The Role of Yeast Rrp6p in Nuclear RNA Turnover

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Declaration

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

Rym Houalla

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Abbreviations

Amp	ampicillin
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
CTP	Cytidine triphosphate
Ci	Curie
cm	centimetre
d	deoxy-
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleicacid
dNTP	desoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylendiaminetetra acetic acid
EtOH	ethanol
ETS	external transcribed spacer
g	gram
GAL	galactose
GDP	guanosine diphosphate
GTP	guanosine triphosphate
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethansulfonic acid
HIS	histidine
H_2O	water, double distilled, sterile
ITS	internal transcribed spacer
IPTG	isopropyl-D-thiogalactopyranoside
kb	kilobase pair
kDa	kilo Dalton
l	litre
LB	luria broth
M	molar
μ	micro (10^{-6})
m	milli (10^{-3})
min	minute
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein
n	nano (10^{-9})
nt	nucleotide
OD	optical density
o/n	overnight
ORF	open reading frame
p	pico (10^{-12})

^{32}P	phosphorus, isotope 32
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylfluoride
RNA	ribonucleic acid
RRM	RNA recognition motif
rRNA	ribosomal RNA
rpm	rotations per minute
RT	room temperature
SD	synthetic dropout
SDS	sodium dodecyl sulphate
sec	second
snoRNA	small nucleolar RNA
snoRNP	small nucleolar ribonucleoprotein
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
TAP	tandem affinity purification
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylendiamine
Tris	Tris-(hydroxymethyl)
tRNA	transfer RNA
TRP	tryptophan
ts	temperature sensitive
TTP	thymidine triphosphate
U	unit
URA	uracil
UTP	uracil triphosphate
UV	ultraviolet
WT	wild-type

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Abstract

In the past few years, a discrete set of pathways for the degradation of mRNAs in eukaryotic cells have been identified, In *Saccharomyces cerevisiae*, two cytoplasmic mRNA decay pathways have been characterised. A major decay pathway that involves mRNA degradation in a 5'-3' direction by the exoribonuclease Xrn1p. the minor 3'-5' degradation pathway depends on a complex of exonucleases termed the exosome. Two distinct forms of the exosome exist, nuclear and cytoplasmic that differ by the presence of Rrp6p in the nucleus. Rrp6p was shown to have functions in nuclear RNA processing that are distinct from those of the "core" components, which are common to nuclear and cytoplasmic complexes. This raises the possibility of Rrp6p being associated with complexes other than the exosome. To gain more insight about the role played by Rrp6p in RNA metabolism, my work aimed at identifying and characterising potential Rrp6p-associated complexes. Density gradient analyses revealed no pool of free Rrp6p, but the majority of Rrp6p was apparently associated with complexes other than the exosome. In addition to the exosome Rrp6p appears to be associated with a 10S complex and another two RNase A sensitive complexes that probably correspond to 40S and 60S pre-ribosomes.

Purification of exosome complexes allowed the identification of a substoichiometric component, which was identified as the nuclear protein Rrp47p. Rrp47p was shown to be required for most Rrp6p functions in stable RNA synthesis but not for Rrp6p function in RNA surveillance. To better understand this similarity, I analysed the Rrp47p-exosome and the Rrp47p-Rrp6p interactions. Around 10-20% of the exosome was found to be associated with both Rrp6p and Rrp47p however Rrp47p did not appear to be part of the observed Rrp6p complexes.

Recent analyses have identified pathways that degrade aberrant nuclear pre-mRNAs and require the nuclear exosome complex. To identify potential endogenous targets for nuclear turnover, microarray analysis were performed on strains lacking the nuclear-specific exosome component Rrp6p or carrying a mutation in Rrp41p, a core exosome component. Microarray data show only limited overlap in mRNAs altered in strains lacking Rrp6p or Rrp41p, supporting the functional distinction between Rrp6p and core exosome components. *NRD1* was found to be increased approximately 3 fold, an observation, which was confirmed by Northern hybridisation and primer extension. *NRD1* expression is subject to auto-regulation involving binding of Nrd1p to the mRNA and the RNA helicase Sen1p. In double mutants, *rrp6*Δ was not clearly synergistic with either *nrd1* or *sen1*, suggesting that they function in the same pathway. In addition, 5' truncated forms of NRD1 mRNA were observed in *rrp6*Δ, *rrp47*Δ and a strain carrying a mutation in Rrp41p. A model was proposed in which the binding of Nrd1p to its mRNA promotes recruitment of the exosome and mRNA degradation.

My results have provided new insights into the function of Rrp6p in nuclear pre-mRNA turnover and into its potential association with complexes other than the exosome.

Chapter one

Introduction

I.A The regulation of gene expression

Regulation of gene expression in eukaryotic cells is the result of a series of complex mechanisms that are initiated in the nucleus by transcription and achieved by translation in the cytoplasm. This regulation is accomplished by mechanisms acting at the level of transcription, pre-mRNA processing, mRNA export, subcellular localisation, translation efficiency, and mRNA degradation (Tourriere et al., 2002). It is becoming evident that the components of the mRNA synthesis machinery interact with each other to establish a distinct surveillance mechanism that determines release of the transcript from the transcription site for further export and utilisation.

RNA degradation must be closely regulated so as to prevent wholesale elimination of all transcripts. The intracellular RNA degradation machinery is largely exonucleolytic allowing RNAs to escape decay by protecting their ends. A key component of the RNA degradation machinery is the nuclear exosome, which comprises a versatile multicomplex of exonucleases and accessory factors (Mitchell et al., 1997). In the budding yeast *Saccharomyces cerevisiae*, the predominant nuclear decay pathway is 3'-5' and involves the activity of the exosome (Bousquet-Antonelli et al., 2000). The exosome also functions in the cytoplasm where it is required for the 3'-5' decay of deadenylated mRNAs (Anderson and Parker, 1998). The nuclear exosome is distinguished from its cytoplasmic counterpart by the presence of the nuclear exonuclease component Rrp6p (Allmang et al., 1999b). Recent studies have focused largely on the role, played by Rrp6p as an additional level of control that decides the fate of the transcript.

I.B The exosome: a processing and degradation machine

The genome of *S cerevisiae* was found to encode at least nineteen 3'-5' exoribonucleases, all of which presumably play roles in RNA processing. Analyses of 3' processing of the 5.8S ribosomal RNA (rRNA) in this organism led to the identification of the exosome complex of 3'-5' exoribonucleases. The exosome is localised in both the nucleus and the cytoplasm and has been described in eukaryotes as diverse *as S. cerevisiae* (Mitchell et al., 1997), *Homo sapiens* (Chen et al., 2001), *Arabidopsis thaliana* (Chekanova et al., 2000), *Drosophila melanogaster* (Andrulis et al., 2002) and the parasitic protozoan *Trypanosoma brucei* (Estevez et al, 2001) and homologous proteins are present in the genomes of Archaea. In yeast, the exosome functions in both the precise processing of 3'-extended precursor molecules to mature stable RNAs and the complete degradation of other RNAs (Mitchell et al., 1996, 1997; Allmang et al., 1999b; Anderson and Parker, 1998). The cytoplasmic complex was shown to function in the degradation of mRNAs in a 3' to 5' direction. In the nucleus, the exosome is involved in the precise 3' processing of precursor molecules to mature ribosomal RNAs, small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) and in nuclear pre-mRNA turnover (Allmang et al., 1999a; van Hoof et al., 2000b; Bousquet-Antonelli et al., 2000). mRNAs, snRNAs and snoRNAs are transcribed by RNA polymerase II whereas the 5.8S, 25S and 18S rRNAs are transcribed by RNA polymerase I (Venema and Tollervey, 1995;Chanfreau et al., 1997;Tollervey and Kiss, 1997). snRNAs and snoRNAs function in pre-mRNA splicing and in the modification and processing of pre-ribosomal RNAs respectively (Chanfreau et al., 1997;Tollervey and Kiss, 1997). The 5.8S and 25S rRNAs are components of the 60S ribosomal subunit whereas the 18S rRNA is a component of the 40S ribosomal subunit (Venema and Tollervey, 1995). Thus, the exosome is required to play a role in the regulated processing and degradation of nearly all types of RNA molecules.

I.B.1 The identification of the exosome

Evidence for the existence of a complex of exoribonucleases involved in RNA processing first emerged from studies on the ribosomal rRNA processing pathway in the budding yeast *S. cerevisiae.* A screen for strains with defects in 5.8S rRNA processing identified a mutant *rrp4-1* (for rRNA processing) that showed a defect in 3' maturation of 5.8S and a temperature sensitive growth phenotype (Mitchell et al., 1996). Upon shift to the nonpermissive temperature the *rrp4-1* strains accumulated a ladder of 3' extended 5.8S molecules, suggesting that Rrp4p is required for a 3'-5' exonuclease activity that generates the 3' end of the 5.8S rRNA. In addition, an in vitro 3'-5' exonuclease activity was observed in immunoprecipitates of an epitope-tagged version of Rrp4p (Mitchell et al., 1996). Subsequent analysis revealed that Rrp4p purified from cells as a complex that exhibits the Rrp4p-associated 3'-5' exonuclease activity in vitro and was designated as "the exosome". Rrp4p-associated proteins were later identified by mass-spectometric analysis and distinct nuclear and cytoplasmic forms of the exosome were characterised (Mitchell et al., 1997;Allmang et al., 1999b).

I.B.2 The cytoplasmic and nuclear forms of the exosome

The exosome complex is present in both the nucleus and the cytoplasm and consists of a core of ten proteins (Rrp4p, Rrp40p, Rrp41p/Ski6p, Rrp42p, Rrp43p, Rrp44p, Rrp45p, Rrp46p, Mtr3p and Csl4p/Ski4p; Table 1.1). Three of these have been demonstrated to have 3'-5' exoribonuclease activity in vitro and a further six have high sequence homology to characterised 3'-5' exoribonucleases (Mitchell et al, 1997;Mitchell and Tollervey, 2000;Table 1.1). Although not formally proven, it is now likely that nine out of the ten proteins are exoribonucleases (Allmang et al., 1999b). The remaining component is Csl4p/Ski4p, which, does not show homology to a known exonuclease but contains a predicted RNA binding domain similar to that present in *E.coli* ribosomal protein S1 (S1 RBD). The nuclear exosome has an additional subunit, Rrp6p, which is yet another 3'-5' exoribonuclease homologous to

Protein	Gene	Activity[1]	Motifs/homologs	Deletion phenotype	Human homolog[2]	Comments
Rrp4p	YHR069c	Hydrolytic, distributive 3′→5′ exonuclease	S1 RBD	Essential	hRrp4p (43%)	hRrp4p complements rrp4-1
Rrp40p	YOL142w	(Hydrolytic, distributive 3′→5′ exonuclease)	S1 RBD	Essential	hRrp40p (30%)	Homologous to Rrp4p
Csl4p/Ski4p csl4-1	YNL232w	?	S1 RBD	Essential	hCsl4p (48%)	hCsl4p complements
Rrp41p/Ski6p	YGR195w	Phosphorolytic, processive 3′→5′ exonuclease	RNase PH	Essential	hRrp41p (35%)	hRrp41p complements GAL:rrp41
Rrp42p	YDL111c	(Phosphorolytic, processive) 3′→5′ exonuclease	RNase PH	Essential		
Rrp43p	YCR035c	(Phosphorolytic, processive 3′→5′ exonuclease)	RNase PH	Essential		
Rrp45p	YDR280w	(Phosphorolytic, processive 3′→5′ exonuclease)	RNase PH	Essential	PM-Scl 75 (38%)	Human KIAA0116 and OIP2 also homologous
Rrp46p	YGR095c	(Phosphorolytic, processive 3′→5′ exonuclease)	RNase PH	Essential	hRrp46p (26%)	
Mtr3p	YGR158c	(Phosphorolytic, processive)	RNase PH	Essential		
Rrp44p/Dis3p	YOL021c	Hydrolytic, processive 3′→5′ exonuclease	RNase R (vacB)	Essential	hDis3p (45%)	hDis3p complements dis3-81
Rrp6p	YOR001w	hydrolytic 3′→5′ exonuclease	RNase D	ts-lethal	PM-Scl 100 (32%)	Component only of nuclear complex
Cofactors						
Mtr4p/Dob1p	YJL050W	(ATP-dependant helicase)	DEAD box	Essential		
Ski2p	YLR398C	(ATP-dependant helicase)	DEAD box	Nonessential	SKIV2L (38%)	
Ski3p	YPR189W		TPR repeat	Nonessential	KIAA0372 (20%)	
Ski7p	YOR076c	(GTPase)		Nonessential		High similarity
Ski8p	YGL213C		WD repeat	Nonessential		to Hbs1p

[1]Demonstrated enzymatic activities are listed; activities in parentheses are predicted from sequence homology.
[2]For the human homologues, the numbers given indicate the percentage identity along the entire length of the protein.

Table 1.1 Exosome components and cofactors (Mitchell and Tollervey, 2000)

E.coli RNase D (Allmang et al., 1999b; Burkard and Butler, 2000). All components of the exosome are essential for viability, with the exception of Rrp6p. Strains lacking Rrp6p are severely impaired in growth and are temperature sensitive (Briggs et al., 1998).

