The central regions of the two proteins are very homologous (73% identity over 363aa) and this homology extends into the minimal interaction region between Trf5p and Mtr4p.

III.B.2 Proteomics in the Literature

In previous systematic analyses, FLAG-tagged Trf4p co-precipitated with Mtr4p as well as two homologous proteins Air1p and Air2p (Ho *et al.* 2002), and TAP-tagged Mtr4p was recently reported to coprecipitate Trf4p and Air2p (Krogan *et al.* 2004). Air2p also interacted with Trf4p in a two-hybrid assay (Ito *et al.* 2001). Air1p and Air2p (Arginine methyltransferase-Interacting RING finger protein) contain RING-type zinc finger domains, and strains deleted for both *AIR1* and *AIR2* accumulate nuclear poly(A)$^+$ RNA (Inoue *et al.* 2000), a phenotype also seen in strains with mutations in the exosome and Mtr4p (Kadowaki *et al.* 1995), consistent with functional interactions.

III.C Mtr4p and the MATPAC

A strain expressing Air2p-TAP was used to identify associated proteins by affinity-purification followed by polyacrylamide gel separation and mass-spectrometry. Only Mtr4p and Trf4p were identified in the precipitate, at apparent stoichiometry (personal communication, Dr. Cosmin Saveneau). To confirm these interactions and determine their salt-sensitivity, Trf4p-TAP, Trf5p-TAP and Air2p-TAP (Open Biosystems, (Ghaemmaghami S *et al.* 2003)) were purified from yeast lysates. During purification the IgG column was washed with either 100mM or 500mM salt and the presence of Mtr4p in the TEV eluate was assessed by Western blotting using anti-Mtr4p (anti-Dob1p) antibodies (de la Cruz *et al.* 1998) (fig. 4). The Western blots were also decorated for the presence of the nucleolar ribosome synthesis factor Nop1p (Schimmang *et al.* 1989) (fig. 5). Mock purifications of the non-tagged control strain P51 at 75, 150 and 500mM salt, conducted in parallel with Trf5-TAP under the same conditions, were probed for the presence of Mtr4p and Nop1p in the eluted cleavage fraction (fig. 6).

Mtr4p was clearly coprecipitated with Trf4p-TAP, Trf5p-TAP and Air2p-TAP in preparations washed with 100mM NaCl and detection was lost following washing at 500mM NaCl (fig. 4), while Mtr4p was not detectably recovered in a mock precipitation from the non-tagged control strain (fig. 6). Nop1p was not

detectably coprecipitated with any of the TAP-tagged proteins or the non-tagged control (fig. 5 and 6) but does appear in a yeast total protein extract (fig. 5).

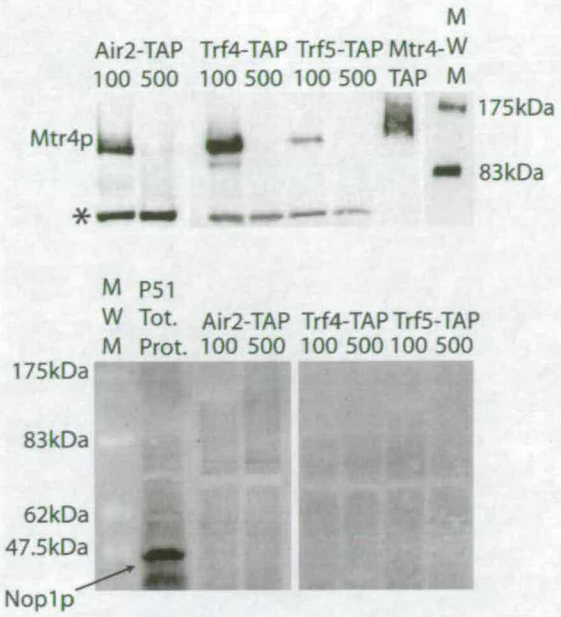


Figure 4: TAP Purifications at 100 and 500mM vs. anti-Mtr4p. 5%/10% discontinuous polyacrylamide gel transferred to nitrocellulose membrane: (Left-Right) Air2-TAP at 100 and 500mM, Trf4-TAP at 100 and 500mM, Trf5-TAP at 100 and 500mM, Mtr4-TAP (500Mm) positive control, molecular weight marker

Figure 5: TAP Purifications at 100 and 500mM salt vs. anti-Nop1p
(Left-Right) molecular weight marker, P51 non-tagged control strain total protein, Air2-TAP at 100 and 500mM, Trf4-TAP at 100 and 500mM, Trf5-TAP at 100 and 500mM

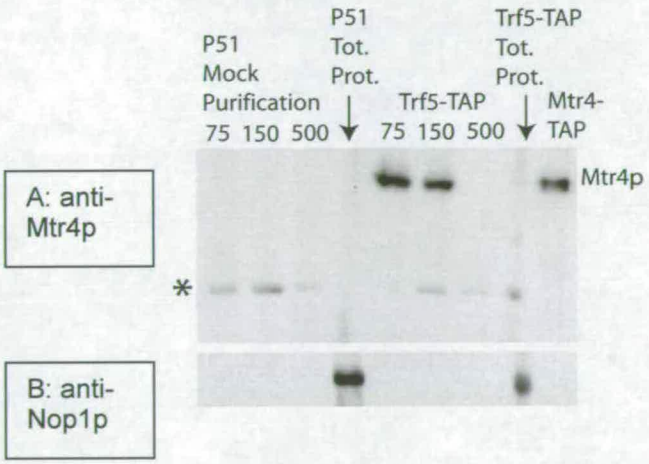


Figure 6: Mock Purification and Trf5-TAP vs. anti-Mtr4p and anto-Nop1p (Left-Right) A&B: P51 mock purification at 75, 150 and 500mM, P51 total protein, Trf5-TAP at 75, 150 and 500mM, Trf5-TAP total protein, Mtr4-TAP (500Mm)

* Band marked with asterisk is an unidentified cross-reacting species

The yield of Mtr4p coprecipitated with Trf4p was higher than with Trf5p, consistent with the estimate (Ghaemmaghami S *et al.* 2003) that Trf4p is ~3-fold more abundant than Trf5p. After collection of the cleavage fractions the IgG columns were cleared of residual protein by washing with 3% glacial acetic acid. Western blotting the acetic acid wash with the peroxidase anti-peroxidase IgG antibody confirmed that all three TAP-tagged proteins were bound to the IgG column and were efficiently cleaved by TEV protease (data not shown). These data, taken

with those presented in section B of this chapter, support the conclusion that Mtr4p interacts with Air2p and Trf4p in a complex *in vivo*. This putative complex is referred to as the MATPAC (Mtr, Air and Trf PolyAdenylation Complex).

III.D *In vitro* Reconstitution Experiments

Armed with the confirmation of RNA stimulated ATPase activity for Mtr4p and the proteomics and Western blot analysis of Mtr4p interacting partners, experiments were conducted to combine affinity purified exosome with affinity purified Mtr4p, Trf4p, Trf5p and Air2p in the presence of 5' {α-^{32}P} labelled RNA. Exosome preparations generally show a weak distributive hydrolytic exoribonuclease activity in the absence of additional cofactors (Mitchell *et al.* 1996).

III.D.1 Affinity purified MATPAC Stimulates Exosome Activity and Exhibits Poly(A) polymerase Activity *in vitro*.

Mtr4-TAP derived MATPAC preparations were combined with affinity-purified exosome (Csl4-TAP, 500mM salt stringency) in the presence or absence of ATP and RNA. The RNA used in these experiments was a 37nt *in vitro* transcript produced from the multiple cloning site of XbaI linearised pBS +/- vector. Figure 7 shows the results of one such experiment. The exosome sample shows the typical signature of distributive activity that has been seen before in other purifications (Mitchell *et al.* 1996). The '*' denotes a commonly observed degradation intermediate which is not present when the exosome is combined with the MATPAC and 1mM ATP. The RNA in the exosome + MATPAC + ATP sample appears to have been elongated, which was an entirely unexpected result. In confirmation of the conclusion, the MATPAC + ATP sample displays strong extension of the input RNA, suggesting that the MATPAC possesses an RNA polymerase activity. When 1mM ADP was combined with the MATPAC only a comparatively slight extension of the input RNA is observed, but this may be due to ATP contamination of the ADP preparation (Roche 127-507, di-sodium powder, stated as typically 82% ADP [enzymatic] and <1% ATP). Finally, Exosome in the presence of MATPAC without added ATP appeared to be repressed.

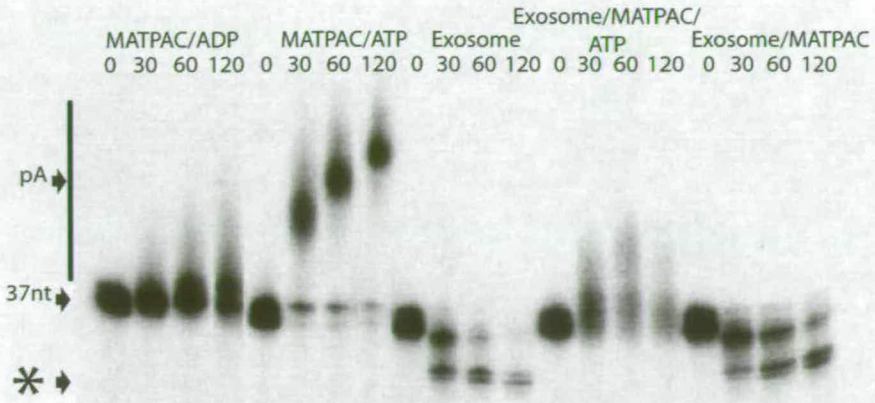


Figure 7: Polymerase and RNase assays
12% polyacrylamide/Urea denauring gel. Time course shown in minutes: (Left-Right) MATPAC + 1mM ADP, MATPAC + 1mM ATP, Exosome, Exosome + MATPAC + ATP, Exosome + MATPAC (No added ATP)

III.D.1.1 The MATPAC Contains a Poly(A) polymerase

MATPAC that was coprecipitated with Mtr4-TAP was tested for its ability to polymerise the different rNTPs, each at 1mM respectively, the results shown in figure 8. Where the MATPAC was able to robustly elongate the input RNA (arrow) in the presence of ATP, it was less active with the other three NTPs. Reduced activity was observed in the presence of GTP, and 1-2nt were added in the presence of UTP. No activity was observed with CTP. Thus the complex shows preferential poly(A) polymerase activity.

The addition of 5mM P_i to the MATPAC + 1mM ATP did not alter or inhibit the observed polymerization reaction (data not shown), indicating that the mechanism of polymerization is probably the same as for the canonical poly(A) polymerases, which hydrolyze ATP, polymerizing AMP and release pyrophosphate (PP_i). PPi is unstable in aqueous solution and rapidly hydrolyses to $2P_i$; hence the polymerization reaction equilibrium is unaffected by high concentrations of P_i.

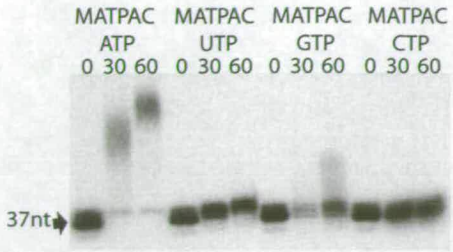


Figure 8: MATPAC Nucleotide Preference 12% polyacrylamide/Urea gel with time course shown in minutes: (Left-Right) MATPAC + 1mM ATP, UTP, GTP, CTP

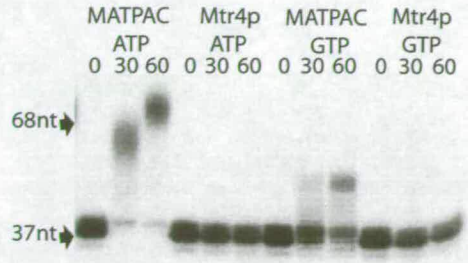


Figure 9: Mtr4-TAP prepared at 150mM (MATPAC) and 500mM salt (Left-Right) 150mM salt Mtr4-TAP preparation + ATP, 500mM salt Mtr4-TAP preparation + ATP, 150mM salt Mtr4-TAP preparation + GTP, 500mM salt Mtr4-TAP preparation + GTP

In order to determine if the observed activity was attributable to Mtr4p or another factor that co-precipitates at this stringency (150mM salt), high stringency (500mM salt) preparations of Mtr4-TAP were subjected to the polymerase assay in parallel (fig. 9). The result indicates that another factor is responsible for the observed polymerase activity. Mtr4p is enzymatically active for RNA stimulated ATPase activity in high stringency preparations, thus it is expected to be structurally intact. These results indicate that the factor responsible for the observed activity is lost from the immuno-precipitated Mtr4-TAP on washing with 500mM salt.

As it was previously demonstrated that Air2p, Trf4p and Trf5p are able to make stable associations with Mtr4p at physiological salt concentrations, but Mtr4p is lost from TAP-purifications of these proteins at 500mM salt, it was investigated whether one of these factors was the salt labile poly(A) polymerase activity observed. For clarity on the figures and in the text, further references to high salt (500mM) preparations will be indicated with the protein name and all else will be indicated as '–TAP' or MATPAC where appropriate. For example, Air2-TAP prepared at 500mM salt will be referred to as Air2p, but Air2-TAP prepared at 75 or 100mM salt will be referred to as either Air2-TAP or MATPAC depending on the context.

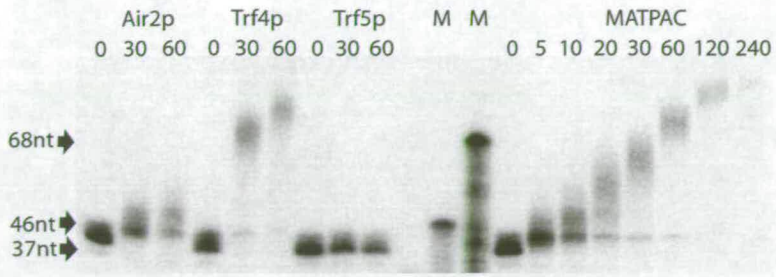


Figure 10: Poly(A) Polymerase Assay for MATPAC and Components
12% polyacrylamide/Urea gel with time in minutes: (Left-Right) Air2p, Trf4p, Trf5p (all high salt preparations), 46nt Molecular Weight Marker, 68nt Molecular Weight Marker (both DNA oligos), MATPAC extended time course.

High stringency affinity-purifications of Air2p, Trf4p and Trf5p were mixed with 5' labeled RNA and ATP and observed for the ability to elongate the input RNA (fig. 10). The results of this assay reveal that the poly(A)-activity co-fractionates with Trf4p at high stringency. Air2p shows a low level activity that may be due to low levels of Trf4p present in this preparation. No activity is observed for Trf5p. Oligo-nucleotide markers (DNA) are provided for approximate size estimation. Based on the sizes shown, the Trf4p sample is able to extend the input RNA by approximately 40nt over a 60-minute time course.

In addition, Mtr4-TAP derived MATPAC preparation was tested for its ability to elongate the input RNA over an extended time course of 4hr. (240 min, fig. 10). The MATPAC achieves a similar but lower polymer length when compared to Trf4p over the same time course (fig. 10), differing by about 10nt over 30 minutes, and less after 60 minutes. The MATPAC also shows a somewhat linear increase in tail size across 30mins, where continued extension begins to taper off thereafter, finally reaching a plateau at 240 minutes. These results are consistent with a distributive mode of action, where the total RNA population undergoes elongation in good synchrony and discrete highly focused bands are not observed.

III.E Chewing Through the Structure

Prominent intermediates in the breakdown of the 37nt substrate (fig. 7, see *) are likely due to stable secondary structure within the molecule (fig. 11). This substrate is predicted by computational RNA modeling to have a 3nt overhang

followed by a 3bp stem structure where the first base pair is a G-U pair. Although these stable structures may slow the ability of the exosome to degrade the substrate they do not present an impenetrable barrier. For the continued analysis of exosome stimulation by the MATPAC, a second substrate was chosen that was predicted to pose greater impediment for the unaided exosome, thus allowing the level of stimulation by the MATPAC to be gauged more accurately. The chosen substrate was a 110nt *in vitro* transcript derived from the serine pre-tRNA of *S. pombe* (*supSI* gene (Krupp *et al.* 1985)). Based on the dG (free energy change) predicted by MFOLD, the 3'-most tRNA stem loop (at –10.4 kcal/mol) is substantially more thermodynamically stable than that of the 37nt transcript (-2.5 kcal/mol, fig. 12). In figure 12 the majority of the respective RNAs outside the stem loop have been replaced by 'A's so as to look at the stability of these structures in isolation. Moreover, the dG of the tRNA stem loop pictured is higher still than even the whole 37nt transcript (-6.6 kcal/mol, fig. 11) and that does not take into account the rest of the tRNA structure, which consists of 4 other stable stem loop structures when properly folded. This pre-tRNA represents a highly ordered and stable molecule. Another major difference between the two is the length of the respective 3' overhangs. The tRNA has a single overhang 'G' at the 3'-end, while the 37nt transcript has a 3nt overhang; thus making the tRNA a potentially more difficult substrate for initial RNA binding.

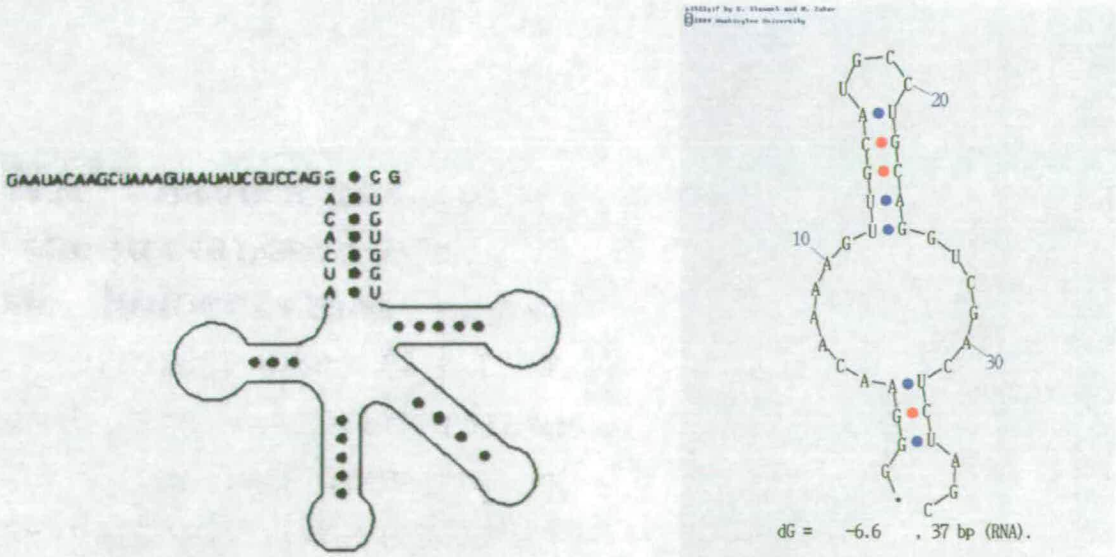


Figure 11: Comparison of Substrate RNA Structures
5' ends on left, 3' ends on right
(Left) pre-tRNA *in vitro* transcript, *supSI*, Figure adapted from pre-tRNA "S" (Krupp *et al.* 1991)
(Right) Structure of the 37nt pBS+/- derived *in vitro* transcript as predicted by MFOLD (Zuker *et al.* 1999)

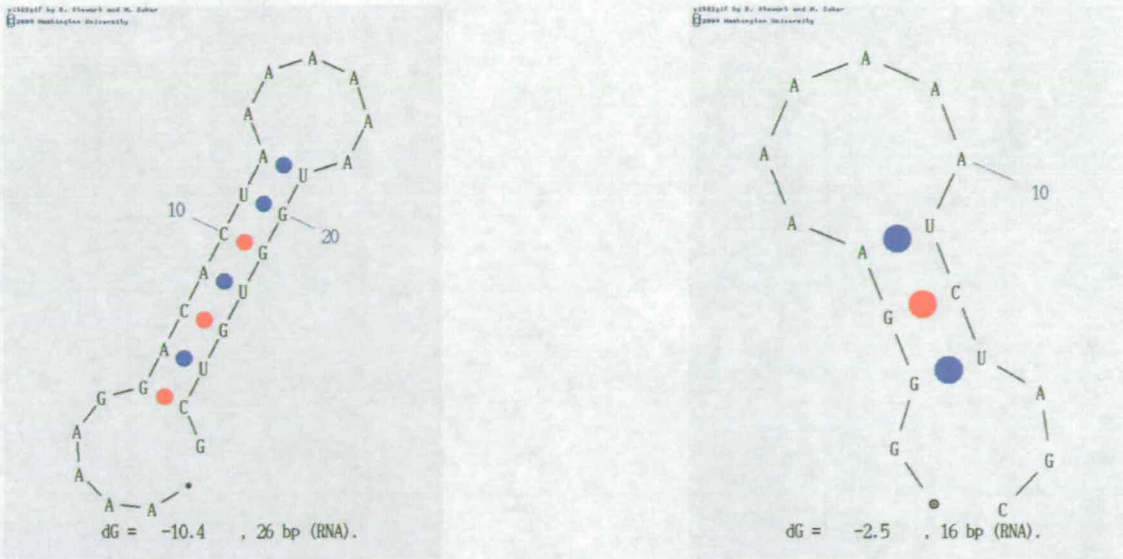


Figure 12: 3'-end Stem Loop Stability
Non-stem structures have been replaced with A-residues
(Left) pre-tRNA substrate 3'-end stem loop structure
(Right) 37nt 3'-end stem loop structure

III.E.1 Exosome and MATPAC vs. tRNA

The exosome was assayed for its ability to degrade the tRNA substrate in the presence or absence of the MATPAC. As shown in figure 13, the exosome has no obvious effect on the level of the full-length tRNA substrate across the 60-minute time course. In contrast, degradative activity is observed when the exosome is combined with the MATPAC. The substrate is initially elongated, then the poly(A)-tails are shortened, accompanied by the loss of the RNA signal. The MATPAC is able to elongate the input RNA in the absence of the exosome.