Rrp41p, Rrp42p, Rrp43, Rrp45p, Rrp46p and Mtr3p are phosphorolytic enzymes using inorganic phosphate as the attacking group and releasing nucleotide 5' diphosphates. However, so far phosphorylytic activity has only been demonstrated for Rrp41p (Mitchell and Tollervey, 2000). Rrp4p, Rrp44p Rrp40p, and Rrp6p are hydrolytic enzymes using water as the attacking group and releasing nucleotide 5' monophosphates. Moreover, Rrp4p and Rrp40 are distributive enzymes whereas Rrp41p, Rrp42p, Rrp43p, Rrp44p, Rrp45 and Rrp46 are processive enzymes. In a distributive activity, the exonuclease repeatedly binds and dissociates from the substrate, removing nucleotides one at a time generating fragments that are progressively shortened from their 3'-end. By contrast, a processive exonuclease once bound remains associated with the RNA digesting the substrate to completion and thus no intermediates can be readily detected during the digestion reaction (Mitchell and Tollervey, 2000).

Furthermore, although the individual proteins are active *in vitro* the exosome purified from yeast lysates exhibited little activity. This activity was a distributive hydrolytic activity characteristic of Rrp4p although the majority of the enzymes comprising the exosome possess a phosphorolytic processive mode of action (Mitchell et al., 1997). This suggests that activation of the complex requires additional cofactors that are not stably associated with the complex and were thus lost during the purification (Mitchell et al., 1997). Candidates for these proteins include the products of the *MTR4, SKI2* and *SKI7* genes. The *MTR4* gene encodes a putative nuclear helicase that is required for the processing of a variety of RNA species by the exosome. and Ski2p and the putative GTPase Ski7p. The *SKI2* gene encodes a homologous cytoplasmic putative RNA helicase and *SKI7* a putative GTPase, which are required for 3'-5'mRNA degradation by the exosome in the cytoplasm (de la Cruz et al., 1998; Anderson and Parker, 1998;Van Hoof et al., 2000c; Table 1.1).

Recently, a speculative model for the structure of the exosome has been proposed based on cryo-electron microscopy and on the similarity between bacterial

polynucleotide phosphorylase (PNPase) and exosome components. PNPase is a single polypeptide with two tandem RNase PH domains (RPD) followed by S1 and KH domains (Aloy et al., 2002) (Figure 1.1). The structure of the PNPase from *Streptomyces antibioticus* shows that the PNPase is a tightly associated trimeric enzyme with a total of six RPDs arranged around a central core (Symmons et al., 2000). This number agrees with the estimated stoichiometry of the yeast exosome in which each particle is thought to contain one copy of the six RPD subunits (van Hoof and Parker, 1999). Based on these studies a model for the exosome structure has been proposed in which the RNase PH-like proteins are arranged in a hexameric ring whereas the three S1 domain proteins are placed on top of the ring structure (Aloy et al., 2002).

I.B.3 Regulation of the exosome

The exosome is involved in both the accurate processing and the complete degradation of RNA substrates. This dual nature of the exosome raises the important issue of how a complex of ten exoribonucleases distinguishes between RNA molecules destined for destruction and those that become mature functional RNAs (Butler, 2002).

Two models for the activation of the exosome have been proposed: the proteosome model and the allosteric model (van Hoof and Parker, 1999;Mitchell and Tollervey, 2000) (Figure 1.2). In the proteosome model, the active sites of the enzymes are directed towards the centre of a hollow cavity and are therefore not freely accessible to macromolecules. Substrates would gain access to the active sites by interacting with adaptor proteins associated with the complex like the RNA helicases Mtr4p and Ski2p and the GTPase Ski7p (Mitchell and Tollervey, 2000;van Hoof et al., 2000c). Mtr4p and Ski2p are closely related members of the superfamily II of RNA helicases, which utilise ATP hydrolysis to promote conformational changes either in RNA structure or possibly in RNA-protein interactions (de la Cruz et al., 1999). Thus, it is thought that displacement or reorganisation of the helicase occurs upon

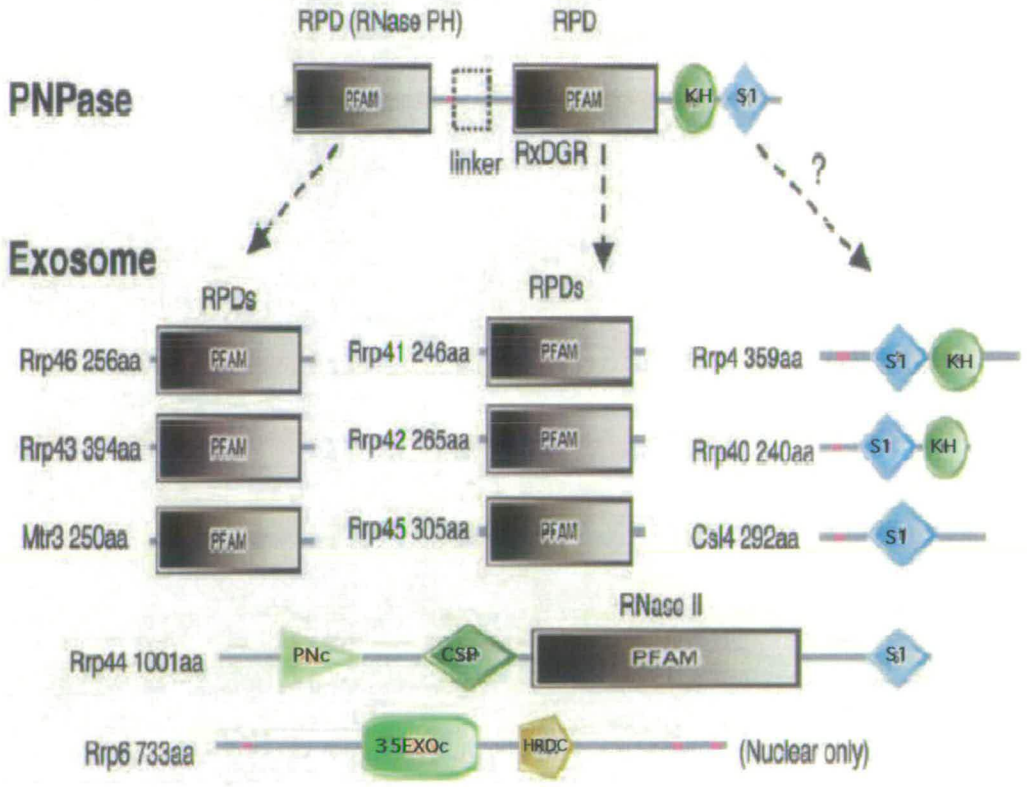


Figure 1.1. Domain structure for the exosome core and PNPase. Three copies of the PNPase form a trimer with a total of six RPD domainss arranged a central pore. This number agrees with the estimated stoichiometry of the yeast exosome. Domains found in Rrp6p and Rrp44p have no PNPase equivalent and may thus differ in location in the exosome. Figure adapted from Aloy et al., (2002).

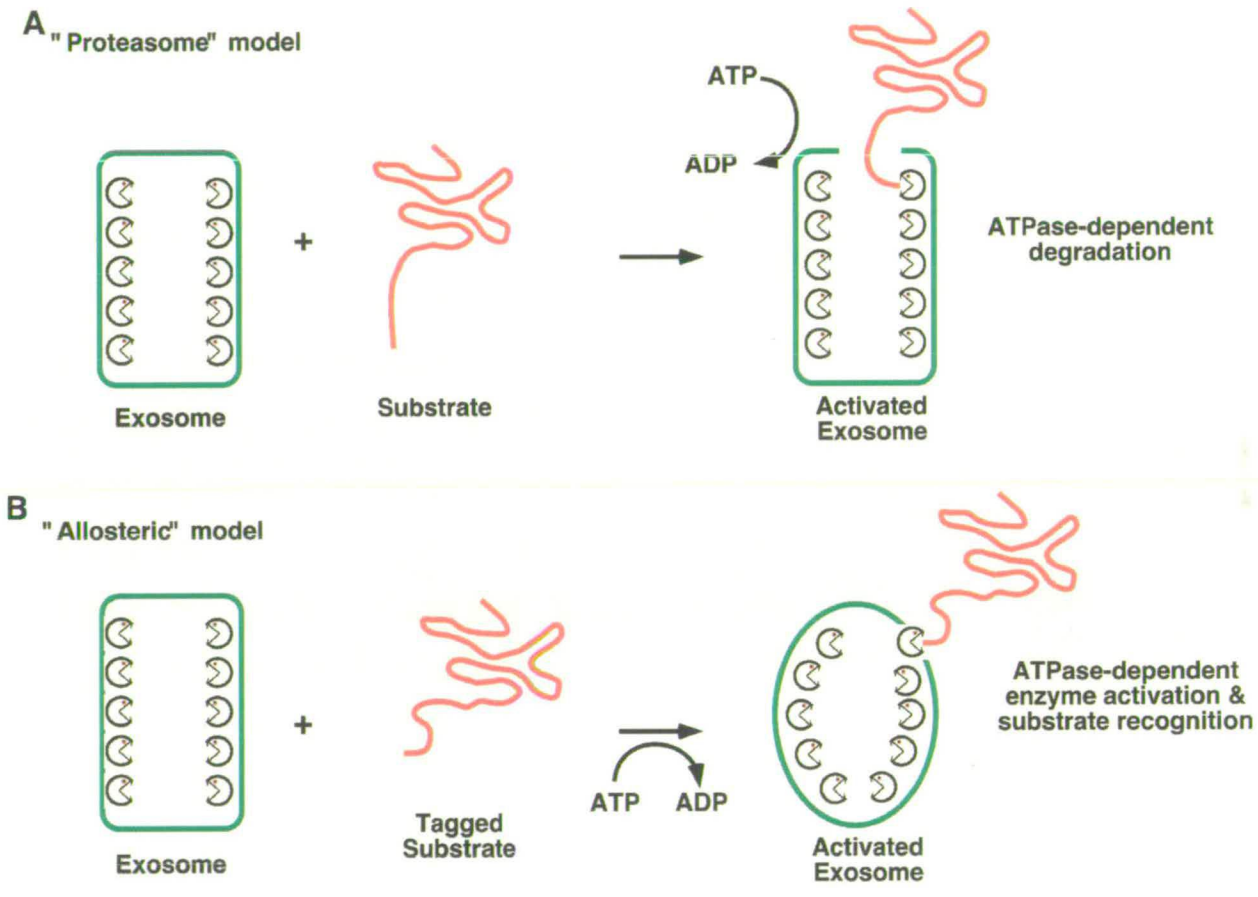


Figure 1.2. Models for the activation of the exosome

A. Proteosome model. the active sites of the exonucleases are arranged within a central cavity. Acess to this cavity is regulated by the RNA helicase Mtr4p or Ski2p.

B. Allosteric model. The RNA helicase interacts with the RNP substrate leading to the remodelling of the exosome into an appropriate active form. Figure adapted from Mitchell and Tollervey, (2000).

interaction with the ribonucleoprotein (RNP) substrate. The activated helicase hydrolyses ATP and is thought to couple the unfolding of the substrate to its delivery to the centre of the exosome (Tollervey and Mitchell, 2000). The exosome cofactor Ski7p is a GTPase that binds GTP and hydrolyses it to GDP and this transition is often associated with large conformational changes in the protein, resulting in alterations of protein-interacting and/or RNA interacting surfaces (van Hoof et al., 2000c). Thus it is possible that Ski7p acts as an exosome cofactor that brings two or more macromolecules together and uses GTPase activity as a source of energy (Benard et al., 1999;van Hoof et al., 2000c). However, there are several important differences between the proteosome and the exosome complex. The proteosome was found as a stable complex with the regulatory ATPase complex whereas the yeast exosome was not isolated with either Mtr4p or Ski2p (Mitchell et al., 1997;Allmang et al., 1999b). In addition, although both complexes degrade various substrates in an apparently processive manner, only the exosome is capable of accurate processing of substrate ends using an exonucleolytic processing mechanism (Mitchell and Tollervey, 2000).

In the alternative allosteric model, specific interaction with the RNA substrates causes the remodelling of the exosome structure and determines whether the substrate undergoes 3' end formation or degradation. In this model, the helicase would interact directly with the RNP substrate recognising specific marker proteins. ATP hydrolysis by the RNA helicase allows the exosome to be remodelled into an appropriately active form. The helicase may interact with the RNP substrate either when associated with the exosome or in its absence. (Mitchell and Tollervey, 2000) The fate of the substrate would also be expected to depend on which of the nucleases is activated. The key difference between these models is that in the proteosome model the enzymes are constutively active and substrate access is strictly regulated. In the allosteric model the enzymes are constutively inactive and are individually activated on substrate binding. However, if each substrate requires a specific " exosome adaptor", more cofactors that have been identified to date would be required. This also raises the issue about the sources of energy available for the activity of the various exoribonucleases in the complex. RNAs are folded and packaged with proteins into RNP particles and the unfolding of these structures

requires a substantial energy cost. Enzymes with a phosphorolytic mode of action (Rrp41p, Rrp42p, Rrp43p, Rrp45p, Rrp46p and Mtr3p) have little free energy available and are dependent on RNA helicases to unwind structured RNA substrates. In contrast, substantial free energy is released by RNA hydrolysis, potentially enabling the hydrolytic enzymes (Rrp4p, Rrp6p, Rrpp40p and Rrp44p) to use this energy to couple RNA degradation to the unfolding of highly structure substrates (Mitchell and Tollervey, 2000).

I.C. Major pathways involving the exosome

I.C.1 rRNA processing

The production of a wide variety of stable RNA species in eukaryotes occurs by post-transcriptional processing from large precursors. This has long been known for the highly abundant cytoplasmic RNAs, tRNAs and rRNAs (Deutscher, 1990;Venema and Tollervey, 1995). Recent studies have shown that this also the case for small nuclear RNAs (snRNAs), which participate in pre-mRNA splicing and the small nucleolar RNAs (snoRNAs), which participate in rRNA processing and modification (Maxwell and Fournier, 1995; Tollervey and Kiss, 1997).

In all eukaryotic cells ribosomal RNAs are largely matured in a special nuclear compartment, the nucleolus. Three of the four mature rRNAs are transcribed by RNA polymerase I as a single precursor, the 35S pre-rRNA that is subsequently cleaved and trimmed to produce the mature 18S, 5.8S and 25S rRNAs (Figure 1.3). In addition to the mature rRNA sequences, the 35S precursor contains two external transcribed spacers, the 5'-ETS and 3'-ETS, and two internal transcribed spacers, ITS1 and ITS2 (Venema and Tollervey, 1995).

Formation of the mature 5.8S from the 7S pre-rRNA occurs via a 3'-5' exonucleolytic processing mechanism that requires the exosome (Mitchell et al.,

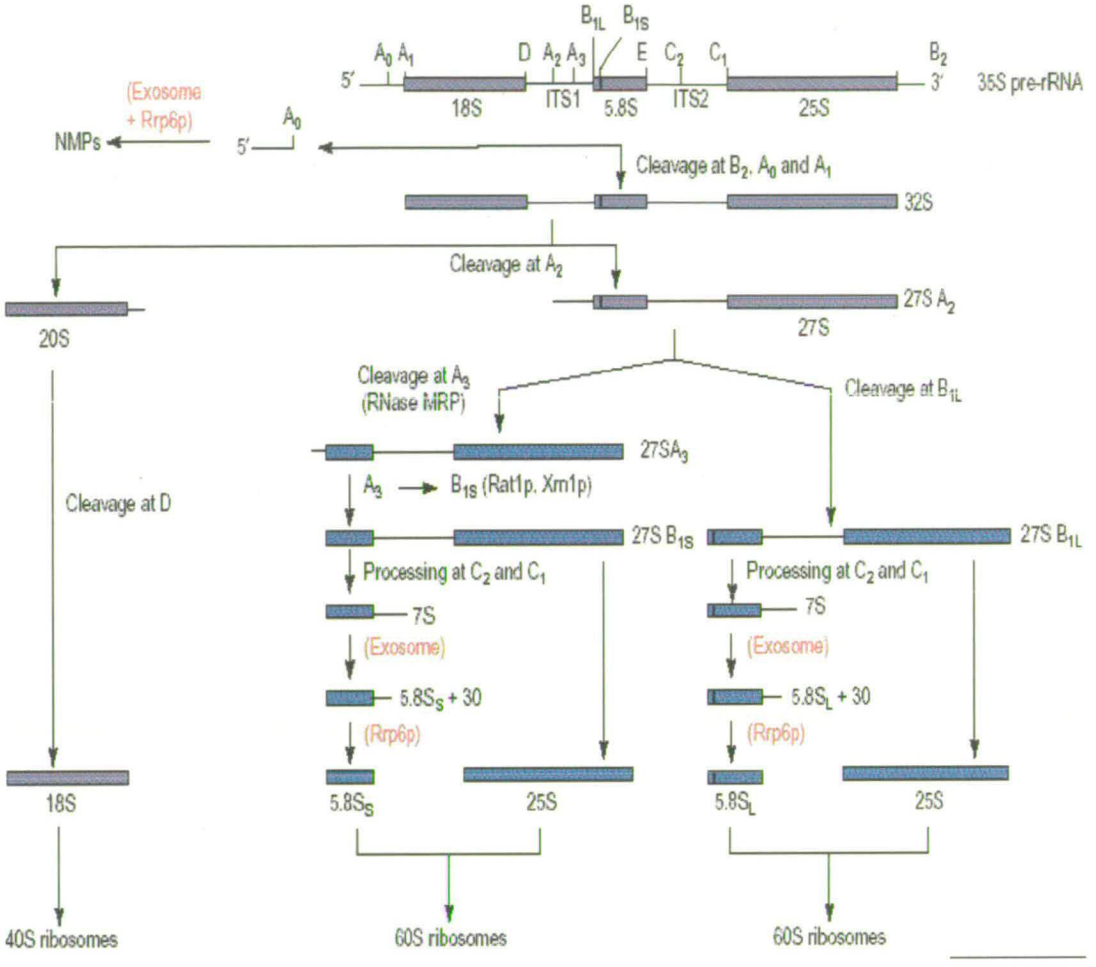


Figure 1.3. The pre-rRNA processing pathway in *Saccharomyces cerevisiae*

The 3' end of 5.8S rRNA is formed by exonucleolytic removal of the 3'end of the 7S pre-rRNA by the exosome and Rrp6p, in which the exosome removes all but the last 30 nucleotides, which are then trimmed away by Rrp6p. 3' end processing of mature 5.8S rRNA involves at least two changes in the nuclease that is active within the exosome (Allmang et al., 2000). Figure adapted from Butler (2002).

1996). The 3' end formation of 5.8S rRNA is a multistep process and two intermediates in the processing of 7S pre-rRNA to mature 5.8S rRNA that are 3' extended by 30nt (5.8S+30) and 8nt (6S pre-rRNA) are detectable in wild type cells. Strains carrying conditional mutations for the 10 essential components of the exosome resulted in the accumulation of 3'extended 5.8S rRNA species forming a ladder up to the position of the 3'-end of the 7S pre-rRNAs. The 6S intermediate accumulates in strains carrying conditional mutation in several of the exosome components whereas the 5.8S + 30 intermediates accumulate specifically in strains lacking the nuclear specific component Rrp6p. This unique accumulation of 5.8S + 30 in Rrp6p mutants is blocked by depletion of Rrp41p or Rrp45p, indicating that the core exosome components normally act upstream of Rrp6p. This also indicates that 3' end processing of the mature 5.8S rRNA involves at least two changes in the nuclease that is active within the exosome complex (Mitchell et al., 1996; Allmang et al., 2000a). Further processing of the 6S pre-rRNA involves two nonessential 3'-5' exonucleases Rex1p and Rex2p, which are homologues to members of the RNase D family of exonucleases (van Hoof et al., 2000a). Both proteins are functionally redundant in the 3'-end formation of the 5.8S rRNA, namely processing of the 6S pre-rRNA, generating a 5.8S rRNA, which is 3'-extended by 5nt. Accumulation of 6S pre-rRNA was only observed in *rex1Δ/rex2Δ* double mutants but not in *rex1Δ* single mutants, indicating Rex2p can functionally replace Rex1p (van Hoof et al., 2000a). Recently, it was shown that removal of the last few nucleotides of this 5.8S+5 rRNA also require the potential endonuclease Ngl2p (Faber et al., 2002). How all of these components interact during the final trimming of the 5.8S rRNA remains unclear, but underlines the true complexity of the apparently simple RNA processing events.

3'- end synthesis of the 5.8S rRNA also requires the DEAD-box putative RNA helicase Dob1/Mtr4p, a cofactor of the exosome. Depletion of Mtr4p results in phenotypes similar to those observed in the absence of Rrp6p and in conditional mutants for exosome components (de la Cruz et al., 1998; Allmang et al., 2000). However, it is not known whether Mtr4p is merely needed to unfold the secondary structure of the pre-rRNA to make it accessible to the exosome, or it has a more specific function in targeting the exosome to its substrate.

Depletion of any exosome component also affects the early cleavages at sites A_0, A_1 and A_2, however, these are each endonuclease cleavages and no direct role for an exonuclease can be readily predicted (Allmang et al., 2000). Many other mutations that result in defects in 60S synthesis also inhibit the early cleavages, presumably via indirect mechanisms. Thus, the nuclear exosome plays a crucial role in the processing of 5.8S rRNA, which is an essential component of the 60S ribosomal subunit. It was shown that *rrp6-1* strains produce 5.8S+30 rRNA 20-fold faster than 5.8S rRNA yet accumulate similar amounts of the two molecules at steady state. Polyribosome analysis revealed that the 5.8S+30 rRNA is probably lost prior to or during, ribosomal subunit assembly. Furthermore, the observed decrease in the amount of free 60S ribosomal subunits is consistent with a defect in ribosome biogenesis (Briggs et al., 1998).

The exosome is also required for degradation of the 5' ETS-A_0 spacer fragment as well as aberrant and truncated RNAs that are products of blocked or delayed pre-rRNA processing events (Allmang et al., 1999a).

I.C.2 snRNA and snoRNA processing

The nuclear exosome plays a complex role in the processing of snRNAs (small nuclear RNAs) and snoRNAs (small nucleolar RNAs) (Figure 1.4). snRNAs U1, U2, U4 and U5 are transcribed by RNA polymerase II and function in mRNA splicing as components of the spliceosome. Each of these snRNAs has a cleavage site for the endonuclease Rnt1p, which cleaves the 3' extended precursors and provides an entry site for the exosome and probably other exonucleases (Chanfreau et al., 1997; Abou Elela and Ares, 1998). snoRNAs function in rRNA processing and modification and can be grouped into two large families, box C/D and box H/ACA snoRNAs. The box C/D snoRNAs select sites for rRNA 2'-O-methylation, whereas the box H/ACA snoRNA select sites of pseudouridine formation (Tollervey and Kiss, 1997; Weinstein and Steitz, 1999;Kiss, 2002). Many yeast snoRNAs are synthesised by post-transcriptional processing, either from the excised introns or from polycistronic transcripts that include multiple snoRNAs (Ooi et al., 1998;Petfalski et al., 1998). All

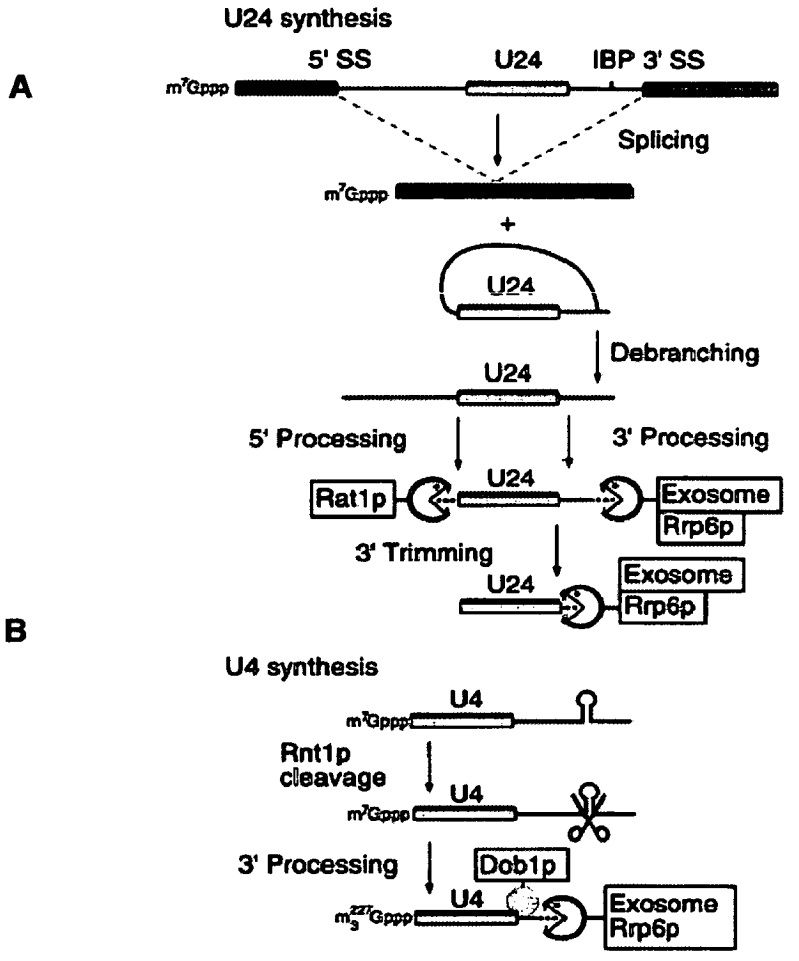


Figure1.4. Functions of the exosome in snRNA and snoRNA processing. A. Processing of the U24 snoRNA from the debranched intron lariat following mRNA splicing. **B.** Processing of the U4 snRNA. An Rnt1p cleavage site lies in the 3' flanking sequence and may act as entry site for the exosome, acting together with the putative helicase Mtr4p/Dob1p. Figure adapted from Allmang et al.,1999EMBO.

characterized yeast polycistronic snoRNAs are initially processed by endonuclease cleavage by Rnt1p (Chanfreau et al., 1998; Qu et al., 1999).

Analysis of selected snRNAs and snoRNAs in strains depleted for exosome components revealed the accumulation of 3'-extended species suggesting that the exosome plays role in the 3'end processing of precursor transcripts (van Hoof et al., 2000b;Allmang et al., 1999a). It was shown that for some snRNAs (U1, U2, U4 and U5) and snoRNAs (U14) processing might involve Rnt1p cleavage followed by exonucleolytic trimming of the 3'end by the exosome. However, the trimming of the last three nucleotides of the snoRNAs specifically requires the activity of Rrp6p (van Hoof et al., 2000b;Allmang et al., 1999a). This activity could not be substituted by other exonucleases since the entire snoRNA population is shifted in size by approximately 3 nt. As seen for 5.8S rRNA synthesis, snoRNA processing is at least biphasic. Initial processing is partially inhibited but not blocked, by different mutations in the exosome, whereas in the case of most box C/D snoRNA trimming of the final 3nt specifically requires Rrp6p (Allmang et al., 1999a;van Hoof et al., 2000b). The specificity of the exonuclease within the exosome appears to change at least twice, a phenomenon referred to as exonuclease hand-over (Allmang et al., 1999a).