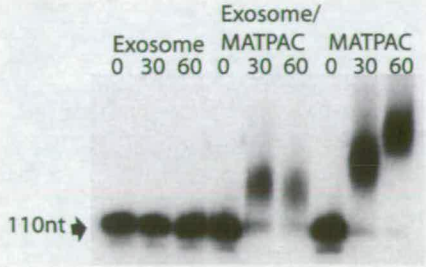


Figure 13: tRNA RNase coupled polymerase assay; 6% polyacrylamide/Urea denauring gel.
Time course shown in minutes:
(Left-Right) Exosome, Exosome + MATPAC, MATPAC

Figure 14 shows the comparative activity of the unaided exosome compared to exosome + MATPAC on a long 12% acrylamide gel. The build-up of a two-nucleotide product is clearly observed in the presence of MATPAC (based on co-migration with an RNA di-nucleotide, pApA, not shown). A ladder of degradation intermediates, starting around 20nt, is also observed (black bar, fig. 14). Figure 15 displays a short exposure of the top of the gel (110nt region) above a long exposure of the bottom of the gel (2nt region). This figure demonstrates how the loss of signal in the 110nt region is directly correlative with the buildup of signal in the 2nt region. Trf4p (fig. 15), Air2p, Mtr4p, and Trf5p (data not shown) from high salt preparations were unable to stimulate exosome digestion of the tRNA substrate. Neither was any combination of these proteins able to achieve similar effect to a MATPAC preparation when combined with the exosome *in vitro,* as shown in figure 15. These data prove the resistance of the tRNA substrate to degradation by the unaided exosome, and the necessity of intact MATPAC complex purified at physiological salt concentrations.

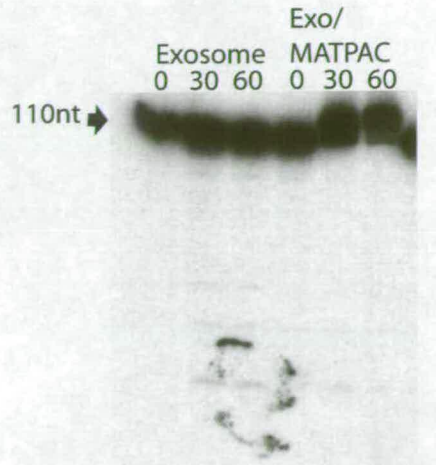


Figure 14: RNase/Polymerase Long Gel 12% polyacrylamide/Urea denauring gel. Time course shown in minutes: (Left-Right) Exosome, Exosome + MATPAC

Bar: Ladder of intermediates visible in Exosome + MATPAC compared to Exosome only.

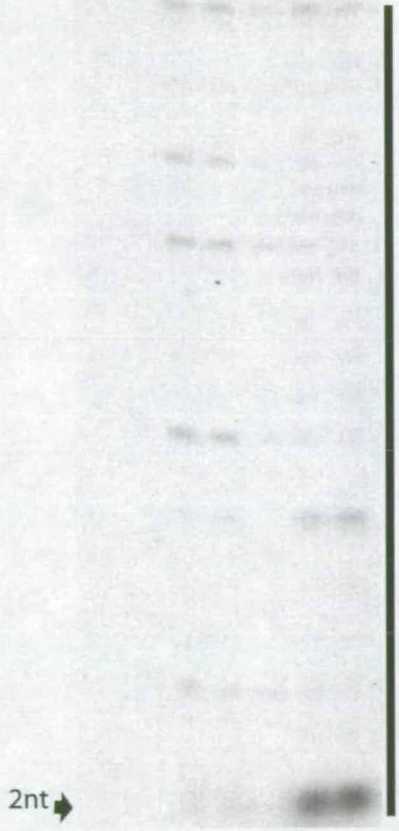
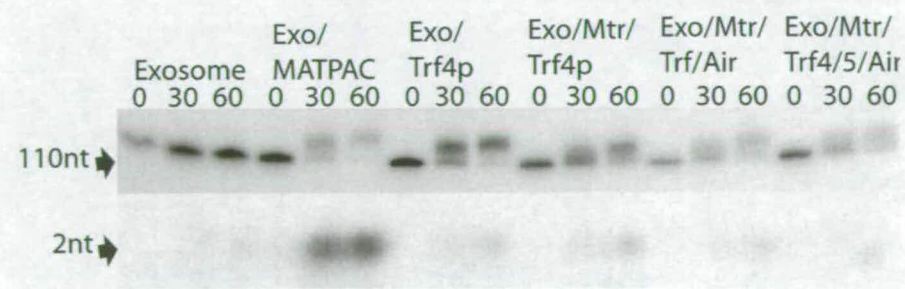


Figure 15: Short (upper) & Long (lower) Exposure MATPAC reconstitution assay. 12% polyacrylamide/Urea denauring gel. Time course shown in minutes: (Left-Right) Exosome (Exo) negative control, Exo + MATPAC positive control, Exo + Trf4p, Exo + Mtr4p +Trf4p, Exo + Mtr4p + Trf4p + Air2p, Exo + Mtr4p + Trf4p + Trf5p + Air2p



To confirm these conclusions, putative MATPAC preparations from the different TAP-tagged strains were purified at 75mM salt stringency and tested for their ability to stimulate the exosome (fig. 16). Air2-TAP, Trf4-TAP, and Mtr4-TAP showed MATPAC activity and all facilitate degradation of the tRNA substrate by the exosome. Each showed the characteristic pattern of elongation followed by degradation in the presence of the exosome as previously shown for Mtr4-TAP derived MATPAC (fig. 16 and 17) and accumulated the 2nt breakdown product (fig. 17). Contrastingly, MATPAC activity was not associated with Trf5-TAP.

The MATPAC associated exosome stimulation resulting from the 75mM TAP preparations does not appear as robust as that obtained from the control preparation (150mM, Mtr4-TAP) in the area of the full length RNA (fig. 17A). Accumulation of the 2nt product is comparable between the Air2-TAP and the control, with the Trf4-TAP and Mtr4-TAP samples accumulating lower amounts (fig. 17B). The Trf5-TAP sample does show some indication of low-level activity but it is does not appear to be greater than that observed for unaided exosome on this substrate (fig. 14 and 15). Importantly the Trf5-TAP preparation does not exhibit the poly(A) polymerase activity and also does not stimulate the exosome in these assays.

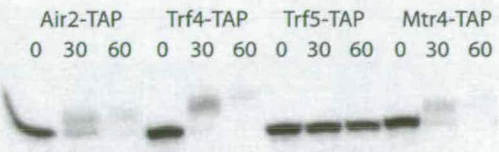


Figure 16: Exosome + MATPAC Polymerase/RNase Assay; 75mM salt preparations of TAP tagged proteins tested for exosome stimulation; 12% polyacrylamide/Urea denauring gel; time in minutes:
(Left-Right) Exo + Air2-TAP, Exo + Trf4-TAP, Exo + Trf5-TAP, Exo + Mtr4-TAP;

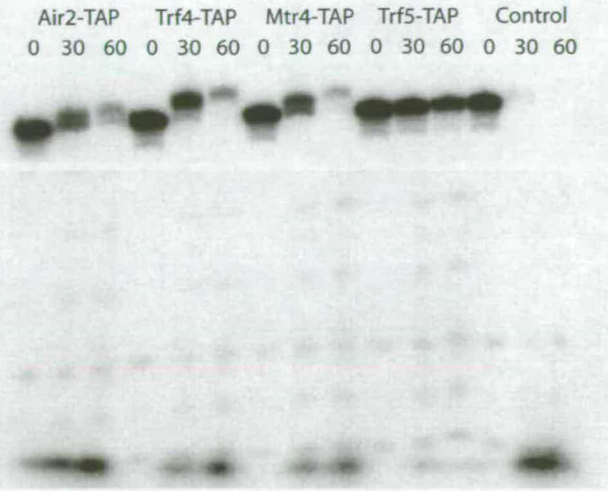


Figure 17: Exosome + MATPAC Polymerase/RNase Assay; same as above with previous 150mM Mtr4-TAP preparation included. Left-Right) Exo + Air2-TAP, Exo + Trf4-TAP, Exo + Mtr4-TAP, Exo + Trf5-TAP, positive Control (Mtr4-TAP, 150mM as fig. 13, 14 & 15)

III.F Is Poly(A) Sufficient?

Mtr4-TAP, Air2-TAP and Trf4-TAP purifications all show poly(A) activity and MATPAC stimulation of the exosome. Experiments were conducted to determine to what extent exosome stimulation was a result of the elongation of substrate RNAs and whether or not intact MATPAC was required for stimulated degradation of these RNAs after elongation.

RNA was pre-polyadenylated by Trf4p purified at high salt, resulting in a mixed population of non-adenylated and polyadenylated RNAs. These samples were then exposed to degradation by the exosome alone and exosome in the presence of Mtr4p and Mtr4-TAP derived MATPAC. Figure 18 shows the polyadenylated species of the pBS +/- transcript (arrow, ~55nt). This figure shows that the polyadenylated species undergoes a similar degradation in the presence or absence of Mtr4p, being characterized by the persistence of species of close to 55nt in length. No stable structures are predicted to be present in the poly(A) tail, yet degradation stalls initially within the first few nucleotides of this substrate, after which no significant level of accumulation of smaller intermediates is observed. Upon addition of the MATPAC, however, increased elongation of the pre-polyadenylated RNAs and a lack of stabilized breakdown intermediates were observed. For all reactions no species were detected in the region corresponding to either the 37nt un-adenylated RNA or the shorter intermediates observed upon its incubation with the exosome (data not shown). These observations indicate that in the absence of the MATPAC the exosome is ineffective in the initiation of degradation on even an unstructured RNA tail *in vitro* and that Mtr4p alone is not sufficient to alleviate this inefficiency.

The tRNA substrate was also pre-polyadenylated prior to incubation with the exosome and the MATPAC. The polyadenylated fraction migrates above the input RNA (110nt, fig. 19 and 20). Phosphorimager analyses (fig. 20, 21, and 22) revealed that the polyadenylated RNA represents 68%, 66%, and 64% of the total RNA detected in the Exosome + MATPAC, Exosome + Mtr4p and Exosome only sample, respectively. When quantified, a decrease in total signal (fig. 20 upper) is observed across samples containing polyadenylated RNA, whereas no decrease is observed in

samples containing non-adenylated RNA and exosome only (qualitative: fig. 19, quantitative: fig. 21 and data not shown).

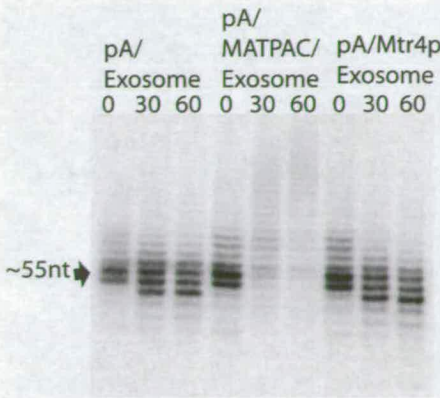


Figure 18: Polyadenylated pBS+/- Transcript Degradation of a prepolyadenylated substrate. 14% polyacrylamide/Urea denauring gel; time in minutes: (Left-Right) poly(A) RNA + Exosome, poly(A) RNA + Exosome + MATPAC, poly(A) RNA + Exosome + Mtr4p

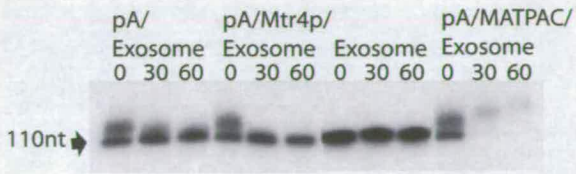


Figure 19: Polyadenylated tRNA Polymerase/RNase Assay Degradation of a prepolyadenylated substrate; 14% polyacrylamide/Urea denauring gel; time in minutes: (Left–Right) poly(A) RNA + Exosome, poly(A) RNA + Exosome + Mtr4p, non-poly(A) RNA + Exosome, poly(A) RNA + Exosome + MATPAC

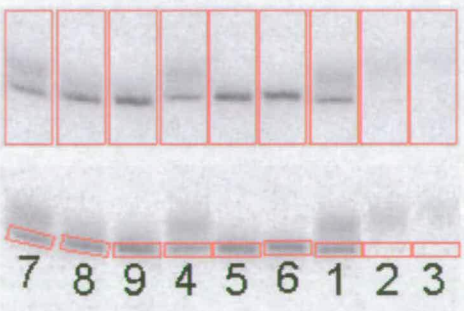


Figure 20: Phosphorimager data Quantification of samples from figure 19: (Left-Right) poly(A) RNA + Exo (7-9), pA + Exo + Mtr4p (4-6), Exo, pA + Exo + MATPAC (1-3)

Upper: Total RNA signal
Lower: poly(A) minus fraction

The region containing the un-adenylated tRNA was separately quantified (fig. 20 lower and 22). An increase in signal is detected in this region for samples that do not contain MATPAC, whereas the signal detected in this region drops by 92% in the presence of the MATPAC. This indicates that samples not containing the MATPAC accumulate tRNAs that have been deadenylated but are stable against further degraded.

While a decrease in total signal strength is observed across all samples, only the sample containing MATPAC shows a net decrease in the 110nt un-adenylated species. Both the exosome only sample and the +Mtr4p sample show an increase in signal strength in the area of the 110nt substrate (fig. 20, 21, and 22), indicating that RNAs from the polyadenylated population are accumulating upon shortening to the length of the un-adenylated species. This effect is more rapid when Mtr4p is present. The proportions of polyadenylated RNA that undergo degradation, and are lost from the total RNA signal measurements (fig. 20 upper), are 39% for the +Mtr4p sample and 20% for the exosome only sample, over the 1hr time course. For the +Mtr4p sample 12% of the polyadenylated RNA remained polyadenylated and the rest was converted to the 110nt species, resulting in a 43% increase in the 110nt species over 1hr. For the exosome only sample 48% of the polyadenylated RNA remained polyadenylated and the rest was converted to 110nt species, resulting in a 36% increase in the 110nt species over 1hr. As the level of polyadenylated RNA present is not measured directly in this study, it is calculated as a function of measured combined signal (fig. 20 upper) minus the signal measured for the 110nt species (fig. 20 lower).

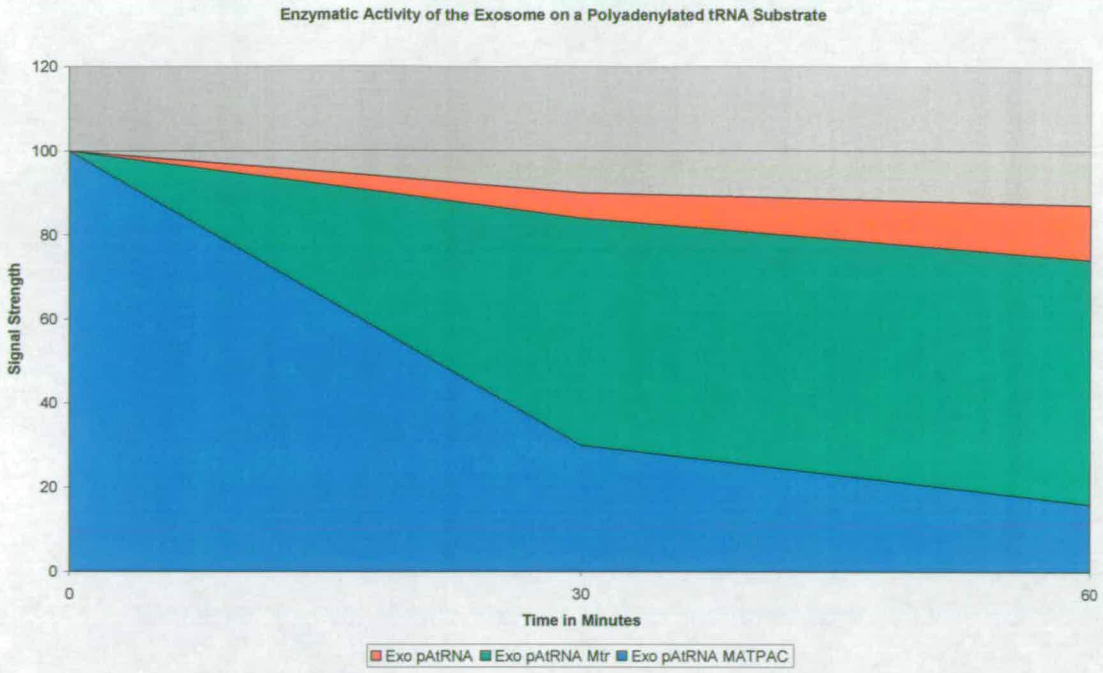


Figure 21: Graph of decay of total RNA (fig. 19, upper)
Red- Exosome only, 10% turnover in 30 min., 13% in 1hr.
Green- Exosome + Mtr4p, 16% turnover in 30min., 26% in 1hr.
Blue- Exosome + MATPAC, 70% turnover in 30min., 94% in 1hr.

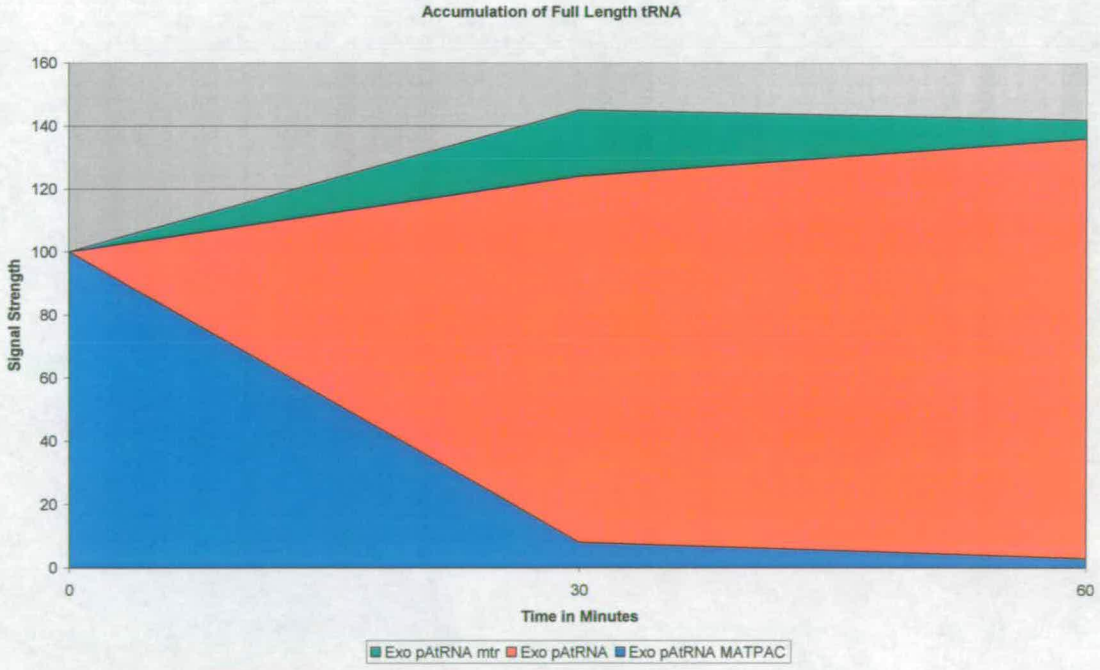


Figure 22: Accumulation of 110nt Substrate (fig. 19, lower)
Red- Exosome only, 24% accumulation in 30 min., 36% in 1hr.
Green- Exosome + Mtr4p, 45% accumulation in 30min., 42% in 1hr.
Blue- Exosome + MATPAC, 92% turnover in 30min, 97% in 1hr.

III.G Discussion of Results

The MATPAC: Here it is reported that Mtr4p exhibits RNA stimulated ATPase activity. The rate of ADP production is increased by more than 2-fold in the presence of RNA. This evidence stands in favor of Mtr4p as an RNA helicase, as its sequence homology suggests. Our two-hybrid data indicate an interaction between Trf4p and Trf5p and published data indicate stable interactions formed between Mtr4p, Trf4p, Air1p and Air2p. Western blot analysis shows salt labile interactions between Trf4-TAP, Trf5-TAP and Air2-TAP with Mtr4p.

Mtr4-TAP purified at physiological salt concentrations stimulates the exosome in the degradation of a 37nt *in vitro* transcript from a plasmid multiple-cloning site and on a stable, highly structured tRNA transcript. Air2-TAP and Trf4-TAP also copurified with an exosome stimulating activity for the degradation of the tRNA substrate. This stimulation is associated with a poly(A) polymerase activity observed explicitly for Mtr4-TAP and in combination with exosome for Mtr4-TAP, Air2-TAP and Trf4-TAP. No such stimulation, nor associated 'poly(A)' activity, is observed in Trf5-TAP preparations when combined with exosome.

Analyses of high stringency preparations of Mtr4-TAP, Air2-TAP, Trf4-TAP and Trf5-TAP indicate that the poly(A) polymerase activity is associated with Trf4p. All TAP-purifications except Trf5-TAP have poly(A) activity at physiological salt, but only Trf4-TAP retains activity upon washing with high salt. Mtr4-TAP shows ATPase activity upon purification in these conditions, so the lack of poly(A) activity in this fraction is not likely to result from disruption of the protein structure. Mtr4p was shown by Western blotting not to associate with Trf4-TAP, Trf5-TAP or Air2-TAP at high stringency thus indicating that these preparations are compromised in the formation of a complex normally formed with this protein.

Trf4p and Trf5p are predicted to be nucleotidyl transferases by sequence homology and Trf4p has been reported to show *in vitro* poly(A) polymerase activity (Aravind *et al.* 1999; Saitoh *et al.* 2002). Trf4-TAP showed poly(A) polymerase activity after purification at high stringency. However, no polymerase activity was detected for Trf5-TAP following purification at high or low stringency. Considering that both Trf4p and Trf5p are highly conserved across the catalytic domain which they share with the poly(A) polymerase enzyme, Pap1p, it seems highly likely that

these proteins are both genuine polymerases. Trf5p may possess greater substrate specificity than Trf4p and therefore was not active under these experimental conditions. Air2p is not predicted to posses poly(A) polymerase activity based on sequence homology but was associated with a low level of this activity even after high salt purification, probably due to residual Trf4p in this preparation. This may be indicative of a stronger affinity of Trf4p and Air2p for each other than either for Mtr4p.

These observations make it likely that that Mtr4p associates *in vivo* with Trf4p, Trf5p, Air2p and probably Air1p. This complex is stable during purification at physiological salt concentrations (75mM-150mM) but is disrupted by treatment with 500mM salt. The two-hybrid data indicates that Mtr4p is likely to interact directly with both Trf4p and Trf5p, whereas the interaction of Air2p and Air1p might be via Trf4p. The complex containing Mtr4p, Trf4p and Air2p, which shows poly(A) polymerase activity and exosome stimulation activity being termed the MATPAC in this study.

Mtr4p and the Trf proteins: Considering the significant homology between Trf4p and Trf5p (57% identical, 74% similar (Sadoff *et al.* 1995)) and that they both form salt labile interactions with Mtr4p, it seems likely that each are in related complexes with perhaps slightly different functions. The fact that Trf5p failed to show polymerase activity *in vitro* may simply be a reflection of greater substrate specificity. In which case Trf4p may form a general pathway MATPAC, and Trf5p may be in a related MATPAC of more specific function.