I.C.3. Cytoplasmic mRNA turnover

I.C.3a. The 3'-5' degradation pathway

Although the exosome was initially assigned nuclear functions like processing of rRNA, snRNAs, and snoRNAs and degradation of spacer fragments, it also functions in the cytoplasm. The cytoplasmic exosome was shown to be required for the 3'-5' decay of deadenylated mRNAs (Figure 1.5) (Anderson and Parker, 1998). Evidence for this pathway arose from studies of the *SKI* genes. The absence of *SKI* gene function leads to enhanced replication of the cytoplasmic LA dsRNA virus and its toxin-expressing satellite virus M1 giving rise to the super killer phenotype (Widner and Wickner, 1993). The phenotype observed in the *ski* mutants is due to the enhanced translation of the LA dsRNA transcripts, which are naturally uncapped and

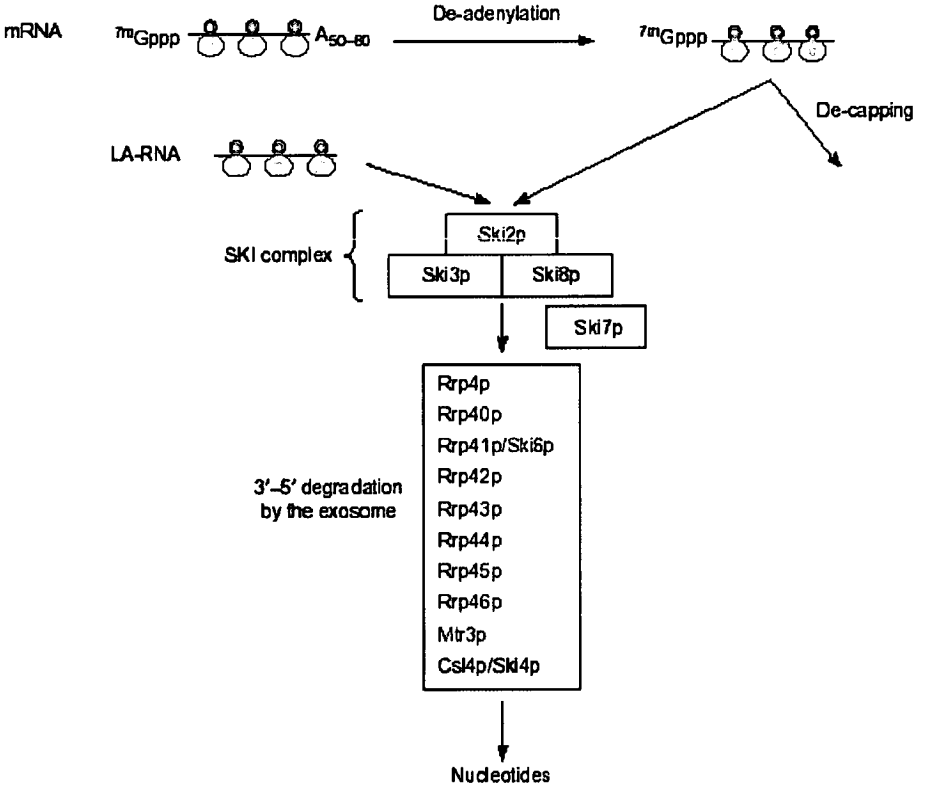


Figure1.5. The cytoplasmic exosome functions in the 3'-5- decay of deadenylated mRNAs. Deadenylated mRNAs are degraded by the exosome as are naturally unadenylated LA RNAs. This function of the cytoplasmic exosome requires the activity of the Ski complex (Ski2p-Ski3p-Ski8p) which is thought to deliver the transcripts to the exosome. The interaction between the exosome and the Ski complex is mediated by the GTPase Ski7p. Degradation of the RNAs requires the activity of the RNA helicase Ski2p. Figure adapted from Butler, (2002).

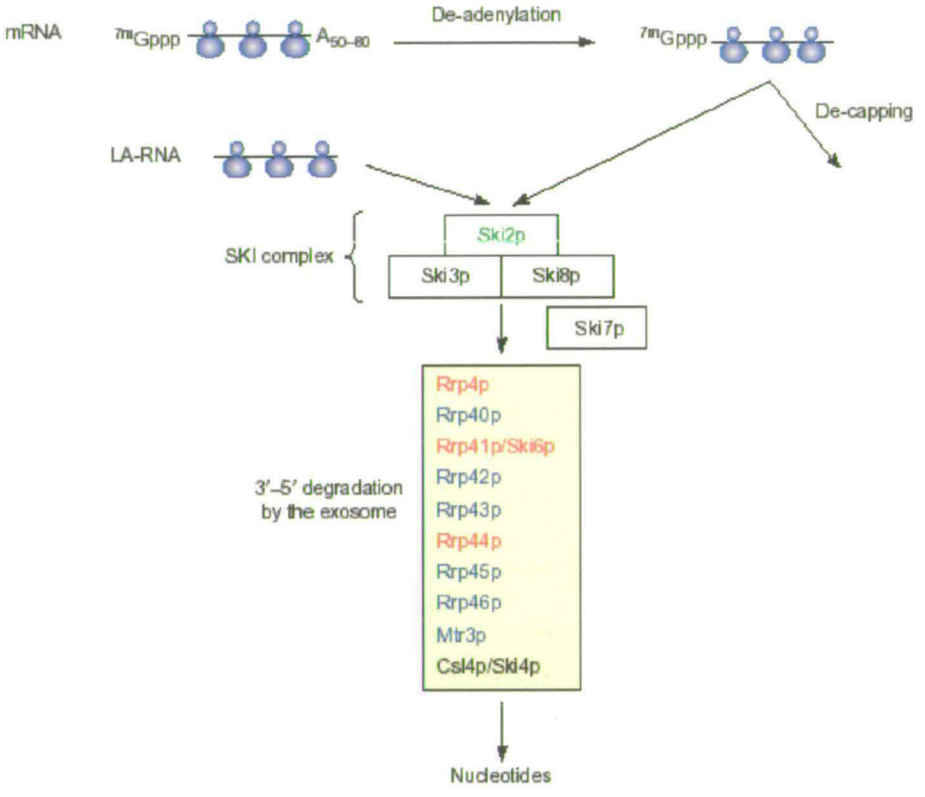


Figure1.5. The cytoplasmic exosome functions in the 3'-5-decay of deadenylated mRNAs. Deadenylated mRNAs are degraded by the exosome as are naturally unadenylated LA RNAs. This function of the cytoplasmic exosome requires the activity of the Ski complex (Ski2p-Ski3p-Ski8p) which is thought to deliver the transcripts to the exosome. The interaction between the exosome and the Ski complex is mediated by the GTPase Ski7p. Degradation of the RNAs requires the activity of the RNA helicase Ski2p. Figure adapted from Butler, (2002).

unadenylated (Widner and Wickner, 1993;Benard et al., 1998,1999). These RNAs are similar to cellular mRNAs that have been deadenylated and decapped and are about to be completely degraded (van Hoof, 2000c). The core exosome contains two *SKI* gene products, Csl4p/Ski4p and Rrp41p/Ski6p. Mutation of Ski6p, as well as Ski2p, Ski3p, Csl4p/Ski4p, Ski7p, Ski8p and Rrp4p, causes a defect in the cytoplasmic 3'-5' mRNA degradation pathway (Anderson and Parker, 1998;van Hoof et al., 2000c). Ski2p, Ski3p and Ski8p form a heterotrimer referred to as the Ski complex. The Ski complex is thought to interact (indirectly) with the cytoplasmic exosome due to the ability of both complexes to bind the separate portions of the N-terminal domain of Ski7p (Brown al., 2000;Araki et al., 2001).

On the basis of these findings a model was proposed in which the Ski complex recruits mRNAs to the exosome via Ski7p which in turn activates the exosome to degrade the mRNAs in a 3'-5' direction. (Anderson and Parker, 1998;van Hoof et al., 2000c). Degradation of the RNAs requires the activity of the putative RNA helicase Ski2p, which might function to unwind the RNA substrates, thereby facilitating their degradation by the exosome (Anderson and Parker, 1998).

I.C.3b. The 5'-3' degradation pathway

In yeast, the major cytoplasmic mRNA turnover pathway is initiated by the 3'-5' shortening of the poly (A) tail to an oligo (A) length of 10 to 15 nucleotides. This is followed by either removal of the 5' cap structure and destruction of the body of the transcript by 5'-3' exonucleolytic digestion of the mRNA body (Tourriere et al., 2002).

Deadenylation is required before degradation of the mRNA body and is therefore a critical step in the modulation of mRNA stability. Differences in the rates of deadenylation between various transcripts make a major contribution to overall differences in mRNA decay rates (Decker and Parker, 1993).

Two distinct deadenylation activities have been identified in yeast. A poly(A) nuclease referred to as PAN (Pan2p/Pan3p) was identified biochemically and shown to physically interact with the poly(A)-binding protein, Pab1p. A second deadenylating activity consists of two previously defined transcriptional regulators Ccr4p (carbon catabolite repression 4) and Ccr4-associated factor (Caf1p) (Tucker et

al., 2001). In the absence of PAN, Ccr4p or Caf1p, residual deadenylation activity was observed, whereas simultaneous disruption of the PAN and Ccr4p/Caf1p complexes abolished deadenylation in vivo (Tucker et al., 2001).

The 5'cap structure is an important determinant of the stability of all messages. However unlike deadenylation, which is progressive, mRNA decapping immediately terminates the cytoplasmic functionality of an mRNA, since subsequent degradation of the body of the transcript is then extremely rapid (Tucker and Parker, 2000). Decapping is carried out by a complex of Dcp1p and Dcp2p and contrary to initial reports, recent analyses have indicated that Dcp2p is actually the catalytic subunit in decapping. Decapping does not normally occur until the poly(A) tail is shortened to oligo(A), which indicates that the poly(A) tail acts as an inhibitor of decapping. Poly(A) tail inhibition of decapping is dependent on the poly(A) binding protein Pab1p (Bernstein et al., 1989; Ross, 1995). Pab1p binds with high-affinity to poly(A) tracts with as few as 12 residues but binds poorly to shorter substrates. It has been proposed that Pab1p inhibition of decapping is mediated by interactions between Pab1 and the cap-binding complex (CBC). This suggests a model in which Pab1p bound to the poly(A) tail interacts with the translation initiation factor eIF4G, which also binds to the cap binding protein eIF4E at the 5' end of the mRNA. The resulting circularisation of the mRNA molecule can promote translation and might simultaneously stabilise mRNAs by preventing access of deadenylating and decapping enzymes to their targets (Tucker and Parker, 2000;Wilusz et al., 2001;Tourriere et al., 2002). Consistent with this observation, eIF4E could act as an inhibitor of decapping and its dissociation from the cap structure is required before mRNA decapping and degradation can occur (Tucker and Parker, 2000).

Following deadenylation and decapping the body of the transcript is trimmed in a 5'-3'direction by the exoribonuclease Xrn1p. In the yeast *S.cerevisiae,* Xrn1p is the primary cytoplasmic RNase responsible for mRNA degradation (Hsu and Stevens, 1993). Xrn1p also degrades other classes of RNAs, including the 5' fragment of the internal transcribed spacer 1 of pre-rRNA (Stevens et al., 1991).

A second 5'-3' exoribonuclease in *S. cerevisiae* is encoded by the *RAT1* gene. Rat1p is primarily nuclear and this is supported by the fact that *rat1* mutants are reported to have defects in 5.8S rRNA processing. Although Rat1p shares considerable

homology with Xrn1p, *RAT1* is essential whereas *XRN1* is nonessential. Several studies provided evidence that Rat1p and Xrn1p are functionally equivalent proteins localized to the nucleus and the cytoplasm and that the essential role of Rat1p is in the nucleus (Johnson, 1997).

I.C.4. Cytoplasmic surveillance

I.C.4a. 5'-3' Nonsense mediated-decay – NMD

An important issue in eukaryotic cells is the accuracy of mRNA biogenesis. To ensure that translation termination occurs at the appropriate location within the mRNA, the cell has evolved a surveillance mechanism known as NMD (nonsense-mediated decay). This cellular process eliminates aberrant mRNAs carrying nonsense mutations within the protein-coding region and thus prevents the synthesis of truncated and deleterious protein that could affect the normal functioning of the cell (Hilleren and Parker, 1999;Gonzalez and Peltz, 2001). The recognition of a premature stop codon triggers rapid deadenylation-independent decapping and subsequent 5'-3'mRNA decay. Additional results have revealed mRNA surveillance to be a system for degrading other types of aberrant mRNAs and not limited to mRNAs with premature stop codons. Other substrates include transcripts with retained introns (He and Jacobson, 1995), upstream reading frames (uORFs) (Cui et al., 1995;Oliveira and McCarthy, 1995), or extended 3' UTRs (Muhlrad and Parker, 1999; Das et al., 2000).

NMD requires several specific trans-acting factors, including Upf1p, Upf2p, Upf3p and Hrp1p. Upf1p was purified from yeast cells and shown to have RNA binding, RNA-dependent ATPase and RNA helicase activities (Czaplinski et al., 1995). The *UPF2* gene encodes an acidic protein and *UPF3* encodes a basic protein harbouring nuclear localisation signals and nuclear export sequences, which has been shown to shuttle from the nucleus to the cytoplasm (Cui et al., 1995;Lee and Culbertson, 1995). The abundance of these factors was found to be approximately 1,600, 160 and 80 molecules of Upf1p, Upf2p and Upf3p respectively (Maderazo et al., 2000).

Although Upf1p, Upf2p and Upf3p were demonstrated to interact and form a complex, these data make it unlikely that these proteins exist in a stable complex or that they associate with all ribosomes (He et al., 1997;Maderazo et al., 2000). Mutations in the *UPF* genes lead to the selective stabilisation of mRNAs containing nonsense codons with no apparent effect on the stability of most wild-type mRNAs (He and Jacobson, 2001).

A critical decision point in the NMD pathway is the discrimination of a premature translation termination event from a normal one. The recognition of a nonsense codon as aberrant is due to loosely conserved downstream sequence elements (DSE) located 3' of a premature termination codon (Peltz et al., 1993; Zhang et al., 1995). DSEs are functional when located within 150 nucleotides 3' of the stop codon and only after the completion of a first round of translation (Ruiz-Echevarria and Peltz, 1996; Ruiz-Echevarria et al., 1998).

More recent results have demonstrated the presence of specific sequences that can protect mRNAs from NMD. These sequences have been defined as stabiliser elements (STEs) and fall into two different classes. The first type is located within the protein-coding region and must be translated to exert its effect (Peltz et al., 1993; Hagan et al., 1995). A second type of STEs can exert its effect when located downstream of the termination codon and upstream of the DSE (Ruiz-Echevarria et al., 1998).

It remains, however, important to explain how the DSEs function to distinguish a normal termination codon from a premature termination codon. Studies have identified Hrp1p/Nab4p, a shuttling, heterologous nuclear ribonucleoprotein (hnRNP like factor) that interacts specifically with the DSE, Upf1 and Upf2 (Gonzalez et al., 2000). A mutation in *HRP1* that resulted in the stabilisation of nonsense-containing transcripts abolished the affinity of Hrp1p for the DSE. These results suggest that the location of a DSE 3' of a premature termination codon promotes NMD because it can promote interactions with proteins such as Hrp1p that mark the mRNA as aberrant (Gonzalez et al., 2000).

Many studies indicate that NMD in yeast is a translational-dependent event and that the recognition of an mRNA as aberrant requires the translation of the mRNA by ribosomes (Beelman and Parker, 1994; Peltz et al., 1992; Muhlard et al., 1995). Thus

it was proposed that failure of the translating ribosome to displace Hrp1p bound to DSE after the first round of translation, signals a premature termination and triggers NMD (Gonzalez et al., 2000).

Three models have been proposed for the mechanism of NMD in *S.cerevisiase*: the scanning surveillance model, the termination/mRNP context model and the Faux UTR model (Hilleren and Parker, 1999; Gonzalez et al., 2001;Maderazo et al., 2003). According to the scanning surveillance model, a surveillance complex (consisting of the Upf proteins) associates with the release factors at translation termination. After translation termination the complex scans the downstream part of the transcript for specific downstream sequence information (DSE). If the surveillance complex encounters a DSE bound to a protein (such as Hrp1p), it communicates a signal to the 5' end of the transcript, initiating deadenylation–independent decapping by Dcp1p. The body of the transcript is then subject to degradation by the 5'-3' exoribonuclease Xrn1p (Hilleren and Parker, 1999).

The second model suggests that concurrent with or following transcription, the mRNA is processed and packaged into an RNP that is transported to the cytoplasm. According to this model, the successful completion of a first round of translation will trigger conformational rearrangements in the RNP such as the 5' and 3'ends become linked. The presence of a nonsense mutation, however, causes the premature termination of translation and the assembly of the surveillance complex. If the surveillance complex encounters a DSE bound to a protein, the transcript is recognised as aberrant due improper remodelling of the RNP and rapidly decapped and degraded by Xrn1p (Gonzalez et al, 2001).

The Faux UTR model postulates that the failure of a ribosome to terminate adjacent to a properly configured 3' UTR triggers mRNA decay. The interactions between a terminating ribosome and a specific RNP domain or set of factors localised 3' to the stop codons decide the fate of the transcript (Maderazo et al., 2003).

I.C.4b. 3'-5' Nonsense-mediated decay

Interestingly, the existence of an NMD pathway involving 3'-5' degradation was recently reported (Mitchell et al., 2003;Cao and Parker, 2003). In contrast to 5'-3' NMD, in 3'-5' NMD mRNAs are initially deadenylated to oligoadenylated

intermediates, followed by 3'-5' degradation by the cytoplasmic exosome. Similar to 5' NMD, this novel turnover pathway required the NMD factor Upf1p and ongoing translation (Mitchell et al., 2003b;Takahashi et al., 2003). Upf1p was shown to interact with an N-terminal domain of Ski7p and that this interaction mediates the degradation of aberrant mRNAs containing premature stop codons (Takahashi et al., 2003). Ski7p also interacts physically through its N-terminal domain with both the exosome and the Ski complex and this interaction is required for the function of the cytoplasmic exosome.

I.C.4c. Non-stop decay

The C-terminal GTPase domain of Ski7p was recently shown to play a key role in the decay of a specific class of mRNAs known as nonstop mRNAs (van Hoof et al., 2002). Nonstop mRNAs are mRNAs that lack termination codons, so that translation continues to the 3'-end of the transcript. These are thought to be recognised and degraded by the exosome in a 3'-5' direction beginning at the 3' end of the poly(A) tail. A *ski7* mutation that prevents its interaction with the exosome resulted in inhibition of the exosome-mediated decay of nonstop mRNAs. A mechanism was suggested in which Ski7p recognises non-stop mRNAs through its C-terminal domain then recruits the exosome via its N-terminal domain to degrade these transcripts (van Hoof et al., 2002). It may be that the GTPase domain of Ski7p is involved in the release of the stalled ribosome from the end of the transcript.

I.C.5. Nuclear surveillance

Nuclear RNA turnover has been best characterised in the budding yeast where the predominant nuclear degradation pathway is 3'to 5'. This pathway was shown to require the activity of Rrp6p, Rrp41p, Rrp44p and Mtr3p, four components of the nuclear exosome and possibly involves the entire exosome.

Studies of exosome mutants in cells defective in splicing provided the first demonstration for the existence of a nuclear turnover pathway (Bousquet-Antonelli

et al., 2000). Mutations in either Rrp6p, Rrp41p, Rrp44p or Mtr3p components of the nuclear exosome lead to the accumulation of pre-mRNAs in the *prp2-1* splicing factor mutant. This lead to the identification of a nuclear turnover pathway that competes with the splicing machinery to degrade aberrant, unspliced transcripts in a 3'-5' direction. Interestingly, the activity of this 3'-5' pre-mRNA degradation pathway was found to be regulated by the carbon source in which the yeast was grown. Media containing glucose, the preferred carbon source for yeast promoted the degradation activity of the exosome. However, when cells were grown in non-fermentable carbon sources like galactose, maltose or acetate this degradation activity was substantially lower. This effect appears to be specific for pre-mRNA degradation since the processing of stable RNAs by the exosome was not affected (Bousquet-Antonelli et al., 2000). Thus, alterations in nuclear RNA turnover in response to different physiological conditions may modulate mRNA synthesis rates.

Although, this the major pathway for pre-mRNA decay in the nucleus, there is another minor 5'-3' pathway involving Rat1p, the nuclear homologue of the 5'-3' exoribonuclease Xrn1p. This pathway appears to play a more important role in pre-mRNA degradation on carbon sources other than glucose (Bousquet-Antonelli et al., 2000).

Recent studies have provided more evidence of the involvement of the exosome in an RNA surveillance mechanism that monitors proper mRNA 3'-end formation (Hilleren et al., 2001). Defects in the nuclear export of mRNAs causes the hyperadenylation of nascent transcripts and their retention at or near their sites of transcription (Hilleren and Parker, 20001; Jensen et al., 2001). In addition, in *pap1-1* mutants, transcripts that fail to acquire poly(A) tails are also sequestered at transcription sites. Deletion of *RRP6* was able to alleviate the transcription site accumulation of both poly(A)$^-$ and poly(A)$^+$ mRNA possibly by allowing the release and the functioning of these aberrant mRNAs. Similar results were obtained with depletion of the core exosome component Rrp41p and the exosome cofactor Mtr4p, suggesting that the retention of these aberrant mRNAs requires the activity of the nuclear exosome (Hilleren et al., 2001).

The role of the exosome in the degradation of nuclear pre-mRNA with defects in 3' end formation was examined in temperature sensitive (ts) mutations in Rna14p and

Rna15p, which are components of the cleavage and polyadenylation factor CF IA (Minivelle-Sebastia et al., 1991, 1994). Yeast mRNA 3' end formation involves coupled cleavage and polyadenylation reactions that are in turn coupled to transcription termination. In the *rna14.1* and *rna15.2* mutant strains both pre-mRNA cleavage and termination reactions are inhibited, as shown by transcription run-on experiments (Birse et al., 1998;Yonaha and Proudfoot, 2000;Minivelle–Sebastia and Keller, 1999; Proudfoot, 2000). As a consequence, long 3'-extended pre-mRNAs were predicted to be synthesised in these mutants but these were not detected in the single mutant strains. Depletion of the core exosome component Rrp41p or the putative helicase Dob1p/Mtr4p from the *rna14.1* and the *rna15.2* strains led to stabilisation of these extended species. These results suggest that the extended transcripts are targeted for degradation by the exosome. Similar to the degradation of unspliced pre-mRNAs, the balance between the potential fate of pre-mRNAs in *rna14.1* and *ran15.2* strains appeared to be regulated by the carbon source. On glucose, the pre-mRNAs are predominantly rapidly degraded, while growth on other carbon sources promotes processing to functional mRNA.

Moreover, in *rna14.1* mutant strains lacking Rrp6p, substantial restoration of synthesis of RNA species similar in size to the wild-type mRNA was seen. These RNA species were polyadenlyated mRNAs with heterogenous 3'-ends and were functional for translation. However, in *rna14.1* mutant strain depleted for both Rrp41p and Rrp6p, only the long extended RNAs were seen indicating that the mRNA-sized species are generated by the activity of the core exosome. It was thus proposed that in the absence of correct cleavage and termination, the 3'-extended transcripts generated *rna14.1* and *rna15.2* are digested by the exosome back to a position close to the normal site of polyadenylation. When strains are grown on glucose, RNA surveillance is activated, exosome activity resumes and the remaining pre-mRNA is degraded. In strains grown on a carbon source other than glucose or in *rna14.1* strains lacking Rrp6p, these products of exosome digestion are stabilised and undergo polyadenylation (Figure 1.6) (Torchet et al., 2002).

So far it appears that the nuclear exosome is involved in the degradation of mRNAs when splicing, polyadenylation or export to the cytoplasm is slow or defective

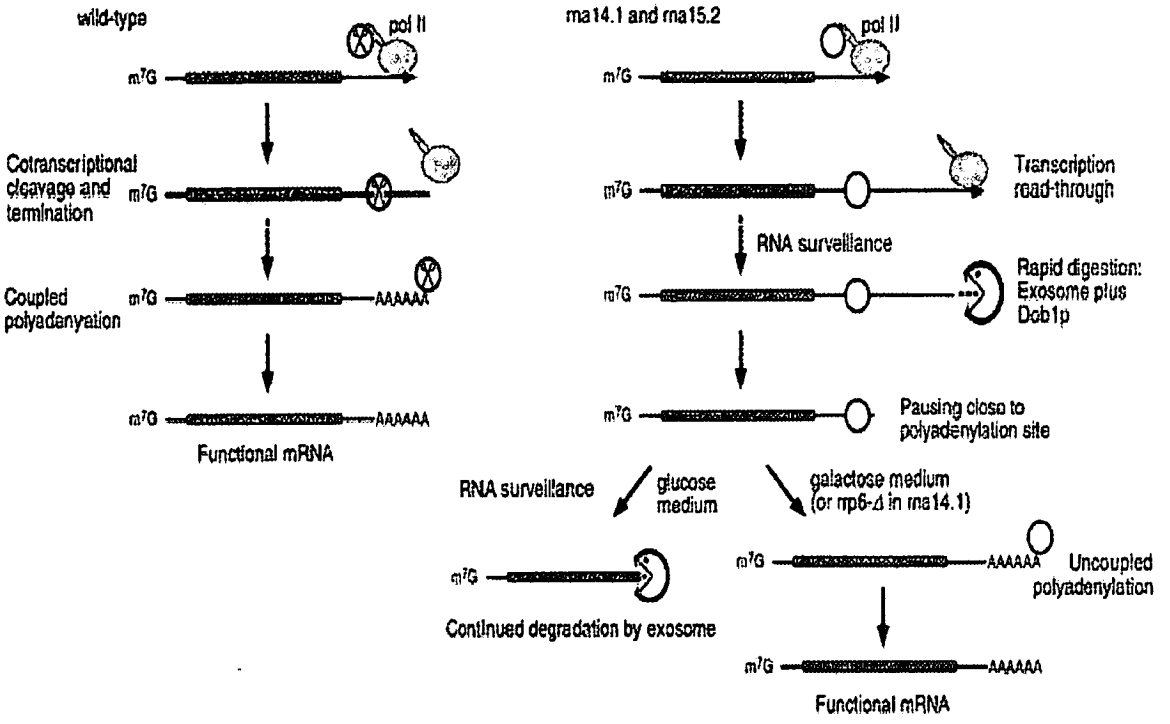


Figure 1.6. Model for the degradation and processing of 3'-extended pre-mRNAs in *rna14.1* and *rna15.2* mutant strains. In *rna14.1* and *rna15.2* cleavage mutants, transcription termination and 3' end formation are abrogated leading to the production of 3'-extended transcripts. These extended transcripts are predicted to be recognised by an RNA surveillance mechanism that targets them for rapid 3' to 5' processing by the exosome complex. In *rna14* or *rna15* strains grown on glucose medium, exosome activity predominantly resumes and the transcript is fully degraded. In strains grown on galactose medium, the products of exosome digestion are predominantly stabilised and undergo polyadenylation. In *rna14.1* strains, the absence of Rrp6p stabilises the products of initial exosome digestion, suggesting a specific requirement for Rrp6p in degrading this substrate Figure adapted from Torchet et al., (2002).