The idea that these proteins are partially redundant is consistent with the synthetic lethality of mutants containing a double deletion in these two genes, but that *trf4Δ*, not *trf5Δ*, is synthetically lethal with *top1Δ* (Castano[a] *et al.* 1996). Over-expression of Trf5p partially complements the *trf4-1* alelle cold sensitive (cs)-phenotype and complements *trf4Δ top1* synthetic lethality. However, Trf4p is reported to be ~3-times more abundant than Trf5p (Ghaemmaghami S *et al.* 2003) and this may be why over-expression is required for suppression, rather than because of a lack of complete functional redundancy.

When analyzed for RNA processing defects *trf4Δ* showed a weak phenotype, *trf5* showed no phenotype, but *trf4D* / Trf5p depleted showed a strong phenotype (J. Houseley unpublished results). These observations give the impression that, as previously observed in *top1* strains, Trf5p can partially substitute for a lack of Trf4p, but that over all a lack of Trf4p has a greater impact than a lack of Trf5p.

Trf4p is reported as being localized to the nucleus whereas Trf5p is reported as being primarily nucleolar as determined by fluorescence microscopy (Ghaemmaghami S *et al.* 2003). The differential compartmentalization of these two proteins further supports the idea of distinct complexes. A recent study has reported detecting polyadenylated rRNA (Kuai *et al.* 2004). As ribosome synthesis and assembly occur in the nucleolus, aberrant molecules and degradation intermediates would be candidates as substrates for an Mtr4p/Trf5p containing exosome recruitment complex, perhaps functioning in an analogous way to the Trf4p containing MATPAC but with substrate specificity for rRNAs.

AMP formation in ATPase assays: In retrospect it is possible that the AMP formation observed in the early ATPase assays was due to the presence of Trf4p or Trf5p. Other Poly(A) polymerases from yeast show a requirement for an RNA primer (Hafe *et al.* 1975) but primer dependence for Trf4p has not been explicitly tested in this *in vitro* system. Furthermore, given the stringency of the purification and the nature of Mtr4p, an RNA helicase, and the MATPAC or Trf5p, which will have undoubtedly been co-purified, it seems likely that some RNA may also have co-purified. Trf4p has previously been reported to show magnesium dependant 3'-5' DNA exonuclease activity (Wang[b] *et al.* 2002) and phylogenetic analysis indicated that it contains an evolutionarily conserved domain potentially capable of harboring nuclease activity (Rogozin *et al.* 2003). While no nuclease activity was observed in any of the polymerase assays conducted with Trf4p in this study it cannot be ruled out. If polymers were formed from the $\{\alpha\text{-}^{32}P\}$ ATP, then $\{\alpha\text{-}^{32}P\}$ AMP would be released upon subsequent hydrolysis, and detected upon TLC analysis. This seems to be an attractive theory since the AMP formation also appeared to be salt labile, as is the interaction between Mtr4p and Trf4p or Trf5p.

The MATPAC Stimulates the Exosome: The MATPAC is able to stimulate the exosome *in vitro* and enhance its processivity through secondary structures. This allows the degradation of a tRNA substrate that is otherwise very stable and generally inert to degradation by the exosome in the absence of MATPAC. The stimulated activity is characterized by the elongation of the input RNA, followed by its disappearance and the accumulation of low molecular weight species. The major product was a 2nt species, indicating that digestion proceeds to a di-nucleotide. This may be due to a very low binding efficiency for the dinucleotide and small oligos in general.

Similar findings have been made in *E. coli* where PNPase and RNase II, the principal enzymes responsible for RNA degradation, primarily release oligonucleotides of between 2-5nt. These oligos are digested to mononucleotides by oligoribonuclease (Ghosh *et al.* 1999). No direct functional homolog of oligoribonuclease has yet been identified in yeast or higher eukaryotes, but the gene family that includes oligoribonuclease, the RNase D family, is well conserved across prokaryotes and eukaryotes (Koonin 1997; Mian 1997) and several members are present in yeast.

A ladder of degradation intermediates was observed starting from the region of ~20nt, which was not detected in the absence of MATPAC. This could indicate a reduced efficiency of degradation of short species by the exosome in association with the MATPAC, thus making them candidates for repeated rounds of adenylation by the MATPAC followed by degradation by the exosome.

MATPAC Stimulation Requires Intact MATPAC: A key question was whether the MATPAC itself or the polyadenylation is responsible for the observed increase in processivity of the exosome. A mixed population including a pre-polyadenylated species and a non-adenylated species was used to assess this. Analysis of the degradation of the pre-polyadenylated substrates indicated that a poly(A)-tail alone or in the presence of Mtr4p is not enough to stimulate the exosome to the level of exonucleolytic potency observed in the presence of intact MATPAC. The exosome + Mtr4p is more efficient than the exosome only sample in both the

degradation of poly(A)$^+$ RNA and its trimming to the 110nt species. However, even so, it is much less efficient than the MATPAC containing sample.

This suggests that a poly(A) tail improves the kinetics of degradation of a structured substrate. In the presence of an appropriate ATP-dependant helicase, kinetics are further improved, but efficiency is not optimized unless the unwinding of secondary structures is properly coupled to degradation. The main factor in the observed stimulation may be the ability to open RNA secondary structures. Mtr4p alone is apparently inefficient in breaking up strong secondary structure. One possibility is that some protein-protein interactions essential for proper "feeding" of unwound RNA to the exosome are lost. Another possibility is that repeat rounds of polyadenylation are required due to an inherent inefficiency of Mtr4p, rendering attempts at degradation ineffective when decoupled from the polyadenylation activity of the MATPAC.

The MATPAC is Active in the Nucleus: The MATPAC represents a nuclear complex. Trf4p and Air2p exhibit nuclear localization and Mtr4p shows nuclear and nucleolar localization ((Huh *et al.* 2003; Liang *et al.* 1996) and observations of the Tollervey lab). Mutations in *MTR4* show an exosome-like phenotype for nuclear RNA substrates and *MTR4* is an essential gene, indicating that it is required for exosome activity and is lethal because of impaired exosome function in the nucleus. Mutations in *MTR4* do not show defects in the function of the cytoplasmic exosome in mRNA turnover (van Hoof[c] *et al.* 2000). Thus the results presented here are of specific significance to the functions of the nuclear exosome, notably in the processing and degradation of stable RNAs. Exosome mutants accumulate 3' extended forms of the 5.8S rRNA as well as many snoRNAs and snRNAs, and are also defective in the degradation of the aberrant 23S pre-rRNA (Allmang *et al.* 2000; Allmang[a] *et al.* 1999; van Hoof[a] *et al.* 2000). To determine whether MATPAC participates in these functions, Dr. Jonathan Houseley conducted extensive Northern blot analysis of yeast strains lacking Trf4p, Trf5p, Air1p and Air2p. These strains were tested for defects in nuclear RNA processing and degradation.

The results of Dr. Houseley's study are summarised here: 3' extended forms of the U14 snoRNA were not seen in the wild-type, *trf5Δ*, *air1Δ* or *air2Δ* strains, but

were detectable in the *air1Δ, air2Δ* double mutant and a *trf4Δ* strain, with elevated levels in a *trf4Δ* strain that was also depleted of Trf5p. The level of accumulation was similar to that seen in an *rrp6Δ* exosome mutant. A low but detectable level of accumulation of 3' extended 5.8S rRNA was also seen in the same strains. Strong accumulation of the 23S pre-rRNA was observed in *air1Δ/air2Δ* strains and in the *trf4Δ* strains. 23S accumulation was most marked in the *trf4Δ* strain that was also depleted of Trf5p. However, clear 23S accumulation was not seen in the *trf5Δ* single mutant. In the case of the U5 snRNA, exosome mutants increase the ratio of the short to long forms of the RNA, probably because the short form is relatively unstable since it lacks a 3' terminal step structure (Allmang[a] *et al.* 1999; Patterson *et al.* 1987). An increase in the $U5_S$:$U5_L$ ratio was also seen in the *air1Δ*, *air2Δ* and *trf4Δ* strains.

Further work: The kinetics of stimulation by the MATPAC require further investigation. A repetition of the experiment that exposed the exosome to pre-polyadenylated tRNA substrate in the presence or absence of Mtr4p and the MATPAC is required, this time with a single population of polyadenylated RNA. In this way the levels of accumulation of the 110nt product can be observed against a low background and the level of poly(A)+ RNA can be measured directly and accurately across all time points. Such data will solidify the extent to which polyadenylation and Mtr4p contribute, both together and respectively, to the stimulation of the exosome as compared to the MATPAC.

As a result of this work a new collaboration has been formed with the laboratory of Dr. James Anderson, who has recently published findings on the role of Trf4p and the exosome in the degradation of hypo-modified $tRNA_i^{met}$ (Kadaba *et al.* 2004). *In vitro* tests similar to those described in this study will be carried out in order to characterise the activity of the mutant gene products of *TRF4* and *RRP44,* which were identified through a genetic screen for suppressor mutations of the ts-phenotype caused by the tRNA hypomodification defect.

It is clear that Trf5p and Mtr4p interact, as determined by yeast two hybrid and co-immunoprecipitation studies. Although *TRF5* deletion does not show a detectable phenotype on the substrates tested by Dr. Houseley, depletion of *TRF5*

does enhance the phenotypes exhibited by *TRF4* deletion mutants. Thus though they may not be entirely redundant homologues, they must have a degree of overlapping function. Trf5p is reported to be nucleolar in localisation, where as Trf4p is nuclear (Huh *et al.* 2003). Recent studies indicate the presence of polyadenylated species on rRNA that may be localised to the nucleolus (Kuai *et al.* 2004). Trf5p may be present in a lower abundance complex with Mtr4p and other factors, which serves a more substrate specific function in combination with the nuclear exosome than the MATPAC described here. Hence elucidating the role Trf5p and the significance of its interaction with Mtr4p is of significant importance to completing the list of exosome activating co-factors.

IV Results and Discussion

Biochemical Activation of the Exosome

IV.A Are PNPase and the Exosome Similar Enzymes?

The range of substrates and assortment of enzymatic components make the topic of exosome activation both curious and attractive. Most theories have concentrated on the need for co-factors to modulate the complex in particular catabolic contexts, thus conferring substrate specific activity on the complex.

However, the topic of biochemical activation is one that has not been addressed in the literature. Given that the exosome bears strong compositional homology to PNPase, and is postulated to bear structural homology as well (section I.E), it seemed logical to examine the behaviour of the complex under conditions derived from PNPase biochemistry.

The first published reference to PNPase dates to 1956 (Grunberg-Manago *et al.* 1956), in a publication describing the enzyme from the bacteria *Azotobacter vinelandii*. This work was a follow-up to previous work by the same group describing the synthesis of nucleic acid polymers (Grunberg-Manago *et al.* 1955). In 1957 R.F. Beers, who, in addition to S. Ochoa, pioneered early studies on the PNPase, conducted a similar study in *Micrococcus lysodeikticus* (now *M. luteus*), which had the added benefit of a less physically damaging lysis method (Beers 1957).

The initial discovery of the enzyme was correlated with the production of nucleic acid polymers, describing it therefore as a synthetase rather than an exonuclease. However, while it has been shown to produce heteropolymeric tails *in vitro*, it had long been assumed that PNPase did not function as a synthetase *in vivo* do to unfavourable thermodynamics. The chemical equation is shown in figure 1.

$$pNpN(pN)_x + P_i \rightleftharpoons ppN + pNpN(pN)_{x-1}$$

Figure 1: PNPase catalysed reaction
Ribonucleotide mono-phosphate polymers are degraded 3'-5' in the presence of excess inorganic phosphate, or polymerised in the presence of excess ADP.
p: 5' phosphate, N: nucleotide, P_i: inorganic phosphate

The equilibrium constant of the PNPase reaction is close to unity and the intracellular concentration of inorganic phosphate exceeds that of NDPs, suggesting that phosphorolysis of RNA polymers is favourable *in vivo*. Ugurbil *et al.* (1978) estimate the P_i concentration ($[P_i]$) to ~9mM in *E. coli* cells beginning anaerobic glycolysis. The $[P_i]$ prior to the initiation of glycolysis is higher as $[P_i]$ is known to drop by 80% as a consequence of the production of sugar phosphates, primarily fructose 1,6-bisphosphate, and NTPs during this process. NDP concentrations remain relatively unchanged (Ugurbil *et al.* 1978). The NMR generated peak intensity of P_i is greater than for NDPs in both anaerobic (Shulman *et al.* 1979; Ugurbil *et al.* 1978) and aerobic glycolytic (respiratory) growth (Navon *et al.* 1977; Ugurbil *et al.* 1982). A separate analysis using other than NMR-based methods puts intracellular [ADP] at 0.4mM under glucose limiting conditions. Intracellular [GDP] is also given, but the calculated concentration is below the stated limit of detection (Buchholz *et al.* 2001). Studies on the activity of PNPase as a polymerase *in vivo* quote the intracellular $[P_i]$ at 10mM (Mohanty *et al.* 2000; Yehudai-Resheff *et al.* 2001), and Monhanty *et al.* (2000) reference Shulman *et al.* (1979) in this calculation, but it is not immediately clear how this precise number was determined. In addition to the assumed thermodynamic barrier to polymerisation, the presence in *E. coli* of hydrolytic exonucleases such as RNase II, which is responsible for 90% of RNA turnover, and oligoribonuclease would scavenge any likely primers for polymerisation. The role of PNPase in RNA metabolism is reviewed by Coburn and Mackie (1999), and Deutscher and Li (2001).

A number of studies now report the activity of PNPase and its homologues as polymerases *in vivo*, most notably studies in *E. coli* (Mohanty *et al.* 2000) and in plant chloroplasts (Rott *et al.* 2003; Yehudai-Resheff *et al.* 2001). Based on the model that the exosome may be included amongst PNPase like molecules, experiments were carried out to determine whether the exosome was also capable of polymerisation of ribonucleic acids or phosphorolytic degradation *in vitro*.

Exosome was prepared as described in section II.L and assayed for the ability to polymerise rADP in the presence of a 37nt RNA primer (II.K.3 by method II.M.4.2). 20µl of exosome preparation, representing 10% of a typical TEV cleavage reaction from 2L of culture grown to between OD2-3, generally gave Coomassie

stainable bands with approximately 100-150ng/band upon SDS-PAGE. Using Rrp4p as the standard band for calculation, with a predicted molecular weight of 39kDa, that is approximately 2.6 – 3.8 x 10^{-12} moles (2.6-3.8 picomoles) of Exosome in 20µl. Hence each reaction conducted with 4µl of standard exosome preparation can be expected to have on the order of 0.5-0.8 picomoles of exosome complex. This approximation works out to ~26nM in a 25µl reaction volume. Exosome yields between different TAP strains were comparable.

IV.A.1 ADP induced exosome stimulation

Varying (r)ADP concentrations were chosen in order to illicit an RNA polymerase activity from the exosome through its phosphorolytic catalytic subunits. This maybe considered as an RNase coupled polymerase assay because the hydrolytic activities of the exosome appear to remain unhindered under the conditions of its predicted polymerase activity.

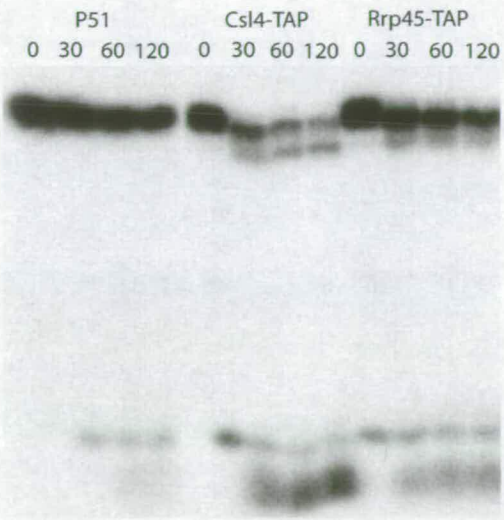


Figure 2: RNase assay
12% polyacrylamide/Urea denauring gel; time course shown in minutes: (Left) -control P51, (Centre) Csl4-TAP, (Right) Rrp45-TAP

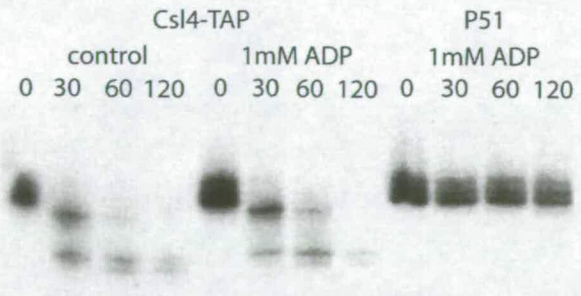


Figure 3: PNPase-like Polymerase assay
RNase assay in the presence of added ADP; time in minutes:(Left) Csl4-TAP control, (Centre) Csl4-TAP +1mM [ADP], (Right) - control P51 +1mM [ADP]

Yeast strains SC1415 and SC1652 containing TAP-tagged Csl4p and Rrp45p respectively were screened for the ability to produce active exosome preparations and tested via an RNA hydrolysis assay. Extracts made from yeast strain P51, which

does not contain a TAP-tagged protein, were prepared in the same fashion and mock-purified over IgG sepharose and TEV eluted. This mock assay shows the background level of RNA hydrolysis in the absence of a TAP-tagged exosome component in the protein extract.

As shown in figure 2, the purified Csl4-TAP is more active than the purified Rrp45-TAP. This may have been the consequence of a structural rearrangement cause by placing the TAP-tag on a core exosome component. Rrp45p is an RNase PH homologue and thus is predicted to make up the PNPase like structural scaffolding upon which the complex is built. Furthermore, since the object of this line of examination was to achieve the reversal of the PH phosphorolysis reaction, it may be crucial that all PH homologues have an appropriate native structure. Thus the Csl4-TAP strain was used for subsequent analysis.

Csl4-TAP was tested for its ability to elongate the same 37nt *in vitro* transcript from linearised pBS+/- used in the RNase assay. This was carried out in the presence 1mM ADP; double the concentration used in early polymerisation assays on PNPase (Beers 1957). However, this did not produce any obvious elongation of the primer and the breakdown pattern is not clearly different from the Csl4p control (figure 3).

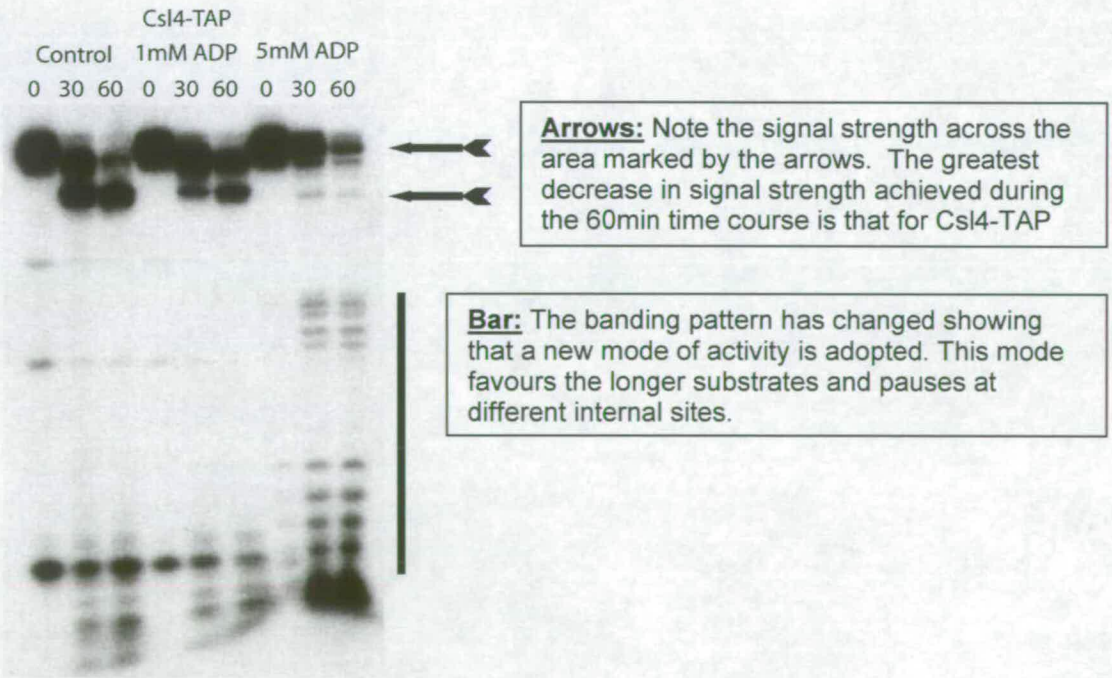


Figure 4: PNPase-like Polymerase Assay RNase assay in the presence of ADP; time in minutes: (Left) control Csl4-TAP, (Centre) +1mM [ADP], (Right) +5mM [ADP]

Interestingly, where adding 1mM ADP failed to manifest any observed effect on exosome activity, the addition of 5mM ADP has had a marked effect. The persistence of the full-length substrate is decreased, whereas degradation intermediates of lower polymer length are accumulated.

IV.A.2 Combinatorial effects and P_i Induced Stimulation

In contrast to the intentions of the ADP addition experiments, complementing the reaction with inorganic phosphate was predicted to stimulate phosphorolytic exonucleolytic activity.