(Butler, 2002). At least in the case of aberrant splicing or polyadenylation this nuclear activity of the exosome appears to be regulated by the carbon source.
However, the exosome was also implicated in the degradation of normal mRNAs in the nucleus (Das et al., 2003). Using the export mutant *nup116*-Δ, it was shown that normal mRNAs are degraded when retained in the nucleus. This degradation involves predominantly a 3'-5' pathway, however, 5'-3' degradation by Rat1p was also observed to a lesser extent. In the 3'-5' pathway, the degradation of the retained mRNAs specifically required Rrp6p and Cbc1p, the large subunit of the CBC. CBC associates with the cap of pre-mRNA and nuclear mRNA and accompanies mRNA to the cytoplasm, and its absence has been predicted to destabilise nuclear mRNAs by exposing them to decapping activities (Das et al., 2000, 2003). The mechanism by which CBC promotes nuclear mRNA turnover is still not clear.

I.C.6. The diverse functions of the nuclear-specific exosome component Rrp6p

There are various major differences between Rrp6p and the core exosome components. Rrp6p is confined to the nucleus in yeast and is associated with the exosome at substoichiometric amounts relative to the core exosome components. Rrp6p is not essential for cell viability, in contrast to the ten core components of the exosome, although *rrp6*Δ strains are severely impaired in growth and are temperature sensitive (Allmang et al., 1999;Briggs et al., 1998). In addition, in both rRNA and snoRNA processing the role played by Rrp6p is distinct from the core exosome components. The lack of Rrp6p results in the accumulation of 5.8S+30 pre-rRNA species and C/D snoRNA precursors with short 3' extensions of approximately 3nt (Briggs et al., 1998;Allmang et al., 1999a).
Furthermore, Rrp6p and the nuclear exosome appear to play a major role in nuclear pre-mRNA turnover that degrades mRNAs when splicing, polyadenylation or transport is defective (Figure 1.7). Studies in the *rna14.1* mutant have revealed a distinct role for Rrp6p in the degradation of 3'-extended read-through transcripts where the fate of these aberrant transcripts was found to be regulated by the carbon

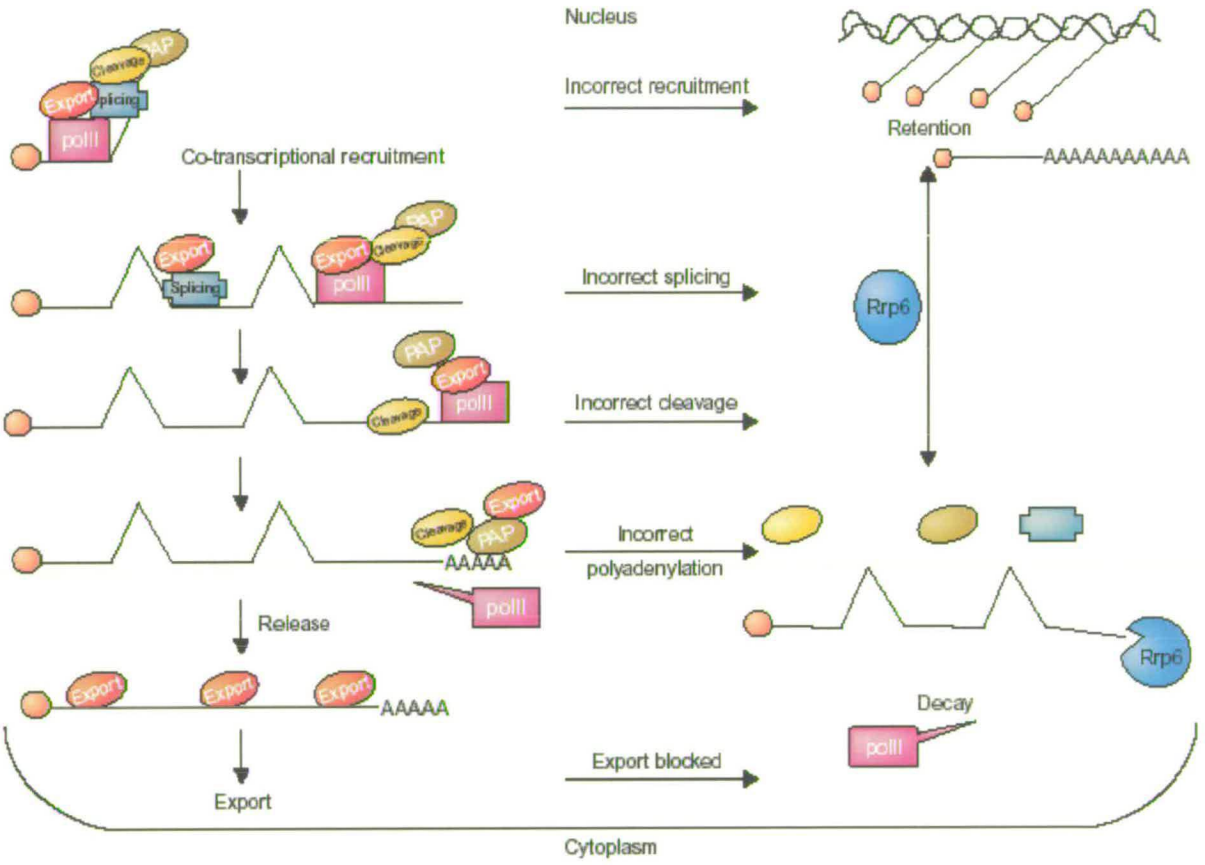


Figure 1.7. The diverse functions of the exosome and Rrp6p in nuclear mRNA surveillance. mRNA synthesis involves a series of processing steps to synthesise mature transcript that is proofread in parallel by nuclear surveillance mechanism at each step. Export competent mRNP is exported and expressed in the cytoplasm. Alternatively, aberrant synthesis or processing leads to retention of the defective transcript at the site of transcription for further exosome-mediated decay. The cap is depicted as a red circle and Rrp6p is representative of nuclear surveillance. Figure adapted from Vasudevan and Peltz, (2003).

source (Torchet et al., 2002). This function of Rrp6p is consistent with its identification as a suppressor of a polyadenylation defect caused by the *pap1-1* temperature-sensitive lethal mutation. The *pap1-1* mutation which results in reduced levels of poly(A)$^+$ mRNAs was suppressed by two cold sensitive alleles of the *RRP6* gene. The deletion of *RRP6* in the *pap1-1* strain was able to partially restore the levels of the poly(A)$^+$ mRNAs without affecting mRNA decay rates. Furthermore, blocking the cytoplasmic 5'-3' decay pathway or the NMD pathway did not restore the levels of the poly(A)$^+$ mRNAs in a *pap1-1* background. These results are in agreement with the localisation of Rrp6p in the nucleus and suggest that Rrp6p caries out this function by a mechanism distinct from the major mRNA decay pathway in yeast. Rrp6p was also reported to interact physically with Pap1p and with Npl3p, an hnRNP protein involved in mRNA processing and export. Since Npl3p genetically interacts with 3' end formation factors, it was proposed that Rrp6p and Npl3p might act together as a checkpoint to ensure that the RNP complex is correctly processed before export. Incorrectly or incompletely processed mRNAs are thought to be targeted for degradation in the nucleus in a 3'-5' pathway involving Rrp6p (Burkard and Butler, 2000).

Rrp6p was also implicated in the retention of 3'-truncated heat-shock messages at the site of transcription in strains carrying mutations in the components of the THO/TREX complex. The THO complex (Tho2p-associated proteins) is a four-protein complex containing Tho2p, Hpr1p, Mft1p and Th2p and deletion of any of these factors leads to accumulation of 3' truncated transcripts (Chavez et al., 2000). Recently, the THO complex was shown to be present in a larger complex, termed TREX (Transcription-Export), together with the components of the export machinery such as Sub2p and Yra1p (Strasser et al., 2002). Deletion of members of the THO/TREX complex leads to accumulation of a pool of 3'-truncated heat shock *HSP104* transcripts and their sequestration at or near transcription sites. However,

deletion of *RRP6* in mutant strains of the components of the THO/TREX complex restores a quasinormal level of full-length transcripts and reverses the transcript sequestration phenotype (Libri et al., 2002). Subsequent analyses have shown that the retained *SSA4* heat shock RNA localises to an area close to the *SSA4* gene (Thomsen et al., 2003). Thus, the nuclear-specific exosome component participates in a quality control mechanism that plays a role in selecting RNAs to be retained for degradation and those to be released for export (Libri et al., 2002).

I.D. The exosome and RNA turnover in other organisms

I.D.1. The conservation of the exosome among various organisms

I.D.1a. The exosome of archae, protozoa and plants

Recent experiments have provided evidence for the existence of an exosome-like complex in the archeon *Sulfolobus solfataricus* (Evguenieva-Hackenburg et al., 2003). The purified 250kDa complex comprises the orthologues of eukayotic exosome subunits Rrp4p, Rrp41p, Rrp42 and Csl4p. In addition, the homologues of DnaG and Cdc48, the chaperone Cpn and ribosomal subunits were found to be associated with the exosome. The *S. solfataricus* proteins Rrp41, Rrp42 and Rrp4 were shown to be present in apparently equimolar amounts in the complex whereas a smaller amount of Csl4p was copurified (Evguenieva-Hackenburg et al., 2003). This suggests that a ring of six RNase PH homologues might be formed from three polypeptides of each of the *S. solfataricus* Rrp41p and Rrp42p subunits, together with 3 copies of Rrp4p. Such a structure would potentially resemble the yeast exosome (Evguenieva-Hackenburg et al., 2003;Aloy et al., 2002). In *S. solfataricus*, Cpn is known to have a protein folding ability, specific RNA-binding and endoribonucleolytic properties and to participate in 16S rRNA maturation. The association of ribosomal subunits with this exosome-like complex suggests that it might function in rRNA maturation (Evguenieva-Hackenburg et al., 2003).

The eukaryotic parasitic protozoan *Trypanosoma brucei* has an exosome complex that is extremely similar to the human and yeast exosomes in composition (Estevez et al., 2003). The complex contains six RNase PH-like subunits, TbRRP41A, TbRRP41B, TbRRP45, TbEAP1, TbEAP2 and TbEAP4. Sequence information and yeast two-hybrid interaction data suggest that these subunits are equivalent to Rrp41p, Rrp46p, Rrp45p, Rrp42p, Rrp43 and Mtr3p respectively. The trypanosome exosome also has three S1 domain proteins, TbRRP4, TbRRP40 and TbCSL4. A combination of RNA interference, complex affinity purification, and yeast two-hybrid approaches have shown that the RNase PH homologues are important for maintenance of complex integrity (Estevez et al., 2003). In contrast, the S1 domain proteins are required for exosome function but not for the association of the remaining exosome components. The TbRRP6 subunit, which is specific for the nuclear exosome in yeast and humans, is part of the cytoplasmic exosome in trypanosomes. Moreover, the homologue of yeast Rrp44p, TbRRP44 could not be detected associated with the exosome. The trypanosome exosome has a sedimentation coefficient of 11S, whereas the yeast complex sediments at 14S which could be due to the absence of the Rrp44p homologue from the Trypanosome complex (Estevez et al., 2003). The yeast Rrp44p was shown to dissociate from the yeast exosome at around 500mM $MgCl_2$ while the remaining components dissociated at around 1.6M $MgCl_2$ (Mitchell et al., 1997). This suggests that the association of both yeast Rrp44p and TbRrp44p with their respective exosomes is weaker than that of the other exosome components. Depletion of any of the trypanosome exosome components results in cell death and defects in 5.8S rRNA maturation similar to those observed in yeast exosome mutants. The complex is predicted to be built around a core composed of the six RNase PH-like proteins (plus TbRRP6 and TbEAP3) similar to the proposed models of the exosome in yeast (Estevez et al., 2001, 2003; Aloy et al., 2002).

The characterisation of the *Arabidopsis thaliana* homologue of the yeast exosome subunit Rrp41p (Ski6p) was recently reported. Purified recombinant AtRrp41p behaves *in vitro* as a processive phosphorolytic 3'-5' exonuclease consistent with it being a member of the RNase PH protein family. Furthermore, the expression of the AtRrp41p cDNA rescued the lethal phenotype of the *rrp41* (*ski6*) null mutant and

corrected the known defects of the partial loss of function mutant *ski6-100* in 5.8S rRNA processing and 3'-5' mRNA degradation (Chekanova et al., 2000).

In *Escherichia coli*, eight 3'-5' exoribonucleases have been identified. In contrast to *S.cerevisiae, E. coli* exoribonucleases function independently of one another and do not appear to assemble into an exosome-like complex. *E.coli* does, however, contain a complex named the degradosome that degrades RNA molecules from their 3' end. This complex includes a fraction of the exoribonuclease PNPase, the endoribonuclease RNase E, the DEAD-box RNA helicase RhIB and an enolase (Carpousis et al., 1999).

The two major exoribonucleases in *E.coli* are RNase II and polynucleotide phosphorymase (PNPase) that degrade all types of RNA *in vitro*. PNPase is a phosphoryltic enzyme whereas RNase II is a hydrolytic enzyme that accounts for most of the poly(A) hydrolytic activity observed in *E. coli* extracts (Deutscher and Reuven, 1991). The residual hydrolytic activity is provided by the related exonuclease RNase R, the homologue of eukaryotic Rrp44p (Kasai et al., 1977;Cheng et al., 1998). The other four characterised enzymes are RNase D, RNase BN, RNase T and RNase PH. RNase T and RNase PH are the major enzymes responsible for final 3' trimming of most tRNAs (Li and Deutscher, 1994, 1996), whereas RNase II and PNPase constitute the major mRNA degradation activities (Donovan and Kushner, 1986)

I.D.1b. The exosome of Drosophila

An exosome complex was also identified in *Drosophila melanogaster*. The *Drosophila* exosome was copurified with the essential elongation factor dSpt6p (Andrulis et al., 2002). dSpt6p was previously shown to be recruited to active sites of transcription and to colocalise with elongating RNA polymerase II (Pol II). Moreover, in yeast, Spt6p interacted genetically and biochemically with transcription elongation factors that regulate the processivity of Pol II. The *Drosophila* exosome comprised nine exosome subunits (dDis3/dRrp44, dRrp6, dMtr3, dRrp42, dRrp4, dRrp41, dRrp46, dRrp40 and dCsl4). However, neither the Rrp43 nor Rrp45 subunits could be detected in the purified complex. An Rrp45 homologue (dRrp45) is present in the *Drosophila* genome whereas a clear Rrp43p homologue is not. It is possible

that *Drosophila* has a yet unidentified Rrp43p that did not purify with the dSpt6-associated exosome fraction. The Rrp45p homologue could be either associated with the *Drosophila* exosome at substoichiometric amounts or lost from the dSpt6-associated exosome complex during purification (Andrulis et al., 2002).

Immunoprecipitation assays of *Drosophila* nuclear extracts revealed the interaction of the exosome with another elongation factor, Spt5p, and with the large subunit of RNA polymerase II. Additional results revealed that dSpt6p and the exosome colocalise at transcriptionally active genes, suggesting that the machinery for transcription elongation and the machinery for pre-mRNA surveillance (exosome) function together in vivo (Andrulis et al., 2002). This observation is in agreement with the established role of the exosome as a quality control mechanism that ensures that only correctly processed pre-mRNA molecules are exported.

I.D.1c. The exosome of humans

Purification of the human homolog of Rrp4p (hRrp4p) in a large complex from HeLa cells suggested the existence of an exosome complex in human cells (Mitchell et al., 1997). This complex was initially identified and characterised using autoimmune antibodies present in patients suffering from polymyositis-scleroderma (PM-Scl) overlap syndrome. The PM-Scl particle was reported to contain 11 to 16 subunits ranging from 20 to 110 kDa and to be localised to the nucleolus (Reimer et al., 1986; Gelpi et al., 1990). Autoantibodies identified two proteins designated PM-Scl100 and PM-Scl75 in most characterised patients, with PM-Scl 100 immunodominant. PM-Scl 100 is homologous to Rrp6p and PM-Scl 75 is homolgous to Rrp45p (Briggs et al., 1998;Allmang et al., 1999b), strongly indicating that the PM-Scl complex is the human exosome. In subsequent studies, full-length cDNAs encoding human homologues of the *S.cerevisiae* Rrp40p, Rrp41p/Ski6p and Rrp46p were cloned. The novel proteins were shown to be part of a large complex cofractionating with hRrp4p and PM/Scl-100. Moreover, expression of hRrp41p and hRrp4p in yeast was able to support the growth of yeast cells deleted for Rrp41p/Ski6p or containing the *rrp4-1* mutation respectively (Brouwer et al., 2001). However, hRrp4p only weakly suppressed the accumulation of 3' extended forms of 5.8S rRNA observed in *rrp4-1* mutants and was unable to complement an *rrp4*Δ null allele (Mitchell et al., 1997).

Human orthologs have been also identified for the Rrp44p/Dis3p and Csl4p components of the exosome and the expression of each of these cDNAs was shown to suppress the phenotypes of mutations in the corresponding yeast genes (Baker et al., 1998; Shiomi et al., 1998).

Subcellular fractionation showed that PM-Scl75 (hRrp45p), hRrp41, hRrp46, hRrp40 and hRrp4p are present in both nuclear and cytoplasmic fractions. Furthermore, all of the PM-Scl100 (hRrp6p) in HeLa cell lysates appeared to be associated with the PM-Scl complex or with complexes with similar sedimentation on sucrose gradients, suggesting that hRrp6p does not function as a free protein *in vivo* (Allmang et al., 1999b). Recent studies have reported that unlike its Rrp6p yeast orthologue, PM-Scl100 is not confined to the nucleus. Using indirect immunofluorescence and confocal microscopy, PM-Scl100 was shown to be present in both nuclei and cytoplasm, although these analyses were performed using a non-purified polyclonal antibody, potentially raising doubts about the specificity of the antibody (Lejeune et al., 2003).

I.D.2. RNA turnover in higher eukaryotes

I.D.2a. ARE-mediated turnover

The pathway of mRNA turnover has been extensively studied in the yeast *S. cerevisiae* but much less is known about this process in other organisms. Activated mRNA turnover plays an important role in regulating the expression of many proto-oncogenes, cytokines and growth factors (Mitchell and Tollervey, 2000;Wliusz et al., 2001). The stability of these transcripts depends on cis -acting elements within the RNA molecule. One of the best studied and most prevalent cis-element is the AU-rich element (ARE) found in the 3' untranslated regions (UTRs) of genes regulated by extracellular stimuli (for example, proto-oncogenes).

Similar to yeast, the first step in mammalian ARE-mediated mRNA decay appears to be poly(A) shortening. AREs were shown to promote the rapid deadenylation by the deadenylase PARN and subsequent decay of the transcripts (Shyu et al., 1991;Xu et al., 1997). Because of the conservation of the exosome and other decay factors

between yeast and mammals, similar decay pathways were thought likely to exist in mammals (Lim and Maquat, 1992; Mitchell et al., 1997;Bashkirov et al., 1997). Recent studies revealed the major pathway for ARE-mediated RNA degradation in mammals to be a 3-5' decay pathway mediated by the exosome. The mammalian exosome may be recruited by ARE-binding proteins to degrade ARE-containing transcripts in 3'-5' direction. Furthermore, a component of the cytoplasmic mammalian exosome, PM-Scl75 (Rrp45p in yeast) was shown to be an ARE-binding protein suggesting that it might be responsible for direct loading of the exosome onto some ARE-containing mRNAs (Mukherjee et al., 2002).

After the complete 3'-5 degradation of the mRNA by the exosome the cap is hydrolysed by a scavenger decapping activity DcpS (Wang and Kiledjian, 2001).

I.D.2b. A 5'-3' turnover pathway

A 5'-3' activity was also observed in HeLa cells cytoplasmic extracts (Mukherjee et al., 2002). A decapping complex (hDcp1, hDcp2) homologous to the yeast complex was recently identified and shown to act in this pathway (Gao et al., 2001). Moreover, homologues of the 5'-3' exoribonuclease Xrn1p were shown to exist in *Drosophila* and mouse. Both homologues were able to complement yeast strains lacking the 5'-3' exoribonuclease (Shobuike et al., 1997;Bashkirov et al., 1997). However, future work will be required to evaluate the relative contribution of both pathways to general RNA degradation.

I.D.2c. Nonsense –mediated decay

Most transcripts containing premature termination codons (PTCs) are rapidly degraded in higher eukaryotes including fungi, plants, *Caenorhabditi elegans* and humans (Pulak and Anderson, 1993;Mendell et al., 2000;Sun and Maquat, 2000). In *C. elegans* seven genes (smg1-smg7) were found to be required for NMD. Mutations in these genes stabilise normally recessive nonsense-containing *unc-54* mRNAs and thereby allowing the synthesis of cytotoxic truncated unc-r4 protein (Pulak and Anderson, 1993). Cloning and characterisation of some of these genes have revealed that smg-2 encodes a phosphoprotein homologous to yeast Upf1p and smg-4 is homologous to the yeast Upf3p.

Human homologues of Upf1 and Upf3 have also been recently identified (Mendell et al., 2000;Serin et al, 2001). While it is still not clear whether NMD in mammalian occurs in the nucleus or in the cytoplasm, the recognition of PTCs was shown to depend on splicing. An intron found 3' of the termination codon functions like a yeast DSE targeting the transcript for rapid degradation. PTCs located more than 50-55 nucleotides upstream of the exon-exon junction were able to trigger NMD. The surveillance complex is thought to recognise this junction through marker proteins, the exon-junction complex (EJC), that are deposited at the exon-exon junction, potentially playing a similar role to the yeast Hrp1p. A model was proposed in which failure of the marker proteins to be removed by the translating ribosome targets the message for rapid degradation by the surveillance complex (Le Hir et al., 2000, 2001). Recent studies revealed that NMD in mammalian cells degrades mRNAs from both 5' and 3' ends. The 3'-5' NMD pathway was shown to take place in both the nucleus and cytoplasm and requires the activity of the human exosome component PM/Scl100 and possibly the rest of the exosome (Lejeune et al., 2003).

I.E. Aims of this Thesis

Many previous elaborate studies have been conducted to unravel the pathways by which the abundance of cellular transcripts in the cell is regulated. The discovery of the exosome has added another layer of complexity to the RNA degradations pathways.

Recent analyses have identified pathways that degrade aberrant nuclear pre-mRNAs and require the nuclear exosome complex and an Rrp6p-specific checkpoint. The role played by Rrp6p in the degradation of mRNAs when splicing, polyadenylation or export to the cytoplasm is slow or defective appears to be distinct from that of other exosome components (Bousquet-Antonelli et al., 2000;Hilleren et al., 2001;Torchet et al., 2002). Furthermore, Rrp6p has been implicated along with the cap-binding complex in the degradation of normal mRNAs in the nucleus (Das et al., 2003). A potential role for nuclear turnover in the regulation of mRNA synthesis is emerging. The diverse functions of the Rrp6p in RNA processing in the nucleus suggest its involvement in the regulation of gene expression at the level of nuclear turnover. Rrp6p might be acting independently of the exosome as a monomeric exonuclease or in conjunction with another set of proteins. The aim of this thesis was to better understand the relationship between Rrp6p and the exosome, the role of Rrp6p in nuclear RNA turnover and to identify potential genes whose expression levels are regulated at the level of nuclear RNA turnover.

Chapter two

Materials and Methods

II A. Materials

Restriction enzymes and other modifying enzymes were obtained from New England Biolabs, Boehringer Mannheim, Roche, Promega 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): 10g bacto-peptone, 5g bacto-yeast extract, 10g NaCl, adjusted to pH 7.2 with NaOh (for 1 litre). Ampicillin was added to media immediately prior to use (100μg/ml). For agar plates, bacto-agar was added to 2% (w/v) prior to autoclaving at 15lb/sq.inch for 20minutes in a liquid cycle.
Standard S. cerevisiae growth and handling techniques were employed (Sherman, 1991). Yeast strains were grown either in rich (YPD) or in minimal media (SD). 1 litre of the following media was composed as follows:
YPD: 10g bacto-peptone, 10g bacto-yeast extract, 20g D-glucose;
SD: 0.67g yeast nitrogen base without amino acids, 20g D-glucose, supplemented with amino acids as required.
To make agar plates, bacto-agar was added prior to autoclaving at 15lb/sq.inch for 20 minutes in a liquid cycle.

II C. Bacterial Strains

E.coli B strain XL10-Gold Ultracompetent cells *Tet^r*Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte {F' proAB lacI^q ZΔM15 Tn10 (Tet^r) Amy Cam^r}* (Stratagene) was used for propagation of all plasmids.
E.coli BL21(DE3)pLysS strain *(F$^-$ ompT hsdS$_B$(r$_B^-$m$_B^-$) gal decm (DE3) pLysS (Cam^R))* was used for the expression of the Rrp6p recombinant protein.

II D. Yeast Strains

The yeast strains used in this study are shown in table 2.1.

Table 2.1. Yeast strains

Strains	Genotype	Reference
BMA38	MAT a/α *ade2-1/ade2-1 his3 Δ200/ his3 Δ200 leu2-3,112/leu2-3,112 trp13-1/ trp13-1, ura3-1/ ura3-1, can1-100/can1-100*	(Baudin et al. 1993)
YCA12	MAT a *ade2-1 his Δ200 leu2-3 112 trp1-1 ura3-1 can1-100 RRP6:: KLTRP*	(C.Allmang et al. 1999)
NOY388	MAT a *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100*	(Tabb et al., 2000)
NOY612	MAT a *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 srp1-31*	(Tabb et al., 2000)
NOY613	MAT a *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 srp1-49*	(Tabb et al., 2000)
YCL004w	MATα his3Δ*1 leu2Δ0 lys25Δ0 ura3Δ0 hrb1Δ::kan^r*	EUROSCARF
YCL011c	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gbp2Δ::kan^r*	EUROSCARF
YRH1	as BMA38 but *Rrp6-TAP-TRP1*	This work
YRH2	as BMA38 but *gbp2Δ::kan^r*	This work
YRH3	as BMA38 but *hrb1Δ::kan^r*	This work
P65	MATα *ade2-1 his3-11 leu2-3 trp1-1 ura3-52 rrp4D::HIS3 [pRS415/N(His6)Rrp4p]*	(Mitchell et al., 2003)

P203	MATa *his3Δ200 leuΔ1 gal2 galΔ108b trp⁻ ura3-52 HIS3-GAL10::zz-rrp44*	(Mitchell et al., 2003)
P246	MATa *ade2-1 his3-11 leu2-3 trp1-1 ura3-52 rrp4Δ::HIS3 p139*	(Mitchell et al., 1997)
P269	MATa *ade1 his4 leu2-3,112 ura3-52 ski7Δ::URA3*	(Mitchell et al., 2003)
P368	as BMA38 but *rrp47Δ::kan^r*	(Mitchell et al., 2003)
P369	as P246 but *rrp47Δ::kan^r*	(Mitchell et al., 2003)
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
P393	as BY4741 but *ski2Δ::kan^r*	EUROSCARF
P394	as BY4741 but *ski3Δ::kan^r*	EUROSCARF
P395	as BY4741 but *ski8Δ::kan^r*	EUROSCARF
P414	as BMA38 but *rrp47-zz::HIS5^{sp}*	(Mitchell et al., 2003)
BY4743	MATa/MATα *ura3Δ0/ura3Δ0 his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 LYS2/lys2Δ0*	(Conrad et al., 2000)
YJC1107	MATα *ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 LYS2 nrd1Δ::KAN (pJC720{LEU2 nrd1-102})*	(Conrad et al., 2000)
D400	MATα *his4-359 leu2-3,112 Lys2-201 trp1-Δ1 ura3-52 ski6::URA3 [ski6-100/LYS2]*	(Anderson and Parker, 1998)
D565	MATα *leu2-3,112 ura3-52 pep4-3*	(Ursic et al., 1997)
D566	as D565 but *sen1-1*	(Ursic et al., 1997)
D710	as D566 but *rrp6::kan^r*	Strain constructed by A. Fatica
D793	as YJC1107 but *rrp6::kan^r*	Strain constructed by J. Kufel

II E. Oligonucleotides and primers

Oligonucleotides used in this study for hybridisation of Northern blots, primer extension, and RNase H analyses are shown in table 2.2.