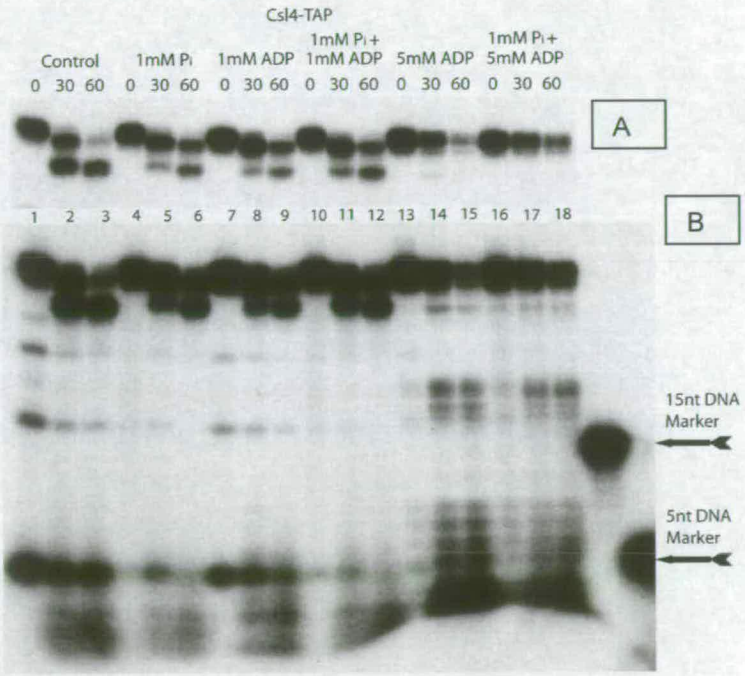


Figure 5: RNase / Polymerase Assay
Carried out in the presence of added ADP and/or phosphate; time in minutes: (Left – Right)
(1-3) control Csl4-TAP; (4-6) +1mM [P_i]; (7-9) +1mM [ADP]; (10-12) +1mM [P_i, ADP]; (13-15) +5mM [ADP]; (16-18) +1mM [P_i] / 5mM [ADP]
A: short exposure B: long exposure

The addition of inorganic phosphate could potentially have changed or accelerated the RNase behaviour of the exosome complex. Under normal assay conditions in the absence of added inorganic phosphate the exosome displays a hydrolytic exonuclease activity (Mitchell *et al.* 1996). Upon the addition of phosphate, if the PH domains are active and accessible to the template RNA and phosphate, an increase in phosphorolytic exonuclease activity is predicted.

Figure 5A shows no obvious difference in the relative activity of either reaction treated with 1mM P_i or 1mM ADP. In fact, both seem to be somewhat inhibitory, as judged by the amount of full-length transcript that is still present after 60 minutes compared to the Csl4-TAP control. Combining these two conditions looks much the same as either 1mM P_i or ADP alone. However, raising the ADP concentration to 5mM leads to a new pattern of processing intermediates.

This new pattern is shown figure 5B, favouring degradation of the full length and the longer breakdown intermediates. Shorter RNAs in the region of ~20nt-16nt and again between ~8 – 2nt are strongly stabilised. The decrease in efficiency is not consistent intensity across all sizes from 20nt and below. Short areas of clearing, representing increased efficiency of degradation of products were seen between ~15-10nt in length. It seems likely that secondary structures in the intermediates are responsible for this pattern. Potential folding of the full-length transcript and the degradation intermediates is shown in figure 6.

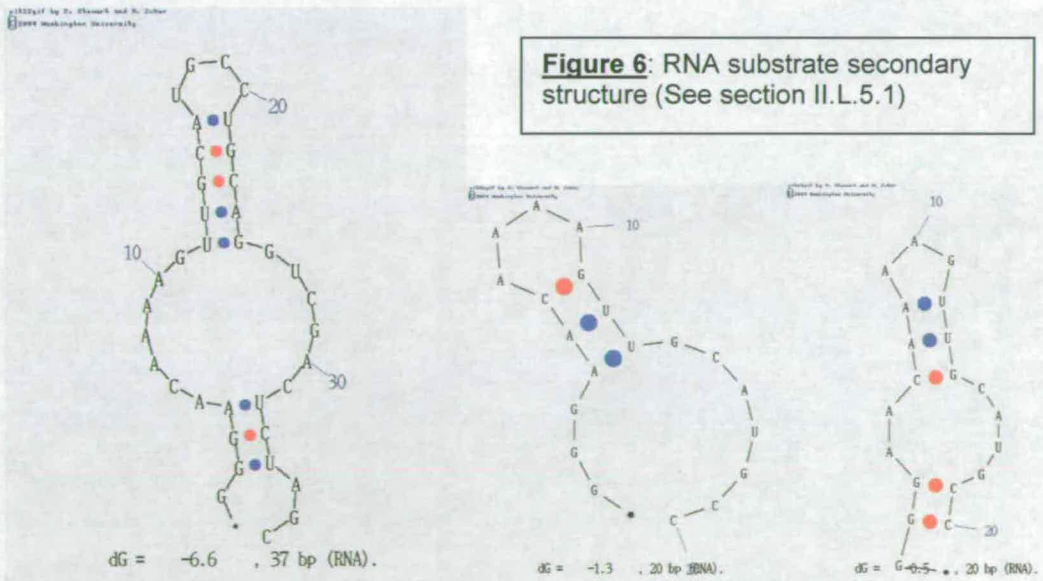


Figure 6: RNA substrate secondary structure (See section II.L.5.1)

(Left) Initial 37nt substrate at 30°C
(Centre) 20nt intermediate structure A
(Right) 20nt structure B

Secondary structure is also likely to be responsible for the intermediates that initially stall the exosome in figure 4A in the area proximal to the full length RNA. Figure 6 demonstrates the predicted structures of the *in vitro* transcript used in these assays. Clearly the stem at 34nt (4nt from 3' end) would present a challenge to exonuclease activity and this may be the reason for the degradation intermediates shown in figure 4 A, across all but the samples containing 5mM ADP.

These results are consistent with a shift in the catalytic activity of the exosome in the presence of 5mM ADP. If this concentration of ADP was sufficient to initiate polymerisation on stems with short primers in the PH active site(s), then such bands might be observed. Degradation normally appears to stall weakly in the region of ~18nt. These intermediates are stabilised and are potential substrates for polymerisation. Similarly for the very small oligonucleotide and dinucleotide products, there is a strong evidence of laddering. Why should this not also be the case for the primary intermediates that are strongly stabilised (panel A, figure 5)? It is possible that these intermediate are the first to be extended, as they are the first to be produced, and as such have been missed by the time course. A finer resolution time course is required in the period of time from 0 to 30 minutes to determine if the initial intermediates normally present during this time are first extended. If so, such extension may contribute to their rapid degradation.

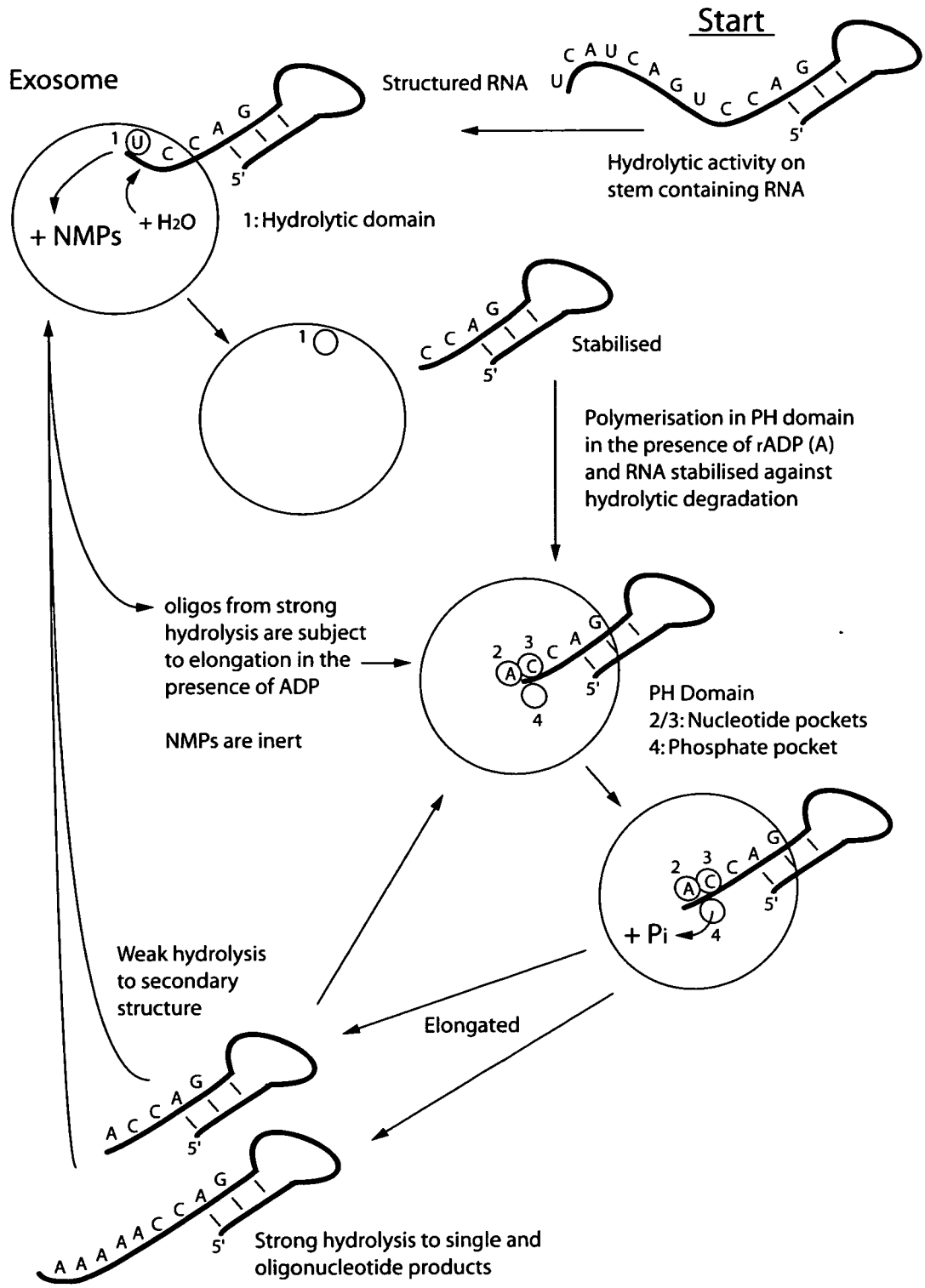


Figure 7: Proposed schematic model for exosome activity in the presence of 5mM ADP Start > Structured RNA with a heteropolymeric tail is subject to hydrolytic degradation by the exosome; Secondary structure leads to stabilisation; Stabilised intermediates are subject to polyadenylation by the Exosome RNase PH homologues; Substrates that acquire short tails are subject to weak hydrolysis back to the secondary structure, substrates that acquire long tails are subject to strong hydrolysis to oligo- and mono-nucleotides; Oligonucleotides are subject to polyadenylation and strong hydrolysis.

Pausing at structural features in the RNA substrate is not simply a question of stem-loop stability, but also active site accessibility. Neither PNPase nor RNase II is effective at degrading small oligonucleotides (Littauer *et al.* 1982; Shen *et al.* 1982). In *E. coli* another exonuclease, the essential protein enzyme oligoribonuclease, is responsible for the complete degradation of RNA to mononucleotides. Upon loss of this enzymatic activity cells accumulate oligoribonucleotides 2 to 5 residues in length ((Ghosh *et al.* 1999) and reviewed in (Deutscher *et al.* 2001)). A stem structure might be sufficient to decrease the efficiency of degradation for enzymes unable to be processive on short oligos. They simply run out of rope, so to speak.

The combined presence of 1mM phosphate / 5mM ADP decreased exosome activity (figure 5). This is predicted to result from the presence of phosphate in some PH active sites preventing ADP polymerisation and promoting phosphorolysis.

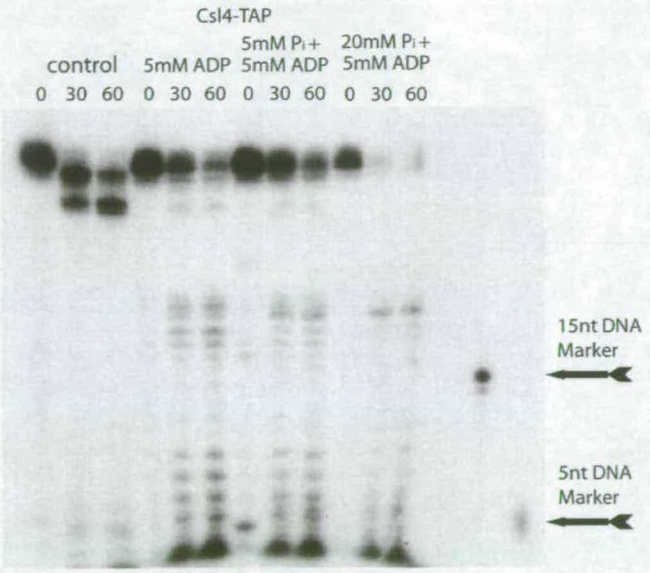


Figure 8: RNase / Polymerase Assay
Carried out in the presence of added ADP and/or phosphate; time in minutes: (Left – Right) control Csl4-Tap; +5mM [ADP]; +5mM [P_i, ADP]; +20mM [P_i] / 5mM ADP

Figure 9A: Phosphorimager Quantification Samples ordered as shown in Figure 8: Control / +5mM ADP / +5mM P_i & ADP

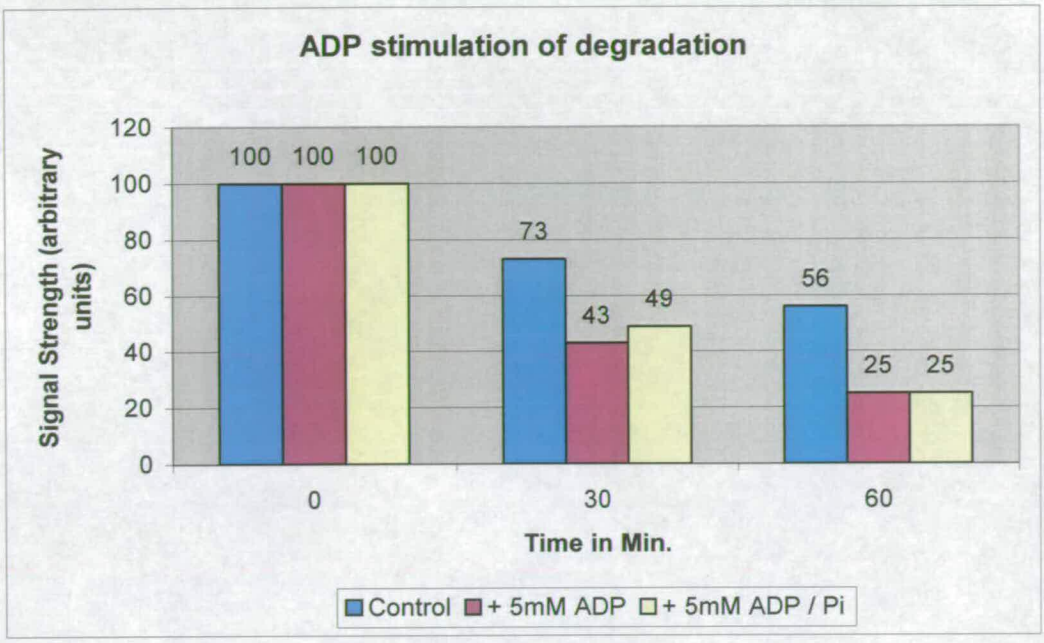


Figure 9B: Quantification data for samples as shown in Figure 8A: Control / +5mM ADP / +5mM P_i & ADP (20mM P_i & 5mM ADP not shown)

As shown in figures 8 and 9, increasing the phosphate concentration to 5mM did little to alter the degradation signature characteristic of 5mM ADP. The result is comparable to that observed for 1mM phosphate + 5mM ADP. Both the 5mM ADP and the 5mM ADP + P_i samples show approximately 2-fold stimulation to degradation through the 30 minute time point (fig. 9B).

A different result was obtained upon incubation with 20mM phosphate and 5mM ADP. It initially appeared that the exosome had been stimulated further than by 5mM ADP alone, as the input RNA all but disappeared across the 60-minute time course. Bands in the intermediate regions also appeared less abundant. This increase in degradation activity is not, however, accompanied by an increase in signal accumulating lower in the gel, and thus it does not appear that the disappearance of high molecular weight signal can be correlated with the accumulation of low molecular weight signal. This casts doubt on the hypothesis that the combination of 20mM phosphate with 5mM ADP confers a heightened state of exonucleolytic stimulation upon the exosome beyond that of 5mM ADP alone. As this result does

not appear to be indicative of a genuine stimulation effect it has been omitted from the quantification analysis presented in figure 9.

It was predicted from the result presented in figure 5 that increasing phosphate concentration might be able to abolish ADP derived stimulation. It was shown in figures 8 and 9 that this does not appear to be the case. Because of the anomalous loss of labelled RNA resulting from the combination of 20mM phosphate + 5mM ADP, this experiment was repeated this time including a 20mM phosphate only control. The result is shown in figure 10. This result confirmed the phenomenon previously observed with the 20mM P_i + 5mM ADP combination (the "disappearing RNA" anomaly), and also shows a potentially novel degradation pattern resultant from 20mM P_i in the absence of additional ADP.

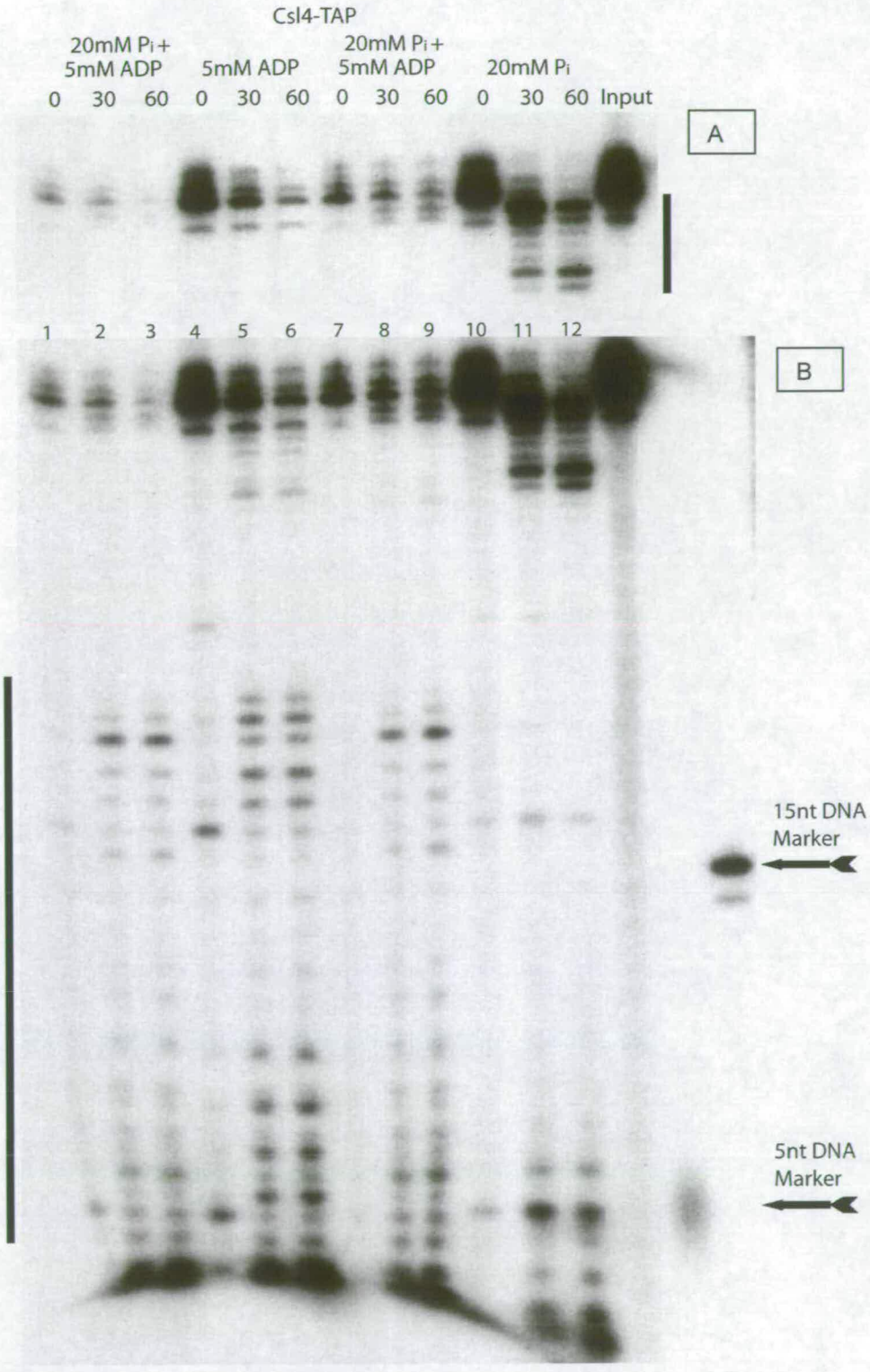


Figure 10: RNase / Polymerase Assay
Time in minutes
(Left – Right)
(1-3) +20mM [P_i] / 5mM ADP from Fig. 7; (4-6) +5mM [ADP]
(7-9) +20mM [P_i] / 5mM ADP; (10-12) +20mM [P_i]; Input RNA

It was observed that some intermediates were accumulating in the "zero" time point of control and experimental samples, which corresponds to samples with exosome added that were left on ice. These series of experiments were therefore repeated but taking the zero time point on ice prior to the addition of enzyme. This was found to alleviate the problem of pre-30 degree incubation intermediate accumulation (fig. 11 and 12)

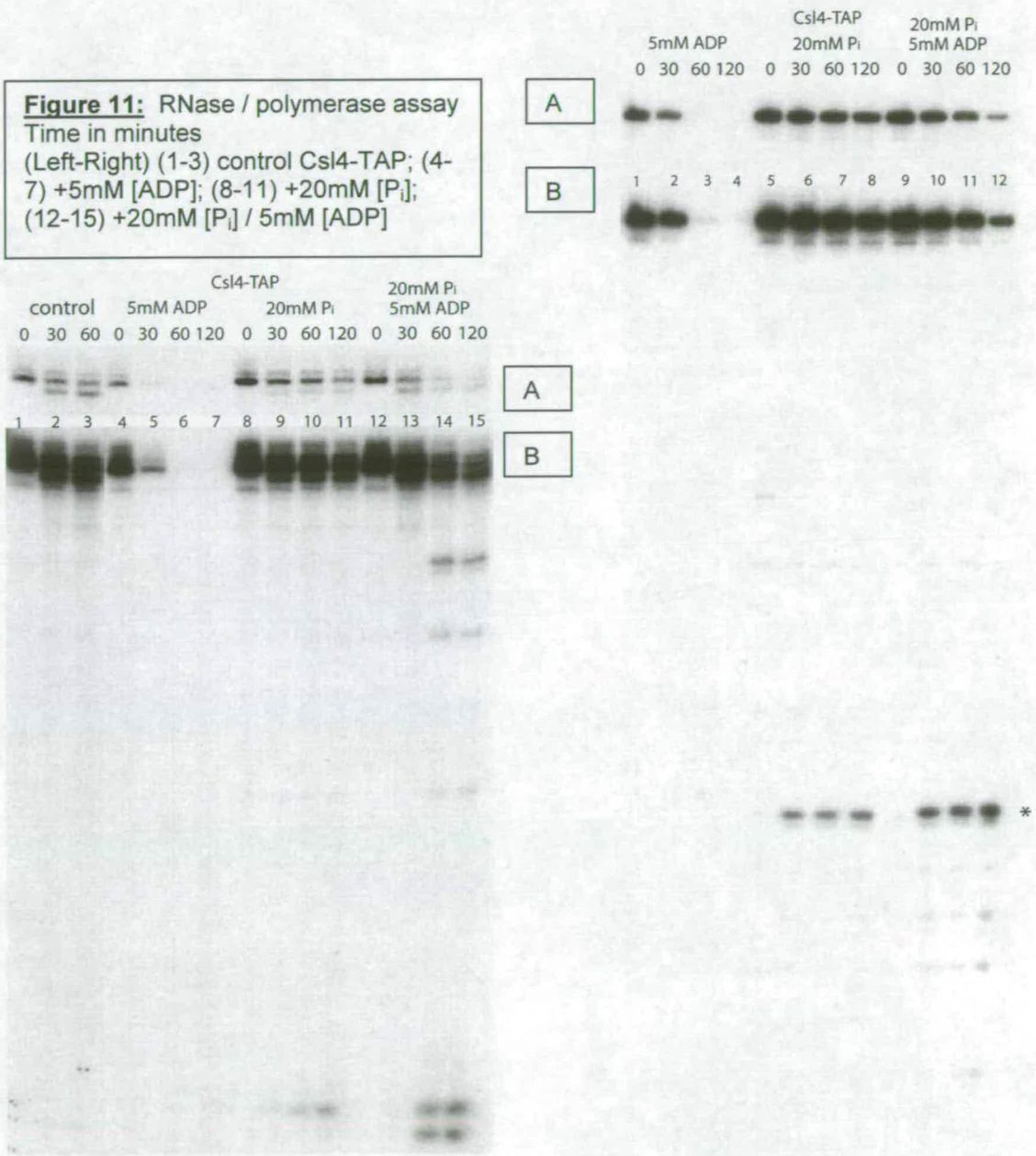


Figure 11: RNase / polymerase assay
Time in minutes
(Left-Right) (1-3) control Csl4-TAP; (4-7) +5mM [ADP]; (8-11) +20mM [P_i]; (12-15) +20mM [P_i] / 5mM [ADP]

Figure 12: RNase / polymerase assay tRNA; separated on a 6% polyacrylamide/Urea gel; time in minutes: (Left-Right) (1-4) +5mM [ADP]; (5-8) +20mM [P_i]; (9-12) +20mM [P_i] / 5mM [ADP]

Figure 11 depicts the result after exclusion of enzyme from the zero time point. The 5mM ADP treated sample appears enormously stimulated but due to the lack of accumulating signal lower in the gel this is reminiscent of the "disappearing RNA" result encountered previously for the phosphate/ADP combination.

On the other hand, the 20mM phosphate sample appears to be mildly stimulated compared to the control with a slightly different pattern of intermediates (panel A), whereas the combined phosphate/ADP sample shows clear stimulation though in a mechanism more resemblant to the control; at least with regards to the initial break down of the full length input RNA.

IV.A.2.1 110nt tRNA substrate

Figure 12 shows the results of these experiments carried out on a 110nt *in vitro* transcript derived from the serine pre-tRNA, *supSI*, of *S. pombe* (see sections II.N.2 and III.E MATPAC). The data shown was collected with the zero time point prior to the addition of exosome to the reaction. These experiments had previously been conducted under standard conditions (enzyme added prior to zero time point collection), and the problem of pre-30 degree incubation intermediate accumulation was observed in the 5mM ADP sample. The combined phosphate/ADP sample exhibited the anomalous, "disappearing RNA", results under these conditions (data not shown).

It seems as though all the intermediates are shared between the 20mM phosphate sample and the phosphate +ADP sample, but the combined sample achieves a significantly higher level of exonucleolytic activity. The accumulation of the strongly stabilised short intermediate in lane 12, with the concurrent decrease in full-length RNA, indicates a preferential stimulation of degradation of the full-length RNA over the smaller intermediate. Lanes 10-12 show a steady increase in this band, in contrast with lanes 6-8 which appear to be more or less in steady state, indicative of an equilibrium between the rate of turnover and the generation of the intermediate in the 20mM phosphate only reaction.

The use of this 110nt transcript gives comparable results to those observed with the 37nt transcript. With either substrate degradation was stimulated in the presence of 20mM phosphate. The 37nt transcript is increasingly unstable compared

to the control in the 5mM ADP reaction condition when enzyme is present prior to t = 0 sample collection. In contrast, the 37nt substrate is less stable in the combined ADP / phosphate conditions when no enzyme is present at t = 0. The tRNA is also less stable in the combined ADP / phosphate condition when no enzyme is present at t = 0.

IV.B "Disappearing RNA"

Figures 8, 10, 11 and 12 display results that I have thus far described as the "disappearing RNA" anomaly. These are seen as follows: figure 8, 20mM P_i + 5mM ADP; figure 10, lanes 1-3 and 7-9 (both are 20mM P_i + 5mM ADP, 1-3 are from the same samples shown in fig. 8); figure 11, lanes 4-7 (5mM ADP); figure 12, lanes 1-4 (5mM ADP).

I have singled out these particular results as anomalous because a decrease in the radio active signal observed in the area of the full length substrate RNA cannot be correlated with a build-up of radio active signal elsewhere. This is not indicative of the full-length substrate having been subjected to 3'-5' exonucleolytic digestion. Looking at the zero time points of these samples it can be seen that signal is vastly decreased (fig. 7 and 9), even in the absence of enzyme (fig. 11).

A variable in these results is the conditions under which the phenomenon is observed. The disappearance of signal is observed in the combined 20mM P_i + 5mM ADP for both the 37nt substrate (fig. 7 and 9) and the 110nt substrate (data not shown), when the exosome (enzyme) is added to the reaction mixture prior to collecting the zero time point sample. However, when the exosome is omitted from the mixture until after collection of the zero time point the phenomenon manifests itself in the 5mM ADP sample (no added phosphate) for both the 37nt substrate (fig. 11) and the 110nt substrate (fig. 12).

An experiment was carried out to determine qualitatively whether precipitation of the labelled substrate RNA in the anomalous test samples might be responsible for the observed phenomenon (methods II.M.4.3). The experiment monitored whether the labelled substrate RNA partitioned with the reaction solution or remained as a pellet in the microfuge tube when the incubation period was followed by centrifugation and the reaction solution transferred to a fresh tube. The

results indicate that under some conditions the input RNA does appear to be precipitated. While the cause of the observed precipitation remains to be determined, this result suggests that it is precipitation of the labelled RNA that is responsible for the observed loss of signal under some experimental conditions.

IV.C Detecting Polymerisation

In order to test the theory that the exosome is able to polymerise short oligo(A)-tails onto the end of primer RNAs *in vitro*, samples of exosome were incubated with [α-32P] ADP in the presence of 5mM unlabelled ADP and unlabelled 37nt and 110nt tRNA primers (data not shown, see section II.L.7 and II.N.4.2). This experiment was aimed at revealing RNAs subjected to oligo-adenylation with [α-32P] ADP. No positive results were obtained. However, this may be due to the low specific activity of the ADP at 5mM.

IV.D Discussion of Results

It is clear that the addition of ADP and phosphate have distinct effects on exosome activity, and that some synergistic effect may result from their combination. There are some technical problems that need to be overcome in the anomalous result characteristic of the phosphate/ADP reaction with enzyme pre-incubation and ADP-only reaction with no enzyme pre-incubation. It is curious that the conditions under which this phenomenon is observed changes dependant upon whether or not the enzyme is added prior to collection of the zero time point sample.

Stimulation by P_i, ADP and P_i + ADP: Despite these technical difficulties it is significant that phosphate, ADP, and combinatorial stimulation effects have been observed. For the 37nt substrate stimulation was achieved by addition of either 5mM ADP or 20mM phosphate. ADP addition (fig. 4, 5, 8, 9 and 10) results in a pronounced increase in the rate of degradation of the full-length product. This is accompanied by the loss of accumulation of the slightly shortened intermediates, a hallmark of the unaided complex, and an increase in accumulation of intermediates of smaller size, both ~20-16nt and ~2-8nt in length. Phosphate alone (fig. 10 and 11)

resulted in less pronounced stimulation, with only a variation in intermediate accumulation intensity.

The results of the experiments on the 110nt tRNA substrate support the observations made on the 37nt substrate and also provide clear evidence of nucleolytic stimulation. Here marked stimulation was seen both for phosphate only and the combined phosphate + ADP reaction (fig. 12). 20mM phosphate alone resulted in the same pattern of accumulated intermediates as seen when combined with 5mM ADP, although the level and speed of accumulation was higher in the combined sample. These intermediates were not observed in exosome only reactions on the tRNA substrate (see MATPAC results III.E).

It appears clear that complex alterations in exosome activity are elicited by the presence of elevated levels of ADP and/or phosphate. In the presence of high ADP concentrations *in vitro* the exosome may be able to polymerise short oligo(A)-tails onto stabilised intermediates (fig. 7), which then become better substrates for degradation. The ADP derived stimulation of RNA degradation reported in this study is found upon incubation of the exosome with 5mM ADP, while the reported intracellular concentrations of ADP in *S. cerevisiae* are between 0.3-0.7mM (Reibstein *et al.* 1986; Saez *et al.* 1976; Williams *et al.* 1993). However, cellular ADP stores in *S. cerevisiae* may be compartmentalised and concentrations may be much higher in the nucleus than the calculated intracellular total (Brindle *et al.* 1990; Williams *et al.* 1993). Moreover, the exosome is predicted to execute phosphorolytic exonucleolysis *in vivo*, so its local [NDP] may be much higher. The phosphate-derived effect was observed at 20mM [P_i]. The intracellular concentration of P_i has been reported at ~3-5mM (Brindle *et al.* 1990; Brindle 1988; Sheldon *et al.* 1996), but also as high as 20mM (Salhany *et al.* 1975).

Given that the forward and reverse reactions are almost energetically equal, at high concentration of inorganic phosphate the PH domain activity can be characterised by competence for phosphorolysis, shortening an RNA polymer, consuming P_i and releasing NDPs; and at high concentration of ADP, the PH domain activity can be characterised by competence for polymerisation, consuming ADP and releasing inorganic phosphate.

Catalytic Synergy between P_i and ADP: It is unclear then how the combined P_i + ADP condition could accelerate exonucleolytic activity beyond either alone as the function of the PH domain in the combined condition should be simply an expression of its equilibrium in that condition, it will either be more likely to phosphorolyse or polymerise. However, in the case that even the addition and removal of one 'A' residue cyclically will in time result in stimulation of exonuclease activity into stable structures via an increase in RNA binding affinity or by allowing numerous attempts, some of which will coincide with the spontaneous opening or rearrangement of the RNA structure in solution (breathing), then we could expect to see a synergistic affect when these reagents are combined.

When substrate RNAs have been shortened to a length proximal to a secondary structure they can become inefficient substrates for hydrolytic or phosphorolytic degradation. Structured substrates with little or no overhang may be poor candidates for enzyme binding, which is required for processivity. When binding interactions between the enzyme and the RNA substrate occur solely in the active site, a distributive mode of activity is likely to be observed because the binding affinity in the active site is abolished at the moment of degradation. However, when a second portion of the substrate upstream from the 3'-end is bound to the enzyme at an accessory motif, then the substrate is not likely to dissociate upon abolition of the RNA-protein binding affinity inside the active site and a processive mode of activity may be achieved.

Moreover, a weak substrate for degradation may be a better substrate for polymerisation if degradation requires deeper penetration of the substrate RNA into the enzymatic active site. As such, under normal conditions where only degradation is favoured, once critical length of a difficult substrate is reached a waiting game begins where repeated cycles of RNA binding and dissociation continue until an RNA becomes a spontaneous substrate, perhaps through breathing of secondary structure or the probabilistic crossing of the activation energy threshold to enter the protein structure with only a short (or no) overhang. Hence the time required for complete degradation of a structured substrate by the exosome is proportional to the thermodynamic stability of that structure. If a particular RNA substrate does not reach deep enough into the active site to undergo degradation, has a highly stable

secondary structure and lacks binding affinity for the enzyme, then it is a very poor substrate for degradation and will be highly stabilised. Such substrates, highly stabilised against degradation, may be effective substrates for polymerisation.

In the presence of exosome, 20mM P_i and 5mM ADP, phosphorolysis is expected to be favoured over polymerisation; but with even a small likelihood of polymerisation some synergy between polymerisation and degradation may be observed. The two modes of PNPase-like activity should be mutually exclusive due to the shared catalytic domain. Only one can occur at a time, dictated by the equilibrium state of products and reactants. Thus, hydrolysis, which is not subject to reversal under these conditions, and phosphorolysis are initially favoured. However, RNA substrates may become stabilised against degradation as the 3' terminus approaches an internal secondary structure during degradation.

As substrates accumulate, and are stabilised against degradation by the presence of the secondary structure, they may become better substrates for polymerisation. For instance, in the case that a substrate must reach two-nucleotides deep into the active site to undergo a hydro- or phosphorolysis, a hairpin loop with a one-nucleotide overhang would be unlikely to undergo cleavage. However, the reverse reaction in a PH active site would only require a one-nucleotide overhang and an ADP bound in the site where the second nucleotide would reside during phosphorolysis. In such circumstance short polymerisations may be able to occur even when phosphorolysis is predicted to be favoured based on the ratio of P_i to ADP concentrations.

In these circumstances the accumulated intermediates stand the possibility of gaining one or more additional 'A' residues at the 3' termini. This 3' extension presents a more efficient substrate to degradation and increases the likelihood of penetrating the stable secondary structure on repeat attempts. After which time the combinatorial effects of the phosphorolytic and hydrolytic activities are resumed.

This hypothetical model assumes that the kinetics of phosphorolysis and hydrolysis combined with low-level mono or oligo(A) incorporation (P_i + ADP), are superior to those of increased efficiency oligo-adenylation and hydrolysis in the absence of phosphorolytic activity (ADP only, as presented in figure 8).

Further work: The answer to whether the nucleolytic stimulation of the phosphate + ADP combination is greater than the stimulation conferred by ADP alone remains to be seen and is dependant on finding appropriate reaction conditions where the two can be compared side by side. It also remains to be determined if the effect observed upon treatment with 20mM [P_i] represents a genuine stimulation of phosphorolytic activity. To test this a continuously labelled substrate transcribed in the presence of an {α-^{32}P} NTP could be subjected to exonucleolytic degradation by the exosome in the presence and absence of added P_i. The nucleotide products of this reaction could be subjected to thin-layer chromatographic analysis for the presence of NMPs and NDPs. The detection of NDPs is expected in the event of a phosphorolytic mode of activity in the presence of P_i.

V Final Discussion

V.A Polyadenylation for Degradation

This study and previous work by Mitchell *et al.* report that purified preparations of yeast exosome show a weak, distributive activity *in vitro*. Here it is reported that the intermediates observed during exosome-catalysed breakdown of a 37nt *in vitro* transcript correlate with positions of predicted secondary structure in the RNA substrate. Accordingly, a highly structured tRNA substrate was almost entirely resistant to degradation. Thus one factor that affects the ability of purified exosome to degrade substrates rapidly and processively *in vitro* is RNA secondary structure.

Subsequent experiments illustrated the potential of oligo- or polyadenylation to compromise the resistance of structured RNA substrates to exonucleolytic degradation by the exosome. If the model presented for ADP stimulated degradation of substrate RNAs proves true, then the ability to polymerize short oligo(A)-tails may be innate to the exosome, as it is for *E. coli* PNPase. Although the exosome achieves an increased level of exonucleolytic activity when the structured substrate RNA has been polyadenylated, its activity is much further stimulated when polyadenylation, RNA unwinding, and degradation are efficiently coupled. The integration of these essential tools for the degradation of structured RNAs is provided to the exosome by the MATPAC.

The results reported herein suggest that the MATPAC complex is responsible for stimulating the exosome *in vivo* towards the rapid degradation of structured RNAs. This is accomplished through initial polyadenylation of substrates by Trf4p, producing a short unstructured docking site for exosome binding, possibly assisted by Mtr4p. Subsequent degradation of the adenylated substrate is then able to proceed rapidly and efficiently through otherwise stable secondary structures.

In bacteria, such as *E. coli,* mRNAs are polyadenylated in order to allow 3'-5' exonucleases to degrade through hairpin structures located at their 3' termini. Two enzymes, PNPase and RNase II, account for all but a small fraction of the detectable exonucleolytic activity. These enzymes have homologues throughout eukaryotes, and indeed both are represented in the exosome, which is itself conserved across all the eukaryotes tested to date. It appears that both the enzymes and the system of polyadenylation as a means of targeting cellular RNAs for degradation, only proven in bacteria in the last 10 years, has also been retained in eukaryotes. However, there was a gain in function in the use of polyadenylation for mRNA stability in eukaryotes. The duality of the function of polyadenylation in eukaryotes is now emerging.

V.B The MATPAC Proteins: Past and Present

The MATPAC complex characterised in this study consists of at least the proteins Mtr4p, the ATP dependant RNA helicase, Air2p, a putative RNA binding protein (Inoue *et al.* 2000), and Trf4p, a poly(A) polymerase (Saitoh *et al.* 2002). The data also support the existence of a separate complex containing the Trf4p homolog Trf5p and Mtr4p, which does not show exosome stimulatory capacity *in vitro.* It remains to be shown if additional proteins including Air1p are also associated with these Mtr4p-containing complexes.

The individual components of the MATPAC had been known and studied for several years prior to this work. However, only in this study has it emerged that these proteins function together in a complex. It may be the case that the proteins of the MATPC serve additional, possibly independent or unrelated roles in the cell. A future aim would be to discover connections between the various other roles the individual proteins play and those of the MATPAC.

V.B.1 Mtr4p

The story of the MATPAC started with Mtr4p. This was the only obvious candidate for a co-factor of the nuclear exosome at this study's commencement. Mtr4p is encoded by an essential gene and is required for exosome function.

Mutants depleted of Mtr4p exhibit defects in exosome function and accumulate 3'-extended processing and degradation intermediates.

Mutations in *MTR4*, mRNA transport-defective, were initially reported to accumulate poly(A)$^+$ RNA in the nucleolus (Kadowaki *et al.* 1995; Liang *et al.* 1996). It now seems likely that these are not polyadenylated mRNAs, but rather are the 3'extended snRNAs and snoRNAs that were shown to be polyadenylated in exosome and Mtr4p mutants (van Hoof[a] *et al.* 2000). These observations indicate that a population of poly(A)$^+$ RNA can be detected in the absence of efficient exosome activity. Considering that the exosome functions in RNA turnover, it seems likely that the accumulated poly(A)$^+$ population observed comprises RNA that would normally have been targeted for degradation.

Mtr4p is as crucial to the degradation of these substrates as the exonucleases involved, highlighting the importance of a "helicase" activity in the function of the exosome. *In vitro* studies reported here showed that Mtr4p is indeed an ATPase, whose activity is stimulated by the presence of cellular RNA. This is in keeping with observations made on other DEAD-box-like helicases, which are broadly considered RNA dependant NTPases when the precise mechanism of their action remains unclear.

V.B.2 Air1p and Air2p

Air1p, arginine methyltransferase-interacting RING finger protein, was also discovered in a screen for factors involved in nuclear export of mRNA (Inoue *et al.* 2000). The screen, a yeast two-hybrid analysis, was designed to identify components that interact with Npl3p, a protein structurally related to human heterogeneous nuclear ribonucleoprotein (hnRNP) components. Npl3p has been characterised as a shuttling protein linked to mRNA export, mutants of which accumulate poly(A)$^+$ RNA in the nucleus (Bossie *et al.* 1992; Flach *et al.* 1994; Lei *et al.* 2001; Singleton *et al.* 1995). Moreover, the screen aimed to identify proteins that interacted with Npl3p through Hmt1p (hnRNP methyltransferase), a protein known to methylate arginine residues in the arginine-glycine-rich (RGG) domain of Npl3p and enhance nuclear export (Henry *et al.* 1996; Inoue *et al.* 2000; McBride *et al.* 2000; Shen *et al.*

1998). Air1p did not interact with Npl3p in a two-hybrid screen conducted in the absence of Hmt1p, confirming that the Air1p/Npl3p interaction is via Hmt1p.

Air2p is highly homologous to Air1p and the proteins are functionally redundant, since double mutants are severely impaired for growth. Both Air1p and Air2p carry the RING finger nucleic acid binding domain (Bohm *et al.* 1997; Inoue *et al.* 2000). Air2p also showed an Hmt1p-dependent interaction with the Npl3p RGG domain in a two-hybrid screen. Air1p has not yet been shown to co-precipitate MATPAC activity, and has not been detected by two-hybrid or other proteomic methods to specifically co-precipitate with Air2p. Air1p was, however, co-precipitated with FLAG-Trf4p in a high throughput mass spectrometry analysis (Ho *et al.* 2002). It currently seems likely that Air1p and Air2p are functionally equivalent and that the MATPAC can contain either protein, but probably not both. Consistent with the proposed redundancy *air1/2Δ* double mutants, but not either single mutant, show a processing phenotype for the RNA species tested by Dr. Houseley. The observed phenotype resembles that reported for *mtr4* or *rrp6Δ* mutants, which accumulate of 3' extend species of small RNAs and rRNAs. (Allmang[a] *et al.* 1999; Mitchell[a] *et al.* 2003).

V.B.2.1 Degradation of Nuclear Retained mRNAs

While Npl3p is essential, Htm1p is not, except in certain Npl3p mutant backgrounds (Henry *et al.* 1996). Hmt1p affects the methylation state and localisation of Npl3p, indicating that methylation enhances its nuclear export (McBride *et al.* 2000). Air1p has been shown to inhibit Npl3p methylation by Hmt1p *in vitro* and *in vivo* (Inoue *et al.* 2000). This model of nuclear export control via methylation by Hmt1p has also been proposed for the hnRNP associated nuclear shuttling proteins Hrp1p (Shen *et al.* 1998) and Nab2p (Green *et al.* 2002). Hmt1p has also been shown to methylate glycine-arginine rich domains of nucleolar proteins, which do not shuttle between the nucleus and cytoplasm and whose cellular localisation is not altered by methylation. It is therefore likely that the role of RGG methylation is protein specific, and may exert its effects by influencing protein-protein interactions and RNP composition (Xu *et al.* 2003; Yu *et al.* 2004).

It is possible that defective hnRNP particles are bound by the MATPAC. Efficient export of Npl3p containing hnRNPs apparently requires methylation of the RGG domain, and the presence of Air1/2p may inhibit methylation by Hmt1p. This would potentially inhibit nuclear export, promoting RNA degradation by the nuclear exosome.

V.B.3 Trf4p and Trf5p

Trf4p (Topoisomerase one-Requiring Function) was originally identified in a screen for mutant alleles whose phenotypes are suppressed by topoisomerase I expression (TOP1), suggesting that the two protein have some overlapping functions (Sadoff *et al.* 1995). Both *top1* and *trf4* single mutants show rDNA hyper-recombination and a failure to halt RNA polymerase II transcription upon entry into stationary phase. Double mutants are defective in the mitotic events of chromosome condensation, spindle elongation, and nuclear segregation, but not in DNA replication (Castano[b] *et al.* 1996). Sporulation is also defective in strains homozygous for a *trf4* disruption, indicating that *TRF4* may be required during meiosis (Iwanejko L 1999).

TRF5 was identified as a high-copy suppressor of the cold-sensitive phenotype conferred by the *trf4-1* allele, and over-expression of *TRF5* has been shown to complement the lethality of *trf4 top1* double mutants (Castano[a] *et al.* 1996). However, *trf5* is not synthetic lethal with *top1*. Given that *trf4(ts) trf5*Δ mutants are synthetic lethal at non-permissive temperature and show defects in nuclear division, it was proposed that *TRF5* function, like *TRF4*, is required for proper mitosis. The relationship between these activities and the functions of Trf4p and Trf5p described here remains unclear.

More recently *TRF4/5* were described as encoding DNA polymerases with homology to the Cid1-related of proteins from *S. pombe* (Wang[a] *et al.* 2000; Wang[b] *et al.* 2000). The new family was classified in the DNA polymerase beta-like nucleotidyltransferase superfamily (Aravind *et al.* 1999). Trf4p was even reported to show DNA polymerase activity *in vitro* (Wang[b] *et al.* 2000). However, an independent study was unable to replicate this finding and instead reported detecting *in vitro* poly(A) polymerase activity for affinity-purified Trf4p from *S. cerevisiae*

and a homolog in *S. pombe*, Cid13 (Saitoh *et al.* 2002). ATP was reported to be converted into polymerized material in the presence of a poly(A) primer, but no further details of the poly(A) polymerase (PAP) activity were provided.

Very recently, a defective tRNA was shown to be sensitive to degradation that was dependent on Trf4p and the exosome (Kadaba *et al.* 2004). The *trm6-504* allele causes the inactivation of a tRNA m^1A58 methyltransferase, encoded by the essential genes *TRM6* and *TRM61*. In the *trm6-504* background $tRNA_i^{met}$ is specifically destabilised at 37°C due to a structural sensitivity resulting from lack of methylation at A58, causing reduced levels of the tRNA and conferring a temperature sensitive-lethal phenotype. Mutant alleles of *TRF4* and *RRP44* were selected as suppressors of the *trm6-504* ts-phenotype, and *trf4Δ* conferred similar suppression. This suppression was linked to increased stability of the hypo-modified tRNA, A strain carrying *trm6-504* and *rrp6Δ* accumulated a polyadenylated form of the hypo-modified tRNA, and this exacerbated by overexpression of Trf4p. The authors concluded that Trf4p is responsible for polyadenylating the defective tRNA in the nucleus and the exosome, possibly via Rrp44p or Rrp6p, is responsible for its degradation. Another suppressor of *trm6-504* has been identified as an allele of *mtr4* (J. Anderson, personal communication), making it probable that the MATPAC is responsible to activating the defective tRNA for degradation by the exosome.

In our studies, strains deleted for *TRF4* showed a mild *air1/2Δ*-like phenotype while strains deleted in *TRF5* did not show any processing defects. However, *trf4Δ, GAL::trf5* strains showed a strong *air1/2Δ*–like phenotype upon shift to glucose containing medium. This indicates that Trf5p is not required for MATPAC function on the substrates analyzed to date, but can partially compensate for the absence of Trf4p. The *trf4/5* phenotype is akin to the *air1/2* phenotype, but *air1/2Δ* strains are viable whereas *trf4/5Δ* are synthetic lethal. This suggests that *TRF4/5* play (an) additional essential role(s) outside of the MATPAC. This may be related to their reported functions as DNA polymerases. Trf5p did not show PAP activity *in vitro* under conditions of clear activity for Trf4p. However both proteins contain the conserved residues of the nucleotidyltransferase active site and are highly homologous to one another. It remains to be established that Trf5p functions as a

PAP *in vivo*, although its ability to partially alleviate the *trf4Δ* phenotype suggests that it does have this activity.

V.C 3'-extended vs. 3'extended and polyadenylated

MATPAC and exosome mutants show a phenotypical accumulation of 3'-extended RNA species, but not all 3'-extended species are created equal. Allmang *et al.* (1999) report that Northern blot analysis revealed mild 3'-extended phenotypes for some small RNA species in "core" exosome mutants such as *rrp45*, *rrp4*, or *rrp42*. Discreet 3'-extended forms of several snoRNAs, for example, accumulated in *GAL::rrp45* strains upon transfer to glucose and in *rrp4-1* strains upon transfer to non-permissive temperature. The same study reported that *rrp6Δ* mutants accumulate 3'-extended forms of snoRNAs, including a smear above and below the position of the discreet 3'-extended species observed in core exosome mutants. The *rrp6Δ* phenotype was exacerbated when combined with a core exosome mutation (such as *GAL::rrp41, rrp6Δ*). RNase H/oligo(dT) cleavage experiments demonstrated that the smears observed in *rrp6Δ* mutants were polyadenylated species of the discreet 3'-extended snoRNAs seen in core exosome mutants.

In a similar study, van Hoof *et al.* (2000) report that heterogeneous 3'-extended forms of snoRNAs and the U4 snRNAs were detected in WT strains but accumulated in strains carrying *mtr4*, core exosome mutations *rrp41* and *mtr3*, or *rrp6Δ*., with the strongest phenotype in the *rrp6Δ* strain. Upon RNase H/oligo(dT) treatment the heterogeneous 3'-extended species collapsed into discreet 3'-extended species, confirming that they were polyadenylated.

The observations of van Hoof *et al.* confirm those of Allmang *et al.* in that both Rrp6p and the core exosome are required for proper 3'-end processing of some small RNAs, but they differ as to whether or not core exosome mutants produce polyadenylated forms. These two studies also do not agree on the reported polyadenylation states of some small RNAs tested by both groups in *rrp6Δ* strains. van Hoof *et al.* report a 3'-extended polyadenylated snRNA species, indicating that snRNAs as well as snoRNAs may be polyadenylated in *rrp6Δ* mutants.

A study by Mitchell *et al.* (2003) showed clear examples of the differences in the 3'-extended snoRNAs accumulated in strains carrying a core exosome mutation, in this case the *rrp4-1* ts-allele, or an *rrp6Δ* or *rrp47Δ* mutation. In *rrp4-1* mutants accumulation of the snoRNA precursors is observed. In *rrp6Δ* or *rrp47Δ* mutants the 3'-extended species observed in the *rrp4-1* mutant are also accompanied by a smear extending above the 3-extended species. These smears are not explicitly shown to be polyadenylated in the Mitchell paper, since such smears in *rrp6Δ* mutants have already been reported as poly(A)$^+$ RNA by Allmang *et al.* and separately by van Hoof *et al.*, albeit that some of the snoRNAs reported were not previously assayed. Based on the results of these three papers, which represent the current state of progress in the understanding of this matter, it appears that the accumulation of poly(A)$^+$ 3' extended small RNA precursors can be readily observed in *rrp6* and *rrp47* mutants, with a weaker, if any poly(A)$^+$ phenotype, in *mtr4* and core exosome mutants.

While *trf4Δ* and *trf4Δ/5* mutants show accumulation of 3' extended RNA species, these are not identical to those observed in *rrp6Δ* strains. In Northern blots the bands appear considerably more discreet in the *trf* background than in the *rrp6Δ* background, indicating that the 3'-extended species in the *trf* background are not polyadenylated. This conclusion should be confirmed by RNase H/oligo(dT) experiments on RNA samples isolated in *trf* and *air* mutant backgrounds.

V.D The Poly(A) Paradox

This study argues for the MATPAC as a stimulator of exosome activity in a polyadenylation dependant degradation pathway. In this model Air2p may mediate the binding of the MATPAC to the RNA substrate, Trf4p adds poly(A) tails, and Mtr4p recruits the exosome and aids in unwinding secondary structures. Studies on *mtr4* and *air1/2* mutants indicate that poly(A)$^+$ RNA is accumulated in the nucleus of these mutants, where a defective MATPAC is expected. However, Trf4p is not expected to lose functionality *in vivo* in the absence of other MATPAC components. It retains *in vitro* activity in the absence of Mtr4p and overexpression of Trf4p alone *in vivo* increase polyadenylation of the defective $tRNA_i^{met}$. Thus Trf4p is a

favourable candidate as the primary factor responsible for polyadenylating defective nuclear RNAs.

V.D.1 Poly(A) Tail Function Depends on How it is Applied

The polyadenylation activity of the MATPAC *in vitro* appears to be distributive. It is likely that this trait is essential for the utilization of polyadenylation as a stimulator of degradation by the exosome. When the MATPAC is combined with the exosome *in vitro*, polymer lengths of approximately 10-15nt are observed over a 30 min time course. We speculate that polymerization by the MATPAC *in vivo* is likely only to achieve only a very modest oligo-adenylation of RNA substrates prior to action by the exosome. This indicates that short tails are generated and the exosome gains access to them shortly after polyadenylation begins.

In contrast, mRNA poly(A)-tails are added by Pap1p, which is reported to show a processive activity and polymerise up to 200nt onto mRNA 3'-ends; although these tails are eventually shortened to an mRNA-specific length of between 50-90nt in yeast (Brown *et al.* 1998; Zhelkovsky *et al.* 1998). This implies that many 'A' residues will be added before the exosome can gain access to the substrate. This would allow time for association of the RNA poly(A) binding protein, Pab1p, potentially stabilising the RNA against exosome mediated degradation. This difference in processivity is postulated to be the key distinction between stabilising mRNA tails added by Pap1p and destabilising poly(A)-tails added by Trf4p.

V.D.1.1 mRNA doppelgangers

The observed number of consecutive 'A' residues polymerized by the MATPAC in the presence of the exosome *in vitro* is at or below the limit of efficient binding by Pab1p, which has been measured *in vitro* at a minimum of 12nt (Sachs *et al.* 1987). *In vivo* studies show that poly(A)-tails are shortened to lengths of 15nt or less prior to the initiation of degradation, in either the decapping dependant 5'-3' pathway (Decker *et al.* 1993) or 3'-5' digestion by the exosome (Anderson *et al.* 1998; van Hoof[c] *et al.* 2000), consistent with a loss of Pab1p binding upon trimming of the poly(A)-tail to this critical length.

However, 30-40nt poly(A) tails are generated *in vitro* across long time courses with MATPAC or high stringency Trf4p-TAP purifications in the absence of the exosome. In MATPAC or exosome mutants, Trf4p polymerized poly(A)-tails may not be properly degraded and may achieve lengths capable of efficiently binding Pab1p, thus becoming stabilized against degradation. Northern blot analyses of the poly(A) tails present on 3' extended small RNA precursors in exosome mutants display lengths of 40nt or greater (Allmang[a] *et al.* 1999; van Hoof[a] *et al.* 2000), approaching lengths typical of yeast mRNA. Similar results are seen in the 3' extension of the defective $tRNA_i^{met}$ in a strain deleted for Rrp6p (Kadaba *et al.* 2004). The presence of stabilized, Pab1p protected oligo(A)-tails on stable RNAs in MATPAC or exosome mutants may mark these RNAs, incorrectly, as mRNA and result in Pap1p dependant elongation of the poly(A)-tail.

V.D.2 Is it Pap1p or Trf4p?

The presented data and lines of reasoning argue that MATPAC, via Trf4p, is normally responsible for polyadenylation of nuclear RNAs targeted for degradation. Likewise, Trf4p is responsible for at least the initial polyadenylation of small RNAs in the presence of exosome or MATPAC lesions. However, van Hoof *et al.* (2000) argue that the poly(A) polymerase, Pap1p, is responsible for the observed polyadenylation of small RNAs in *rrp6Δ* mutants analyzed by Northern blot in their work. Kuai *et al.* (2004) make similar claims for polyadenylated rRNAs.

However, the conclusion that inactivation of Pap1p has resulted in loss of $poly(A)^+$ small RNAs is somewhat badly drawn on the data provided by van Hoof *et al.* The presence of the 3'-extended non-adenylated species is expected in the *rrp6Δ pap1-1* double mutants at non-permissive temperature but it is not clearly present in any of the small RNAs tested except one, and this species was not previously shown to be polyadenylated by Allmang *et al.* (1999) in *rrp6Δ* single mutants. Certainly in the absence of a clear 3'-extended precursor signal in the *rrp6Δ pap1-1* double mutants, no obvious conclusions can be draw about the polyadenylation state of these molecules, by Trf4p or otherwise. Moreover, the Northern blot data for the small RNAs tested in the *rrp6Δ pap1-1* at permissive temperature generally show both

reduced levels of polyadenylation and smaller polymers lengths. This might be expected for the tails polymerized by MATPAC in an exosome mutant, but without the enhanced polyadenylation by Pap1p.

The poly(A)$^+$ rRNAs were identified by Kuai *et al.* by reverse transcription and subsequent PCR amplification, both carried using a poly(dT)$_{30}$ as a primer. Polyadenylated rRNA with oligo(A) tails catalysed only by the MATPAC would probably go undetected in this screen.

V.E Connecting the PAPs

Poly(A) and oligo(A) homopolymers were elongated more efficiently than others by purified Pap1p, suggesting that a short 'A'-tail may encourage Pap1p catalysed elongation *in vivo*. Furthermore, partial and complete purifications of the Pap1p enzyme do not show specificity for genuine mRNA 3'-ends *in vitro*, indicating that specificity factors are required for substrate discrimination *in vivo* (Lingner *et al.* 1991). Thus it is predicted that the binding of Pab1p and mRNA associated factors (Mangus[a] *et al.* 2004; Mangus[b] *et al.* 2004) to stable RNAs is likely to promote Pap1p catalysis. This is backed by reports that mutant genes which present altered or inauthentic 3'-ends can still be effectively polyadenylated *in vivo* (Osborne *et al.* 1989; Zaret *et al.* 1982). Hence, it is not implausible that the 3'-ends of polyadenylated stable RNAs might attract Pap1p activity.

V.F MATPAC Across the Board

Mtr4p has a homologue in humans that also coprecipitates with the exosome (Chen *et al.* 2001), and proteins with strong similarity to Mtr4p are predicted in mouse, *C. elegans* and *S. pombe*. A cross-species comparison of the Air proteins does not produce meaningful results due to low identity hits and the widespread occurrence of RING-finger domain containing proteins. Trf4p has been reported to be homologous to the Cid family of proteins from *S. pombe*. It has been compared to Cid13p (Saitoh *et al.* 2002), a cyptoplasmic poly(A) polymerase which shares homology with GLD-2, a cytoplasmic poly(A) polymerase from *C. elegans* (Wang[a] *et al.* 2002). However, Trf4p is most homologous to other members of the Cid

family, Cid14p and Cid12p, whose localisation remains uncharacterised, with lower similarity to the cytoplasmic polymerases, Cid13p and Cid1p. Likewise, Trf4p shows greatest homology to the *C. elegans* protein ZK858.1, not GLD-2, and ZK858.1 shares the highest homology with Cid14p and Cid12p rather than Cid13p. In Humans the protein POLS shares greatest homology with Trf4p and Cid14p and POLS was initially designated as human TRF4-1 (hTRF4-1) along with its closest homolog in humans PAPD5 (designated hTRF4-2) (Walowsky *et al.* 1999). Given the persistence of Mtr4p and Trf4p across species, and specifically in humans where an exosome complex that shares significant homology with the yeast exosome has been identified, it is likely that a MATPAC complex also exists across species and that findings reported here represent a conserved mechanism for RNA processing.

V.G Other Tails Other Activities, *E. Coli* to *S. Cerevisiae*

While the exosome component(s) that is stimulated to enzymatic activity by the MATPAC has not been established, the *in vitro* activity showed no phosphate dependence, strongly arguing against the participation of any of the 6 homologues of the phosphorolytic enzyme RNase PH. In contrast, simulation of *in vitro* activity by addition of nucleotide diphosphates is predicted to involve one or more of these enzymes, at least at the nucleotide addition steps, and probably also during subsequent degradation. While we are far from understanding the distinct roles of the different exonucleases in the complex, these observations indicate that this will be at least partially amenable to analyses *in vitro*. Recently, we have initiated two collaborative projects for structural analyses of the exosome: one using cryo-electron microscopy and the other protein cross-linking and mass spectrometry. It is anticipated that these studies will also contribute greatly to understanding the mechanism and specificity of exosome activation.

The features of nuclear RNA turnover described here show striking similarities to RNA degradation in *E .coli*. Combined with the potential structural similarity of the exosome to a complex between PNPase and RNase II, the major degradative exoribonuclease in *E .coli*, these observations serve to unify two systems that appeared to be quite distinct.

Allmang, C. and D. Tollervey (1998). "The role of the 3' external transcribed spacer in yeast pre-rRNA processing." Journal of Molecular Biology **278**(1): 67-78.

Allmang, C., P. Mitchell, E. Petfalski and D. Tollervey (2000). "Degradation of ribosomal RNA precursors by the exosome." Nucl. Acids. Res. **28**(8): 1684-1691.

Allmang[a], C., J. Kufel, G. Chanfreau, P. Mitchell, E. Petfalski and D. Tollervey (1999). "Functions of the exosome in rRNA, snoRNA and snRNA synthesis." EMBO J. **18**: 5399-5410.

Allmang[b], C., E. Petfalski, A. Podtelejnikov, M. Mann, D. Tollervey and P. Mitchell (1999). "The yeast exosome and human PM-Scl are related complexes of 3' right-arrow 5' exonucleases." Genes Dev. **13**(16): 2148-2158.

Aloy, P., F. D. Ciccarelli, C. Leutwein, A. C. Gavin, G. Superti-Furga, P. Bork, B. Bottcher and R. B. Russell (2002). "A complex prediction: three-dimensional model of the yeast exosome." EMBO Rep **3**(7): 628-35.

Amberg, D. C., A. L. Goldstein and C. N. Cole (1992). "Isolation and characterization of RAT1: an essential gene of Saccharomyces cerevisiae required for the efficient nucleocytoplasmic trafficking of mRNA." Genes Dev **6**(7): 1173-89.

Anderson, J. S. J. and R. P. Parker (1998). "The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex." EMBO J. **17**(5): 1497-1506.

Andrulis, E. D., J. Werner, A. Nazarian, H. Erdjument-Bromage, P. Tempst and J. T. Lis (2002). "The RNA processing exosome is linked to elongating RNA polymerase II in Drosophila." Nature **420**(6917): 837-41.

Araki, Y., S. Takahashi, T. Kobayashi, H. Kajiho, S. Hoshino and T. Katada (2001). "Ski7p G protein interacts with the exosome and the Ski complex for 3'-to-5' mRNA decay in yeast." EMBO J. **20**(17): 4684-4693.

Aravind, L. and E. Koonin (1999). "DNA polymerase beta-like nucleotidyltransferase superfamily: identification of three new families, classification and evolutionary history." Nucl. Acids. Res. **27**(7): 1609-1618.

Baudin, A., O. Ozier-Kalogeropoulos, A. Denouel, F. Lacroute and C. Cullin (1993). "A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae." Nucleic Acids Res **21**(14): 3329-30.

Beers, R. F., Jr. (1957). "Polynucleotides; partial purification and properties of a polynucleotide phosphorylase from Micrococcus lysodeikticus." Biochem J **66**(4): 686-93.

Beese, L. and T. Steitz (1991). "Structural basis for the 3'-5' exonuclease activity of Escherichia coli DNA polymerase I: a two metal ion mechanism." EMBO J. **10**(1): 25-33.

Benard, L., K. Carroll, R. C. Valle and R. B. Wickner (1998). "Ski6p is a homolog of RNA-processing enzymes that affects translation of non-poly(A) mRNAs and 60S ribosomal subunit biogenesis." Mol Cell Biol **18**(5): 2688-96.

Benard, L., K. Carroll, R. C. Â. P. Valle, D. C. Masison and R. B. Wickner (1999). "The Ski7 Antiviral Protein Is an EF1-alpha Homolog That Blocks Expression of Non-Poly(A) mRNA in Saccharomyces cerevisiae." J. Virol. **73**(4): 2893-2900.

Boeck, R., S. Tarun, Jr., M. Rieger, J. A. Deardorff, S. MÃ¼ller-Auer and A. B. Sachs (1996). "The Yeast Pan2 Protein Is Required for Poly(A)-binding Protein-stimulated Poly(A)-nuclease Activity." J. Biol. Chem. **271**(1): 432-438.

Bohm, S., D. Frishman and H. Mewes (1997). "Variations of the C2H2 zinc finger motif in the yeast genome and classification of yeast zinc finger proteins." Nucl. Acids. Res. **25**(12): 2464-2469.

Bossie, M., C. DeHoratius, G. Barcelo and P. Silver (1992). "A mutant nuclear protein with similarity to RNA binding proteins interferes with nuclear import in yeast." Mol. Biol. Cell **3**(8): 875-893.

Bousquet-Antonelli, C., C. Presutti and D. Tollervey (2000). "Identification of a Regulated Pathway for Nuclear Pre-mRNA Turnover." Cell **102**(6): 765-775.

Briggs, M. W., K. T. Burkard and J. S. Butler (1998). "Rrp6p, the yeast homologue of the human PM-Scl 100-kDa autoantigen, is essential for efficient 5.8 S rRNA 3' end formation." J. Biol. Chem. **273**(21): 13255-13263.

Brindle, K., P. Braddock and S. Fulton (1990). "31P NMR measurements of the ADP concentration in yeast cells genetically modified to express creatine kinase." Biochemistry **29**(13): 3295-302.

Brindle, K. M. (1988). "31P NMR magnetization-transfer measurements of flux between inorganic phosphate and adenosine 5'-triphosphate in yeast cells genetically modified to overproduce phosphoglycerate kinase." Biochemistry **27**(16): 6187-96.

Brown, C., S. Tarun, Jr, R. Boeck and A. Sachs (1996). "PAN3 encodes a subunit of the Pab1p-dependent poly(A) nuclease in Saccharomyces cerevisiae." Mol. Cell. Biol. **16**(10): 5744-5753.

Brown, C. E. and A. B. Sachs (1998). "Poly(A) Tail Length Control in Saccharomyces cerevisiae Occurs by Message-Specific Deadenylation." Mol. Cell. Biol. **18**(11): 6548-6559.

Brown, J. T., X. Bai and A. W. Johnson (2000). "The yeast antiviral proteins Ski2p, Ski3p, and Ski8p exist as a complex in vivo." RNA **6**(3): 449-457.

Buchholz, A., R. Takors and C. Wandrey (2001). "Quantification of Intracellular Metabolites in Escherichia coli K12 Using Liquid Chromatographic-Electrospray Ionization Tandem Mass Spectrometric Techniques." Analytical Biochemistry **295**(2): 129-137.

Burd, C. G. and G. Dreyfuss (1994). "Conserved structures and diversity of functions of RNA-binding proteins." Science **265**(5172): 615-21.

Burkard, K. T. D. and J. S. Butler (2000). "A Nuclear 3'-5' Exonuclease Involved in mRNA Degradation Interacts with Poly(A) Polymerase and the hnRNA Protein Npl3p." Mol. Cell. Biol. **20**(2): 604-616.

Butler, J. S. (2002). "The yin and yang of the exosome." Trends in Cell Biology **12**(2): 90-96.

Bycroft, M., T. J. P. Hubbard, M. Proctor, S. M. V. Freund and A. G. Murzin (1997). "The Solution Structure of the S1 RNA Binding Domain: A Member of an Ancient Nucleic Acid-Binding Fold." Cell **88**(2): 235-242.

Caponigro, G. and R. Parker (1996). "Mechanisms and control of mRNA turnover in Saccharomyces cerevisiae." Microbiol. Rev. **60**(1): 233-249.

Carpousis, A. J., G. Van Houwe, C. Ehretsmann and H. M. Krisch (1994). "Copurification of E. coli RNAase E and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation." Cell **76**(5): 889-900.

Carpousis, A. J. (2002). "The Escherichia coli RNA degradosome: structure, function and relationship in other ribonucleolytic multienzyme complexes." Biochem Soc Trans **30**(2): 150-5.

Castano[a], I., S. Heath-Pagliuso, B. Sadoff, D. Fitzhugh and M. Christman (1996). "A novel family of TRF (DNA topoisomerase I-related function) genes required for proper nuclear segregation." Nucl. Acids. Res. **24**(12): 2404-2410.

Castano[b], I. B., P. M. Brzoska, B. U. Sadoff, H. Chen and M. F. Christman (1996). "Mitotic chromosome condensation in the rDNA requires TRF4 and DNA topoisomerase I in Saccharomyces cerevisiae." Genes Dev **10**(20): 2564-76.

Chanfreau, G., S. A. Elela, M. Ares, Jr. and C. Guthrie (1997). "Alternative 3'-end processing of U5 snRNA by RNaseÂ III." Genes Dev. **11**(20): 2741-2751.

Chanfreau, G., P. Legrain and A. Jacquier (1998). "Yeast RNase III as a key processing enzyme in small nucleolar RNAs metabolism." Journal of Molecular Biology **284**(4): 975-988.

Chekanova, J. A., J. A. Dutko, I. S. Mian and D. A. Belostotsky (2002). "Arabidopsis thaliana exosome subunit AtRrp4p is a hydrolytic 3'->5' exonuclease containing S1 and KH RNA-binding domains." Nucl. Acids. Res. **30**(3): 695-700.

Chen, C.-Y., R. Gherzi, S.-E. Ong, E. L. Chan, R. Raijmakers, G. J. M. Pruijn, G. Stoecklin, C. Moroni, M. Mann and M. Karin (2001). "AU Binding Proteins Recruit the Exosome to Degrade ARE-Containing mRNAs." Cell **107**(4): 451-464.

Chen, J., Y.-C. Chiang and C. L. Denis (2002). "CCR4, a 3'-5' poly(A) RNA and ssDNA exonuclease, is the catalytic component of the cytoplasmic deadenylase." EMBO J. **21**(6): 1414-1426.

Coburn, G. A. and G. A. Mackie (1998). "Reconstitution of the degradation of the mRNA for ribosomal protein S20 with purified enzymes." Journal of Molecular Biology **279**(5): 1061-1074.

Coburn, G. A. and G. A. Mackie (1999). "Degradation of mRNA in Escherichia coli: an old problem with some new twists." Prog Nucleic Acid Res Mol Biol **62**: 55-108.

Collart, M. A. (2003). "Global control of gene expression in yeast by the Ccr4-Not complex." Gene **313**: 1-16.

Danin-Kreiselman, M., C. Y. Lee and G. Chanfreau (2003). "RNAse III-Mediated Degradation of Unspliced Pre-mRNAs and Lariat Introns." Molecular Cell **11**(5): 1279-1289.

Darnell, J. E., R. Wall and R. J. Tushinski (1971). "An adenylic acid-rich sequence in messenger RNA of HeLa cells and its possible relationship to reiterated sites in DNA." Proc Natl Acad Sci U S A **68**(6): 1321-5.

Daugeron, M.-C., F. Mauxion and B. Seraphin (2001). "The yeast POP2 gene encodes a nuclease involved in mRNA deadenylation." Nucl. Acids. Res. **29**(12): 2448-2455.

de la Cruz, J., D. Kressler, D. Tollervey and P. Linder (1998). "Dob1p (Mtr4p) is a putative ATP-dependent RNA helicase required for the 3' end formation of 5.8S rRNA in Saccharomyces cerevisiae." EMBO J. **17**(4): 1128-1140.

de la Cruz, J., D. Kressler and P. Linder (1999). "Unwinding RNA in Saccharomyces cerevisiae: DEAD-box proteins and related families." Trends in Biochemical Sciences **24**(5): 192-198.

Decker, C. J. and R. Parker (1993). "A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation." Genes Dev **7**(8): 1632-43.

Denis, C. L., Y.-C. Chiang, Y. Cui and J. Chen (2001). "Genetic Evidence Supports a Role for the Yeast CCR4-NOT Complex in Transcriptional Elongation." Genetics **158**(2): 627-634.

Denis, C. L. and J. Chen (2003). "The CCR4-NOT complex plays diverse roles in mRNA metabolism." Prog Nucleic Acid Res Mol Biol. **73**: 221-50.

Deutscher, M. P. and N. B. Reuven (1991). "Enzymatic basis for hydrolytic versus phosphorolytic mRNA degradation in Escherichia coli and Bacillus subtilis." Proc Natl Acad Sci U S A **88**(8): 3277-80.

Deutscher, M. P. and Z. Li (2001). "Exoribonucleases and their multiple roles in RNA metabolism." Prog Nucleic Acid Res Mol Biol. **66**: 67-105.

Dlakic, M. (2000). "Functionally unrelated signalling proteins contain a fold similar to Mg2+-dependent endonucleases." Trends in Biochemical Sciences **25**(6): 272-273.

Duvel, K., R. Pries and G. H. Braus (2003). "Polyadenylation of rRNA- and tRNA-based yeast transcripts cleaved by internal ribozyme activity." Curr Genet **43**(4): 255-62.

Edmonds, M. and M. G. Caramela (1969). "The Isolation and Characterization of Adenosine Monophosphate-rich Polynucleotides Synthesized by Ehrlich Ascites Cells." J. Biol. Chem. **244**(5): 1314-1324.

Edmonds, M., M. H. Vaughan, Jr. and H. Nakazato (1971). "Polyadenylic acid sequences in the heterogeneous nuclear RNA and rapidly-labeled polyribosomal RNA of HeLa cells: possible evidence for a precursor relationship." Proc Natl Acad Sci U S A **68**(6): 1336-40.

Elela, S. A., H. Igel and M. Ares, Jr. (1996). "RNase III cleaves eukaryotic preribosomal RNA at a U3 snoRNP-dependent site." Cell **85**(1): 115-24.

Estevez, A. M., T. Kempf and C. Clayton (2001). "The exosome of Trypanosoma brucei." EMBO J. **20**(14): 3831-3839.

Estevez, A. M., B. Lehner, C. M. Sanderson, T. Ruppert and C. Clayton (2003). "The Roles of Intersubunit Interactions in Exosome Stability." J. Biol. Chem. **278**(37): 34943-34951.

Evguenieva-Hackenburg, E., P. Walter, E. Hochleitner, F. Lottspeich and G. Klug (2003). "An exosome-like complex in Sulfolobus solfataricus." EMBO Rep **4**(9): 889-93.

Flach, J., M. Bossie, J. Vogel, A. Corbett, T. Jinks, D. A. Willins and P. A. Silver (1994). "A yeast RNA-binding protein shuttles between the nucleus and the cytoplasm." Mol. Cell. Biol. **14**(12): 8399-8407.

Fraser[a], R. S. (1975). "Studies on messenger and ribosomal RNA synthesis in plant tissue cultures induced to undergo synchronus cell division." Eur J Biochem **50**(3): 529-37.

Fraser[b], R. S. (1975). "Turnover of polyadenylated messenger RNA in fission yeast. Evidence for the control of protein synthesis at the translational level." Eur J Biochem **60**(2): 477-86.

Fromont-Racine, M., B. Senger, C. Saveanua and F. Fasiolob (2003). "Ribosome assembly in eukaryotes." Gene **313**: 17.

Gavin, A.-C., M. Bosche, R. Krause, P. Grandi, M. Marzioch, A. Bauer, J. Schultz, J. M. Rick, A.-M. Michon, C.-M. Cruciat, M. Remor, C. Hofert, M. Schelder, M. Brajenovic, H. Ruffner, A. Merino, K. Klein, M. Hudak, D. Dickson, T. Rudi, V. Gnau, A. Bauch, S. Bastuck, B. Huhse, C. Leutwein, M.-A. Heurtier, R. R. Copley, A. Edelmann, E. Querfurth, V. Rybin, G. Drewes, M. Raida, T. Bouwmeester, P. Bork, B. Seraphin, B. Kuster, G. Neubauer and G. Superti-Furga (2002). "Functional organization of the yeast proteome by systematic analysis of protein complexes." Nature **415**(6868): 141-147.

Geerlings, T. H., J. C. Vos and H. A. Raue (2000). "The final step in the formation of 25S rRNA in Saccharomyces cerevisiae is performed by 5'-->3' exonucleases." RNA **6**(12): 1698-1703.

Gerlt, J. A. (1993). Nuclease Mechanisms. Nucleases. New York, Cold Spring Harbor Laboratory Press: 1-34.

Ghaemmaghami S, W.-K. H., Kiowa Bower, A. B. Russell W. Howson, Noah Dephoure, and E. K. O. S. J. S. Weissman (2003). "Global analysis of protein expression in yeast." Nature **425**(6959): 737-41.

Ghosh, S. and M. P. Deutscher (1999). "Oligoribonuclease is an essential component of the mRNA decay pathway." PNAS **96**(8): 4372-4377.

Green, D. M., K. A. Marfatia, E. B. Crafton, X. Zhang, X. Cheng and A. H. Corbett (2002). "Nab2p Is Required for Poly(A) RNA Export in Saccharomyces cerevisiae and Is Regulated by Arginine Methylation via Hmt1p." J. Biol. Chem. **277**(10): 7752-7760.

Grosshans, H., K. Deinert, E. Hurt and G. Simos (2001). "Biogenesis of the Signal Recognition Particle (SRP) Involves Import of SRP Proteins into the Nucleolus, Assembly with the SRP-RNA, and Xpo1p-mediated Export." J. Cell Biol. **153**(4): 745-762.

Grunberg-Manago, M., P. J. Oritz and S. Ochoa (1955). "Enzymatic synthesis of nucleic acidlike polynucleotides." Science **122**(3176): 907-10.

Grunberg-Manago, M., P. J. Ortiz and S. Ochoa (1956). "Enzymic synthesis of polynucleotides. I. Polynucleotide phosphorylase of azotobacter vinelandii." Biochim Biophys Acta **20**(1): 269-85.

Grunberg-Manago, M. (1963). "ENZYMATIC SYNTHESIS OF NUCLEIC ACIDS." Prog Biophys Mol Biol **13**: 175-239.

Hafe, L. A. and E. B. Keller (1975). "The polyadenylate polymerases from yeast." J Biol Chem **250**(5): 1838-46.

Harlow, L. S., A. Kadziola, K. F. Jensen and S. Larsen (2004). "Crystal structure of the phosphorolytic exoribonuclease RNase PH from Bacillus subtilis and implications for its quaternary structure and tRNA binding." Protein Sci **13**(3): 668-677.

HCPF 2D Gel Sample Preparation: TCA/Acetone Precipitation, Harvard Center for Environmental Health Proteomics Facility.

Hector, R. E., K. R. Nykamp, S. Dheur, J. T. Anderson, P. J. Non, C. R. Urbinati, S. M. Wilson, L. Minvielle-Sebastia and M. S. Swanson (2002). "Dual requirement for yeast hnRNP Nab2p in mRNA poly(A) tail length control and nuclear export." EMBO J. **21**(7): 1800-1810.

Henry, M. F. and P. A. Silver (1996). "A novel methyltransferase (Hmt1p) modifies poly(A)+-RNA-binding proteins." Mol Cell Biol **16**(7): 3668-78.

Henry, Y., H. Wood, J. P. Morrissey, E. Petfalski, S. Kearsey and D. Tollervey (1994). "The 5' end of yeast 5.8S rRNA is generated by exonucleases from an upstream cleavage site." EMBO J **13**(10): 2452-63.

Hilleren[a], P., T. McCarthy, M. Rosbash, R. Parker and T. H. Jensen (2001). "Quality control of mRNA 3'-end processing is linked to the nuclear exosome." Nature **413**(6855): 538-42.

Hilleren[b], P. and R. Parker (2001). "Defects in the mRNA export factors Rat7p, Gle1p, Mex67p, and Rat8p cause hyperadenylation during 3'-end formation of nascent transcripts." Rna 7(5): 753-64.

Ho, Y., A. Gruhler, A. Heilbut, G. D. Bader, L. Moore, S. L. Adams, A. Millar, P. Taylor, K. Bennett, K. Boutilier, L. Yang, C. Wolting, I. Donaldson, S. Schandorff, J. Shewnarane, M. Vo, J. Taggart, M. Goudreault, B. Muskat, C. Alfarano, D. Dewar, Z. Lin, K. Michalickova, A. R. Willems, H. Sassi, P. A. Nielsen, K. J. Rasmussen, J. R. Andersen, L. E. Johansen, L. H. Hansen, H. Jespersen, A. Podtelejnikov, E. Nielsen, J. Crawford, V. Poulsen, B. D. Sorensen, J. Matthiesen, R. C. Hendrickson, F. Gleeson, T. Pawson, M. F. Moran, D. Durocher, M. Mann, C. W. Hogue, D. Figeys and M. Tyers (2002). "Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry." Nature **415**(6868): 180-3.

Hoffman, C. S. (1993). Rapid Isolation of Yeast Chromosomal DNA. Current Protocols in Molecular Biology. R. B. Fred M. Ausubel, Robert E. Kingston, David D. Moore, J.G. Seidman, John A. Smith, Kevin Struhl. Boston, John Wiley & Sons, Inc. **2:** 13.11.1 â€“ 13.11.4.

Hopper, A. K. and E. M. Phizicky (2003). "tRNA transfers to the limelight." Genes Dev. **17**(2): 162-180.

Hsu, C. L. and A. Stevens (1993). "Yeast cells lacking 5'-->3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure." Mol Cell Biol **13**(8): 4826-35.

Huh, W. K., J. V. Falvo, L. C. Gerke, A. S. Carroll, R. W. Howson, J. S. Weissman and E. K. O'Shea (2003). "Global analysis of protein localization in budding yeast." Nature **425**(6959): 686-91.

Inoue, K., T. Mizuno, K. Wada and M. Hagiwara (2000). "Novel RING Finger Proteins, Air1p and Air2p, Interact with Hmt1p and Inhibit the Arginine Methylation of Npl3p." J. Biol. Chem. **275**(42): 32793-32799.

Ishii, R., O. Nureki and S. Yokoyama (2003). "Crystal Structure of the tRNA Processing Enzyme RNase PH from Aquifex aeolicus." J. Biol. Chem. **278**(34): 32397-32404.

Ito, T., T. Chiba, R. Ozawa, M. Yoshida, M. Hattori and Y. Sakaki (2001). "A comprehensive two-hybrid analysis to explore the yeast protein interactome." Proc Natl Acad Sci U S A **98**(8): 4569-74.

Iwanejko L, S. K., Loeillet S, Nicolas A, Fabre F (1999). "Disruption and functional analysis of six ORFs on chromosome XV: YOL117w, YOL115w (TRF4), YOL114c, YOL112w (MSB4), YOL111c and YOL072w." Yeast **14**: 1529-39.

Jankowsky, E. and A. Jankowsky (2000). "The DExH/D protein family database." Nucl Acids Res **28**(1): 333-334.

Jarrige, A.-C., D. Brechemier-Baey, N. Mathy, O. Duche and C. Portier (2002). "Mutational Analysis of Polynucleotide Phosphorylase from Escherichia coli." Journal of Molecular Biology **321**(3): 397-409.

Jensen, T. H., K. Patricio, T. McCarthy and M. Rosbash (2001). "A block to mRNA nuclear export in S. cerevisiae leads to hyperadenylation of transcripts that accumulate at the site of transcription." Mol Cell **7**(4): 887-98.

Johnson, A. (1997). "Rat1p and Xrn1p are functionally interchangeable exoribonucleases that are restricted to and required in the nucleus and cytoplasm, respectively." Mol. Cell. Biol. **17**(10): 6122-6130.

Kadaba, S., A. Krueger, T. Trice, A. M. Krecic, A. G. Hinnebusch and J. Anderson (2004). "Nuclear surveillance and degradation of hypomodified initiator tRNAMet in S. cerevisiae." Genes Dev. **18**(11): 1227-1240.

Kadowaki, T., R. Schneiter, M. Hitomi and A. Tartakoff (1995). "Mutations in nucleolar proteins lead to nucleolar accumulation of polyA+ RNA in Saccharomyces cerevisiae." Mol. Biol. Cell **6**(9): 1103-1110.

Kelly, K. and M. Deutscher (1992). "Characterization of Escherichia coli RNase PH." J. Biol. Chem. **267**(24): 17153-17158.

Koonin, E. V. (1997). "A conserved ancient domain joins the growing superfamily of 3'-5' exonucleases." Current Biology **7**(10): R603-R607.

Koonin, E. V., Y. I. Wolf and L. Aravind (2001). "Prediction of the archaeal exosome and its connections with the proteasome and the translation and transcription machineries by a comparative-genomic approach." Genome Res **11**(2): 240-52.

Kreivi, J.-P., S. Petersen-Mahrt and G. Akusja rvi (2001). Messenger RNA in Eukaryotes. Nature Encyclopedia of Life Sciences, http://www.els.net/, Nature Publishing Group.

Kressler, D., P. Linder and J. de la Cruz (1999). "Protein trans-Acting Factors Involved in Ribosome Biogenesis in Saccharomyces cerevisiae." Mol Cell Biol **19**(12): 7897-7912.

Krogan, N. J., W. T. Peng, G. Cagney, M. D. Robinson, R. Haw, G. Zhong, X. Guo, X. Zhang, V. Canadien, D. P. Richards, B. K. Beattie, A. Lalev, W. Zhang, A. P. Davierwala, S. Mnaimneh, A. Starostine, A. P. Tikuisis, J. Grigull, N. Datta, J. E. Bray, T. R. Hughes, A. Emili and J. F. Greenblatt (2004). "High-definition macromolecular composition of yeast RNA-processing complexes." Mol Cell **13**(2): 225-39.

Krupp, G., P. Thurianx, I. Willis, V. Gamulin and D. Soll (1985). "First identification of an amber nonsense mutation in Schizosaccharomyces pombe: major differences in the efficiency of homologous versus heterologous yeast suppressor tRNA genes." Mol Gen Genet. **201**(1): 82-7.

Krupp, G., D. Kahle, T. Vogt and S. Char (1991). "Sequence changes in both flanking sequences of a pre-tRNA influence the cleavage specificity of RNase P." J Mol Biol **217**(4): 637-48.

Kuai, L., F. Fang, J. S. Butler and F. Sherman (2004). "Polyadenylation of rRNA in Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **101**(23): 8581-6.

Kuhn, U. and E. Wahle (2004). "Structure and function of poly(A) binding proteins." Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression **1678**(2-3): 67-84.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**: 680-685.

Larimer, F. W. and A. Stevens (1990). "Disruption of the gene XRN1, coding for a 5'----3' exoribonuclease, restricts yeast cell growth." Gene **95**(1): 85-90.

Larimer, F. W., C. L. Hsu, M. K. Maupin and A. Stevens (1992). "Characterization of the XRN1 gene encoding a 5'-->3' exoribonuclease: sequence data and analysis of disparate protein and mRNA levels of gene-disrupted yeast cells." Gene **120**(1): 51-7.

Lee, S. Y., J. Mendecki and G. Brawerman (1971). "A polynucleotide segment rich in adenylic acid in the rapidly-labeled polyribosomal RNA component of mouse sarcoma 180 ascites cells." Proc Natl Acad Sci U S A **68**(6): 1331-5.

Lei, E. P., H. Krebber and P. A. Silver (2001). "Messenger RNAs are recruited for nuclear export during transcription." Genes Dev. **15**(14): 1771-1782.

Liang, S., M. Hitomi, Y. Hu, Y. Liu and A. Tartakoff (1996). "A DEAD-box-family protein is required for nucleocytoplasmic transport of yeast mRNA." Mol. Cell. Biol. **16**(9): 5139-5146.

Li[b], Z., S. Pandit and M. P. Deutscher (1998). "Polyadenylation of stable RNA precursors in vivo." PNAS **95**(21): 12158-12162.

Lingner, J., I. Radtke, E. Wahle and W. Keller (1991). "Purification and characterization of poly(A) polymerase from Saccharomyces cerevisiae." J Biol Chem **266**(14): 8741-6.

Lisitsky, I. and G. Schuster (1999). "Preferential degradation of polyadenylated and polyuridinylated RNAs by the bacterial exoribonuclease polynucleotide phosphorylase." Eur J Biochem **261**(2): 468-474.

Littauer, U. Z. and H. Soreq (1982). Polynucleotide Phosphorylase. The Enzymes. P. D. Boyer. New York, Academic Press. **XV, part B:** 518-553.

Lorsch[a], J. R. and D. Herschlag (1998). "The DEAD box protein eIF4A. 1. A minimal kinetic and thermodynamic framework reveals coupled binding of RNA and nucleotide." Biochemistry **37**(8): 2180-2193.

Lorsch[b], J. R. and D. Herschlag (1998). "The DEAD box protein eIF4A. 2. A cycle of nucleotide and RNA-dependent conformational changes." Biochemistry **37**(8): 2194-2206.

Mangus[a], D. A., M. M. Smith, J. M. McSweeney and A. Jacobson (2004). "Identification of factors regulating poly(A) tail synthesis and maturation." Mol Cell Biol **24**(10): 4196-206.

Mangus[b], D. A., M. C. Evans, N. S. Agrin, M. Smith, P. Gongidi and A. Jacobson (2004). "Positive and negative regulation of poly(A) nuclease." Mol Cell Biol **24**(12): 5521-33.

Marfatia, K. A., E. B. Crafton, D. M. Green and A. H. Corbett (2003). "Domain Analysis of the Saccharomyces cerevisiae Heterogeneous Nuclear Ribonucleoprotein, Nab2p. DISSECTING THE REQUIREMENTS FOR Nab2p-FACILITATED POLY(A) RNA EXPORT." J. Biol. Chem. **278**(9): 6731-6740.

Masison, D. C., A. Blanc, J. C. Ribas, K. Carroll, N. Sonenberg and R. B. Wickner (1995). "Decoying the cap- mRNA degradation system by a double-stranded RNA virus and poly(A)- mRNA surveillance by a yeast antiviral system." Mol Cell Biol **15**(5): 2763-71.

McBride, A. E., V. H. Weiss, H. K. Kim, J. M. Hogle and P. A. Silver (2000). "Analysis of the yeast arginine methyltransferase Hmt1p/Rmt1p and its in vivo function. Cofactor binding and substrate interactions." J Biol Chem **275**(5): 3128-36.

McLaughlin, C. S., J. R. Warner, M. Edmonds, H. Nakazato and M. H. Vaughan (1973). "Polyadenylic Acid Sequences in Yeast Messenger Ribonucleic Acid." J. Biol. Chem. **248**(4): 1466-1471.

Mead, D. J. and S. G. Oliver (1983). "Purification and properties of a double-stranded ribonuclease from the yeast Saccharomyces cerevisiae." Eur J Biochem **137**(3): 501-7.

Messias, A. C. and M. Sattler (2004). "Structural basis of single-stranded RNA recognition." Acc Chem Res **37**(5): 279-87.

Mian, I. (1997). "Comparative sequence analysis of ribonucleases HII, III, II PH and D." Nucl. Acids. Res. **25**(16): 3187-3195.

Mitchell, P., E. Petfalski and D. Tollervey (1996). "The 3'-end of yeast 5.8S rRNA is generated by an exonuclease processing mechanism." Genes Dev. **10**: 502-513.

Mitchell, P., E. Petfalski, A. Shevchenko, M. Mann and D. Tollervey (1997). "The Exosome: A Conserved Eukaryotic RNA Processing Complex Containing Multiple 3'->5' Exoribonucleases." Cell **91**(4): 457-466.

Mitchell, P. and D. Tollervey (2000). "Musing on the structural organization of the exosome complex." Nat. Struct. Biol. **7**(10): 843-846.

Mitchell[a], P., E. Petfalski, R. Houalla, A. Podtelejnikov, M. Mann and D. Tollervey (2003). "Rrp47p Is an Exosome-Associated Protein Required for the 3' Processing of Stable RNAs." Mol. Cell. Biol. **23**(19): 6982-6992.

Mitchell[b], P. and D. Tollervey (2003). "An NMD Pathway in Yeast Involving Accelerated Deadenylation and Exosome-Mediated 3'->5' Degradation." Molecular Cell **11**(5): 1405-1413.

Mitsis, P. G. (2001). Exonucleases. Nature Encyclopedia of Life Sciences, http://www.els.net/, Nature Publishing Group.

Mohanty, B. K. and S. R. Kushner (2000). "Polynucleotide phosphorylase functions both as a 3' right-arrow 5' exonuclease and a poly(A) polymerase in Escherichiacoli." PNAS **97**(22): 11966-11971.

Mohanty[a], B. K. and S. R. Kushner (1999). "Analysis of the function of Escherichia coli poly(A) polymerase I in RNA metabolism." Mol Microbiol **34**(5): 1094-108.

Moser, M., W. Holley, A. Chatterjee and I. Mian (1997). "The proofreading domain of Escherichia coli DNA polymerase I and other DNA and/or RNA exonuclease domains." Nucl. Acids. Res. **25**(24): 5110-5118.

Muhlrad, D. and R. Parker (1994). "Premature translational termination triggers mRNA decapping." Nature **370**(6490): 578-81.

Musco, G., G. Stier, C. Joseph, M. A. Castiglione Morelli, M. Nilges, T. J. Gibson and A. Pastore (1996). "Three-dimensional structure and stability of the KH domain: molecular insights into the fragile X syndrome." Cell **85**(2): 237-45.

Navon, G., S. Ogawa, R. G. Shulman and T. Yamane (1977). "High-resolution 31P nuclear magnetic resonance studies of metabolism in aerobic Escherichia coli cells." Proc Natl Acad Sci U S A **74**(3): 888-91.

O'Hara, E., J. Chekanova, C. Ingle, Z. Kushner, E. Peters and S. Kushner (1995). "Polyadenylylation Helps Regulate mRNA Decay in Escherichia coli." PNAS **92**(6): 1807-1811.

Osborne, B. I. and L. Guarente (1989). "Mutational analysis of a yeast transcriptional terminator." Proc Natl Acad Sci U S A **86**(11): 4097-101.

Patterson, B. and C. Guthrie (1987). "An essential yeast snRNA with a U5-like domain is required for splicing in vivo." Cell **49**(5): 613-24.

Petfalski, E., T. Dandekar, Y. Henry and D. Tollervey (1998). "Processing of the Precursors to Small Nucleolar RNAs and rRNAs Requires Common Components." Mol. Cell. Biol. **18**(3): 1181-1189.

Proudfoot, N. (2000). "Connecting transcription to messenger RNA processing." Trends in Biochemical Sciences **25**(6): 290.

Proudfoot[b], N. J., A. Furger and M. J. Dye (2002). "Integrating mRNA Processing with Transcription." Cell **108**(4): 501-512.

Py, B., H. Causton, E. A. Mudd and C. F. Higgins (1994). "A protein complex mediating mRNA degradation in Escherichia coli." Mol Microbiol **14**(4): 717-29.

Py, B., C. F. Higgins, H. M. Krisch and A. J. Carpousis (1996). "A DEAD-box RNA helicase in the Escherichia coli RNA degradosome." Nature **381**(6578): 169-172.

Qu, L.-H., A. Henras, Y.-J. Lu, H. Zhou, W.-x. Zhou, Y.-Q. Zhu, J. Zhao, Y. Henry, M. Caizergues-Ferrer and J.-P. Bachellerie (1999). "Seven Novel Methylation Guide Small Nucleolar RNAs Are Processed from a Common Polycistronic Transcript by Rat1p and RNase III in Yeast." Mol. Cell. Biol. **19**(2): 1144-1158.

Ragg, H., J. Schroeder and K. Hahlbrock (1975). "Poly(A)-containing RNA from Petroselinum hortense: isolation, properties and messenger function in vitro." Mol Biol Rep **2**(2): 119-27.

Raijmakers, R., W. V. Egberts, W. J. van Venrooij and G. J. M. Pruijn (2002). "Protein-Protein Interactions between Human Exosome Components Support the Assembly of RNase PH-type Subunits into a Six-membered PNPase-like Ring." Journal of Molecular Biology **323**(4): 653-663.

Reed, R. (2003). "Coupling transcription, splicing and mRNA export." Curr Opin Cell Biol **15**(3): 326.

Reibstein, D., J. A. den Hollander, S. J. Pilkis and R. G. Shulman (1986). "Studies on the regulation of yeast phosphofructo-1-kinase: its role in aerobic and anaerobic glycolysis." Biochemistry **25**(1): 219-27.

Rhee, S. K., T. Icho and R. B. Wickner (1989). "Structure and nuclear localization signal of the SKI3 antiviral protein of Saccharomyces cerevisiae." Yeast **5**(3): 149-58.

Rogozin, I. B., L. Aravind and E. V. Koonin (2003). "Differential Action of Natural Selection on the N and C-terminal Domains of 2'-5' Oligoadenylate Synthetases and the Potential Nuclease Function of the C-terminal Domain." Journal of Molecular Biology **326**(5): 1449-1461.

Rosenberg, I. M. (1996). Protein Analysis and Purification: Benchtop Techniques. Boston, Birkhauser.

Rott, R., G. Zipor, V. Portnoy, V. Liveanu and G. Schuster (2003). "RNA Polyadenylation and Degradation in Cyanobacteria Are Similar to the Chloroplast but Different from Escherichia coli." J. Biol. Chem. **278**(18): 15771-15777.

Ryan, K., O. Calvo and J. L. Manley (2004). "Evidence that polyadenylation factor CPSF-73 is the mRNA 3' processing endonuclease." RNA **10**(4): 565-573.

Sachs, A. B., R. W. Davis and R. D. Kornberg (1987). "A single domain of yeast poly(A)-binding protein is necessary and sufficient for RNA binding and cell viability." Mol Cell Biol **7**(9): 3268-76.

Sachs, A. B. and J. A. Deardorff (1992). "Translation initiation requires the PAB-dependent poly(A) ribonuclease in yeast." Cell **70**(6): 961-73.

Sadoff, B. U., S. Heath-Pagliuso, I. B. Castano, Y. Zhu, F. S. Kieff and M. F. Christman (1995). "Isolation of Mutants of Saccharomyces cerevisiae Requiring DNA Topoisomerase I." Genetics **141**(2): 465-479.

Saez, M. J. and R. Lagunas (1976). "Determination of intermediary metabolites in yeast. Critical examination of the effect of sampling conditions and recommendations for obtaining true levels." Mol Cell Biochem **13**(2): 73-8.

Saitoh, S., A. Chabes, W. H. McDonald, L. Thelander, I. Yates, John R. and P. Russell (2002). "Cid13 Is a Cytoplasmic Poly(A) Polymerase that Regulates Ribonucleotide Reductase mRNA." Cell **109**(5): 563-573.

Salhany, J. M., T. Yamane, R. G. Shulman and S. Ogawa (1975). "High resolution 31P nuclear magnetic resonance studies of intact yeast cells." Proc Natl Acad Sci U S A **72**(12): 4966-70.

Sambrook, J., E. F. Fritsch and T. Maniatis (1989). Molecular Cloning. New York, CSHL Press.

Schimmang, T., D. Tollervey, H. Kern, R. Frank and E. C. Hurt (1989). "A yeast nucleolar protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability." Embo J **8**(13): 4015-24.

Schmitt, C., C. von Kobbe, A. Bachi, N. Pante, J. P. Rodrigues, C. Boscheron, G. Rigaut, M. Wilm, B. Seraphin, M. Carmo-Fonseca and E. Izaurralde (1999). "Dbp5, a DEAD-box protein required for mRNA export, is recruited to the cytoplasmic fibrils of nuclear pore complex via a conserved interaction with CAN/Nup159p." EMBO J **18**(15): 4332-47.

Schmitt, M. and D. Clayton (1993). "Nuclear RNase MRP is required for correct processing of pre-5.8S rRNA in Saccharomyces cerevisiae." Mol. Cell. Biol. **13**(12): 7935-7941.

Sheldon, J. G., S. P. Williams, A. M. Fulton and K. M. Brindle (1996). "31P NMR magnetization transfer study of the control of ATP turnover in Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **93**(13): 6399-404.

Shen, E. C., M. F. Henry, V. H. Weiss, S. R. Valentini, P. A. Silver and M. S. Lee (1998). "Arginine methylation facilitates the nuclear export of hnRNPÂ proteins." Genes Dev. **12**(5): 679-691.

Shen, V. and D. Schlessinger (1982). RNase I, II, and IV of Esterichia coli. The Enzymes. P. D. Boyer. New York, Academic Press. **XV, part B:** 501-515.

Sherman, F., Fink, G.R., and Hicks, J.B.N. (1986). Methods in Yeast Genetics. New York, CSHL Press.

Shulman, R. G., T. R. Brown, K. Ugurbil, S. Ogawa, S. M. Cohen and J. A. den Hollander (1979). "Cellular applications of 31P and 13C nuclear magnetic resonance." Science **205**(4402): 160-6.

Singleton, D., S. Chen, M. Hitomi, C. Kumagai and A. Tartakoff (1995). "A yeast protein that bidirectionally affects nucleocytoplasmic transport." J Cell Sci **108**(1): 265-272.

Siomi, H., M. Choi, M. C. Siomi, R. L. Nussbaum and G. Dreyfuss (1994). "Essential role for KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome." Cell **77**(1): 33-9.

Sommer, S. S. and R. B. Wickner (1987). "Gene disruption indicates that the only essential function of the SKI8 chromosomal gene is to protect Saccharomyces cerevisiae from viral cytopathology." Virology **157**(1): 252-6.

Soreq, H. and U. Z. Littauer (1977). "Purification and characterization of polynucleotide phosphorylase from Escherichia coli. Probe for the analysis of 3' sequences of RNA." J. Biol. Chem. **252**(19): 6885-6888.

Stams, T., S. Niranjanakumari, C. A. Fierke and D. W. Christianson (1998). "Ribonuclease P Protein Structure: Evolutionary Origins in the Translational Apparatus." Science **280**(5364): 752-755.

Steitz, T. A. and J. A. Steitz (1993). "A general two-metal-ion mechanism for catalytic RNA." Proc Natl Acad Sci U S A **90**(14): 6498-502.

Stevens, S. W. and J. Abelson (1999). "Purification of the yeast U4/U6.U5 small nuclear ribonucleoprotein particle and identification of its proteins." Proc Natl Acad Sci U S A **96**(13): 7226-31.

Symmons, M. F., G. H. Jones and B. F. Luisi (2000). "A Duplicated Fold Is the Structural Basis for Polynucleotide Phosphorylase Catalytic Activity, Processivity, and Regulation." Structure **8**(11): 1215-1226.

Symmons, M. F., M. G. Williams, B. F. Luisi, G. H. Jones and A. J. Carpousis (2002). "Running rings around RNA: a superfamily of phosphate-dependent RNases." Trends in Biochemical Sciences **27**(1): 11-18.

Thore, S., F. Mauxion, B. Seraphin and D. Suck (2003). "X-ray structure and activity of the yeast Pop2 protein: a nuclease subunit of the mRNA deadenylase complex." EMBO Rep **4**(12): 1150-5. Epub 2003 Nov 14.

Toh, E. A., P. Guerry and R. B. Wickner (1978). "Chromosomal superkiller mutants of Saccharomyces cerevisiae." J Bacteriol **136**(3): 1002-7.

Tollervey, D. and I. W. Mattaj (1987). "Fungal small nuclear ribonucleoproteins share properties with plant and vertebrate U-snRNPs." Embo J **6**: 469-476.

Torchet, C., C. Bousquet-Antonelli, L. Milligan, E. Thompson, J. Kufel and D. Tollervey (2002). "Processing of 3'-Extended Read-Through Transcripts by the Exosome Can Generate Functional mRNAs." Molecular Cell **9**(6): 1285-1296.

Tourriere, H., K. Chebli and J. Tazi (2002). "mRNA degradation machines in eukaryotic cells." Biochimie **84**(8): 821-837.

Tucker, M., M. A. Valencia-Sanchez, R. R. Staples, J. Chen, C. L. Denis and R. Parker (2001). "The Transcription Factor Associated Ccr4 and Caf1 Proteins

Are Components of the Major Cytoplasmic mRNA Deadenylase in Saccharomyces cerevisiae." Cell **104**(3): 377-386.

Tucker, M., R. R. Staples, M. A. Valencia-Sanchez, D. Muhlrad and R. Parker (2002). "Ccr4p is the catalytic subunit of a Ccr4p/Pop2p/Notp mRNA deadenylase complex in Saccharomyces cerevisiae." EMBO J. **21**(6): 1427-1436.

Ugurbil, K., H. Rottenberg, P. Glynn and R. G. Shulman (1978). "31P nuclear magnetic resonance studies of bioenergetics and glycolysis in anaerobic Escherichia coli cells." Proc Natl Acad Sci U S A **75**(5): 2244-8.

Ugurbil, K., H. Rottenberg, P. Glynn and R. G. Shulman (1982). "Phosphorus-31 nuclear magnetic resonance studies of bioenergetics in wild-type and adenosinetriphosphatase(1-) Escherichia coli cells." Biochemistry **21**(5): 1068-75.

van Hoof, A. and R. Parker (1999). "The Exosome: A Proteasome for RNA?" Cell **99**(4): 347-350.

van Hoof[a], A., P. Lennertz and R. Parker (2000). "Yeast Exosome Mutants Accumulate 3'-Extended Polyadenylated Forms of U4 Small Nuclear RNA and Small Nucleolar RNAs." Mol. Cell. Biol. **20**(2): 441-452.

van Hoof[b], A., P. Lennertz and R. Parker (2000). "Three conserved members of the RNase D family have unique and overlapping functions in the processing of 5S, 5.8S, U4, U5, RNase MRP and RNase P RNAs in yeast." EMBO J. **19**(6): 1357-1365.

van Hoof[c], A., R. R. Staples, R. E. Baker and R. Parker (2000). "Function of the Ski4p (Csl4p) and Ski7p Proteins in 3'-to-5' Degradation of mRNA." Mol. Cell. Biol. **20**(21): 8230-8243.

Wahle[b], E. and U. Ruegsegger (1999). "3'-End processing of pre-mRNA in eukaryotes." FEMS Microbiology Reviews **23**(3): 277-295.

Walowsky, C., D. J. Fitzhugh, I. B. Castano, J. Y. Ju, N. A. Levin and M. F. Christman (1999). "The Topoisomerase-related Function Gene TRF4 Affects Cellular Sensitivity to the Antitumor Agent Camptothecin." J. Biol. Chem. **274**(11): 7302-7308.

Wang[a], L., C. R. Eckmann, L. C. Kadyk, M. Wickens and J. Kimble (2002). "A regulatory cytoplasmic poly(A) polymerase in Caenorhabditis elegans." Nature **419**(6904): 312-316.

Wang[a], S.-W., T. Toda, R. MacCallum, A. L. Harris and C. Norbury (2000). "Cid1, a Fission Yeast Protein Required for S-M Checkpoint Control when DNA

Polymerase delta or varepsilon Is Inactivated." Mol. Cell. Biol. **20**(9): 3234-3244.

Wang[b], Z., I. B. Castano, A. De Las Penas, C. Adams and M. F. Christman (2000). "Pol kappa: A DNA Polymerase Required for Sister Chromatid Cohesion." Science **289**(5480): 774-779.

Wang[b], Z., I. B. Castano, C. Adams, C. Vu, D. Fitzhugh and M. F. Christman (2002). "Structure/Function Analysis of the Saccharomyces cerevisiae Trf4/Pol {sigma} DNA Polymerase." Genetics **160**(2): 381-391.

Wickner, R. B. (1996). "Prions and RNA viruses of Saccharomyces cerevisiae." Annu Rev Genet. **30**: 109-39.

Williams, S. P., A. M. Fulton and K. M. Brindle (1993). "Estimation of the intracellular free ADP concentration by 19F NMR studies of fluorine-labeled yeast phosphoglycerate kinase in vivo." Biochemistry **32**(18): 4895-902.

Wu, P., J. S. Brockenbrough, A. C. Metcalfe, S. Chen and J. P. Aris (1998). "Nop5p Is a Small Nucleolar Ribonucleoprotein Component Required for Pre-18 S rRNA Processing in Yeast." J. Biol. Chem. **273**(26): 16453-16463.

Xiao, S., F. Houser-Scott and D. R. Engelke (2001). "Eukaryotic ribonuclease P: increased complexity to cope with the nuclear pre-tRNA pathway." J Cell Physiol **187**(1): 11-20.

Xiao, S., F. Scott, C. A. Fierke and D. R. Engelke (2002). "EUKARYOTIC RIBONUCLEASE P: A Plurality of Ribonucleoprotein Enzymes." Annual Review of Biochemistry **71Annual Review of Biochemistry 1**: 165-189.

Xu, C., P. A. HENRY, A. SETYA and M. F. HENRY (2003). "In vivo analysis of nucleolar proteins modified by the yeast arginine methyltransferase Hmt1/Rmt1p." RNA **9**(6): 746-759.

Xu, F., S. Lin-Chao and S. Cohen (1993). "The Escherichia coli pcnB Gene Promotes Adenylylation of Antisense RNAI of ColE1-Type Plasmids in vivo and Degradation of RNAI Decay Intermediates." PNAS **90**(14): 6756-6760.

Xu, F. and S. N. Cohen (1995). "RNA degradation in Escherichia coli regulated by 3' adenylation and 5' phosphorylation." Nature **374**(6518): 180-3.

Yehudai-Resheff, S., M. Hirsh and G. Schuster (2001). "Polynucleotide Phosphorylase Functions as Both an Exonuclease and a Poly(A) Polymerase in Spinach Chloroplasts." Mol. Cell. Biol. **21**(16): 5408-5416.

Yu, M. C., F. Bachand, A. E. McBride, S. Komili, J. M. Casolari and P. A. Silver (2004). "Arginine methyltransferase affects interactions and recruitment of mRNA processing and export factors." Genes Dev. **18**(16): 2024-2035.

Zanchin, N. and D. Goldfarb (1999). "The exosome subunit Rrp43p is required for the efficient maturation of 5.8S, 18S and 25S rRNA." Nucl. Acids. Res. **27**(5): 1283-1288.

Zaret, K. S. and F. Sherman (1982). "DNA sequence required for efficient transcription termination in yeast." Cell **28**(3): 563-73.

Zarudnaya, M. I., I. M. Kolomiets and D. M. Hovorun (2002). "What nuclease cleaves pre-mRNA in the process of polyadenylation?" IUBMB Life **54**(1): 27-31.

Zhelkovsky, A., S. Helmling and C. Moore (1998). "Processivity of the Saccharomyces cerevisiae Poly(A) Polymerase Requires Interactions at the Carboxyl-Terminal RNA Binding Domain." Mol. Cell. Biol. **18**(10): 5942-5951.

Zorio, D. A. R. and D. L. Bentley (2004). "The link between mRNA processing and transcription: communication works both ways." Experimental Cell Research **296**(1): 91-97.

Zuker, M., D. H. Mathews and D. H. Turner (1999). Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide in RNA Biochemistry and Biotechnology, Kluwer Academic Publishers.

Zuo, Y. and M. P. Deutscher (2001). "Exoribonuclease superfamilies: structural analysis and phylogenetic distribution." Nucl. Acids. Res. **29**(5): 1017-1026.