Table 2.2. Oligonucleotides

Oligonucleotide	Sequence	Remarks
007 (25S)	CTCCGCTTATTGATATGC	Complementary to pre-rRNA 5' of mature 25S
008 (18S)	CATGGCTTAATCTTTGAGAC	Complementary to mature 18S rRNA
017 (5.8S)	GCGTTGTTCATCGATGC	Complementary to mature 5.8S rRNA
020 (ITS2-5'B)	TGAGAAGGAAATGACGCT	Complementary to pre-rRNA 3' of mature 5.8S rRNA
205 (U18)	GTCAGATACTGTGATAGTC	Complementary to *U18* snRNA
250 (SCR1)	ATCCCGGCCGCCTCCATCAC	Complementary to *SCR1* mRNA
405 (CYH2)	GTGCTTTCTGTGCTTACCGATACGAC CTTTACCG	Complementary to *CYH2* mRNA
482 (PGK1)	GATCTATCGATTTCAATTCA	Complementary to *PGK1* mRNA
RH1(NRD1)	GCTCATCGGGGTATAAGTGGTGATT GTTTGTGC	Complementary to *NRD1* mRNA
RH2(NRD1)	CAAAGATATGATTTTCGATTCAATAT CAATG	Complementary to 5'end of *NRD1* mRNA
RH3 (NRD1)	TGGTACATTACGATAATACGATGCG ATCAG	Complementary to 5' end of *NRD1* mRNA
RH4 (NRD1)	CCATACGGTTGCTGCATCATAGGTTG C	Complementary to 3' end of *NRD1* mRNA
RH5 (NRD1)	GTGGAGTAAAGATCTTAGC	Complementary to 3' end of *NRD1* mRNA
RH6 (MRPL17)	GACCTATCTGCTTCCGTTATCCTGTC G	Complementary to *MRPL17* mRNA
RH7 (YPL245w)	GAAATTGTCACCACATTCAACGTAAT AGTCTTTTCCG	Complementary to *YPL245w* mRNA
RH8 (NOG2)	GCAACTGTGAGAACTGACGTAGCAA CTGAATTAACG	Complementary to *NOG2* mRNA
RH9 (BAG7)	CGATGACAGTTCTGGAAGCCTCATA CTCTTTC	Complementary to *BAG7* mRNA

Primers used in this study for amplification by Polymerase Chain Reaction are shown in table 2.3; sequence complementary to tagging cassettes are shown in upper case, chromosomal sequences in lower case.

Table 2.3. Primers

Primer	Sequence
YOR001w-TAP	aaagaggaggcctgccgccaaaggtaagaatctgtcatttaaaaggTCCATGGAAA AGAGAAG
YCL011c-BMA5'	gcaaacctagcaaggaaatag
YCL011c-BMA 3'	cttctcttaattggtggcag
YNL004w-BMA5'	gcattgctaatgtttatctactg
YNL004w-BMA3'	cttctgaggcctcttgtctg

II F. Radiolabelled compounds

All radiolabelled compounds were purchased from Amersham (UK): {γ-^{32}P}ATP (500Ci/ mmol) and{α-^{32}P} dCTP (500Ci/ mmol).

II G. Plasmids

The plasmids used in this study are listed in table 2.4.

Table 2.4. Plasmids

Plasmid	Description	Reference/ Remarks
PRS1479-TAP	CEN-TAP-TRP1	(Rigaut et al.,1999)
pRSET B	T7promoter-His-AMP	Invitrogen

II H. Antibodies

The antibodies used in this study are listed in table 2.4.

Table 2.5. Antibodies

Antibody	Description	Supplier
Peroxidase-anti-Peroxidase (PAP)	rabbit IgG	Sigma, UK
anti-rabbit IgG	anti-rabbit IgG conjugated to horseradish peroxidase, secondary	Amersham, UK
anti-Rrp6p	rabbit IgG, primary	This work
anti-Rrp4p	rabbit IgG, primary	(Mitchell et al., 1997)
anti-Npl3p	rabbit IgG, primary	(Russell and Tollervey, 1992)

II I. Bacterial techniques

II I.1. Preparation of competent cells

Cells were prepared for transformation by the following procedure. *E.coli* were grown in LB to an optical density of 0.4 at 600nm. The bacterial cultures were transferred to centrifuge bottles and chilled on ice for 10 minutes. Then the cells were pelleted at 4000rpm for 10 minutes in a Model Avanti^{TM} J-25 Centrifuge (Beckman), resuspended in ice-cold sterile 0.1M$CaCl_2$ and left on ice for 5 minutes. The bacteria were recovered by centrifugation at 4000rpm and resuspended in ice-cold, sterile 0.1M calcium chloride/10% (v/v) glycerol (2ml suspension for each 50ml original culture). This suspension was incubated on ice for 15 minutes, aliquoted, then frozen and stored at –80°Celsius.

II I.2. Transformation of competent cells

Competent *E.coli* were transformed with DNA plasmid by the following method. 2µl plasmid {500ng/µl} were added to 100µl competent cells. The mixture was incubated on ice for 30 minutes, heat shocked at 42°C for 90 seconds. After the addition of 900µl of pre-warmed LB medium (37°C), the mixture was incubated for 1 hour at 37°C on a rotary shaker at 225rpm before plating on LB plates containing ampicillin.

II I.3. Expression of recombinant proteins

Following successful cloning into an expression vector, the plasmid was then transformed into competent BL21 cells following the protocol described in section II.I.2. A single colony was then grown overnight at 37°C. The following day this culture was diluted 1:100 in broth medium and grown at 37°C to an OD_{600} of 0.5. At this time, a 1ml sample was collected for analysis of the expression pattern before induction by IPTG. Expression was then induced by the addition of IPTG to the culture followed by growth of the culture for a further 3-4 hours at 30°C. In order to optimise the expression of the recombinant protein, the concentration of IPTG used (range 0.05 – 1mM), the temperature (18 - 37°C), and the time of induction can be varied. After induction, a further 1ml sample was collected and the reminder of the cells centrifuged at 4000rpm for 15 minutes in a Model Avanti™ J-25 Centrifuge (Beckman), the supernatant removed and stored frozen at -80°C if required.

II I.4. Purification of His fusion proteins

The bacterial cell pellet from a 500ml culture was re-suspended in BufferA (6M GuHCl, 0.1M Na-phosphate, 0.01 M Tris/HCl, pH 8.0) at 5ml per gram wet weight and incubated on a rotating wheel for 1 hour at room temperature. Following this incubation, the lysate was centrifuged at 10,000 g for 15 min at 4°C and the supernatant was collected. 8ml of an 50% slurry of Ni-NTA superflow resin previously equilibrated in bufferA was added to the supernatant and this was then incubated on a rotating wheel for 45min at room temperature. Then the resin was loaded carefully on a poly-prep

chromatography column and washed with 10 column volumes of Buffer A, 5 column volumes of Buffer B (8 M urea, 0.1M Na-phosphate, 0.01 M Tris/HCl, pH 8.0), and Buffer C (8 M urea, 0.1M Na-phosphate, 0.01 M Tris/HCl, pH 6.3). The fusion protein was then eluted from the beads with 10 - 20 ml Buffer D ((8 M urea, 0.1M Na-phosphate, 0.01 M Tris/HCl, pH 5.9) followed by 10 – 20 ml Buffer E (8 M urea, 0.1M Na-phosphate, 0.01 M Tris/HCl, pH 4.5). 3 ml fractions were collected from each elution. The eluted protein was quantified using BioRad Protein Assay at OD_{595}.

II.I.5. Preparation of recombinant protein as antigen for Immunisation

Recombinant protein was purified as described in section II.1.4 and run on SDS-polyacrylamide gel. A comb with only one wide tooth was used so the largest possible amount of protein could be run. Initially the gel was washed in dH_2O, 3 times for 10 minutes each. The gel was then stained with aqueous Coomassie Brilliant blue G-250. After visualisation of the band, the gel was destained with dH_2O. The protein was then cut out using a scalpel and ground up in liquid nitrogen using a pestle and a mortar. The gel band was ground to a powder fine enough that it would pass through a syringe so that it could be injected into the rabbit. The ground powder was then placed in a tube and an equal volume of PBS was added. This was stored at -80°C until required.

II J. Yeast techniques

II J.1. Transformation of Yeast and selection

Yeast strains were transformed using a lithium acetate method (Gietz et al., 1992). An overnight yeast culture was grown YPD to an optical density of 1.0 at 600nm (OD_{600}). The cells were diluted to an OD_{600} of 0.1 in 50ml medium and grown to an OD_{600} of 0.5 (10^7 cells/ml). After harvesting, the cells were washed with 50ml sterile water, centrifuged again and resuspended in 1ml of water. Then the cells were washed in 1ml

LiTE (10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0, 100mM lithium acetate). The cells were centrifuged again and, after two more washes in 500µl LiTE, resuspended in 500µl of LiTE. 1 to 5µg transforming DNA was added to 50µl of yeast suspension, as well as 5µl carrier DNA (herrings sperm DNA, Boehringer) and 300µl fresh PEG solution (40% Polyethylenglycol 4000 in LiTE). The suspension was thoroughly mixed and incubated at 30°C under constant shaking for 30 minutes. DMSO was added to a final concentration of 10% and the cells were heat shocked at 42°C for 15 minutes without shaking, centrifuged for 5 seconds and resuspended in 100µl 1xTE (10mM Tris-HCl pH 7.5, 1mM EDTA, pH 8.0), plated on selective plates and incubated at 30°C.

II K. Recombinant DNA techniques

Standard recombinant DNA techniques were carried out according to (Sambrook et al., 1989). This includes agarose gel electrophoresis of DNA, ethidium bromide fluorescence, phenol/chloroform extraction and alcohol precipitation.

II K.1. Restriction enzyme digests

All restriction enzymes and molecular weight markers were obtained from New England Biolabs, UK. Restriction digests were performed in total volumes of 20µl, using approximately 0.5µg DNA per digest, and appropriate amounts of enzyme (usually 1µl) in 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 37°C for 1-4 hours and then analysed on an agarose gel with appropriate molecular weight markers.

II K.2. Plasmid preparation

Medium scale preparations of DNA were performed using the Quiagen plasmid preparation kit and following manufacturer's instructions for midi/maxi preparations.

II K.3. DNA sequencing

All sequencing reactions were performed on double strand plasmid DNA using the United States Biochemical (USB) Sequenase kit BIG DYE KIT (Amersham) and following the manufacturer's instructions. The sequencing reactions were then sent to the sequencing facility in ICAPB at Edinburgh University.

II K.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, to generate DNA fragments and to identify recombinants. All applications followed the general protocol described below or modifications upon it.

PCR reactions in a volume of 50 to 100µl contained 1.5mM magnesium chloride, 0.2mM of each dNTP, 0.25µM of two oligonucleotide primers, 1x of the supplied reaction buffer, 10 U/µl 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:

94°C for 5 min, 1 cycle

94°C for 30 sec, 45°C for 1 min, 72°C for 3 min, 35 cycles

72°C for 10 min, 1 cycle

II K.5. Polyacrylamide gel electrophoresis

Standard PAGE techniques were employed (Sambrook et al., 1989). Gels were normally 6 – 8% acrylamide-bis-acrylamide (30:1 cross-linking), 8M urea, 1x TBE. DNA sequencing gels were 40cm long and 0.4 mm thick; gels for separating low molecular

weight RNAs were 20cm long and 1.5mm thick. Following electrophoresis sequencing gels were dried on Whatman 3MM paper before exposure to X-ray film.

II L. DNA techniques

II L.1. Preparation of total genomic yeast DNA

10ml of a yeast culture were grown to an OD_{600} of 0.1, harvested and washed once with 1.5ml 1 M Sorbitol. The cell pellet was then resuspended in 800µl of spheroplast buffer (0.9 M sorbitol, 50mM NaH_2PO_4, 10mM β-mercaptoethanol) and incubated 20min at 37°C. Then 15µl of zymolase 100T and the mixture incubated for 45 minutes at 30°C. After a 10min centrifugation at 2500rpm in a bench top centrifuge (Eppendorf), the cell pellet was resuspended in 500µl lysis buffer (50mM EDTA pH 8.0, 0.3% (w/v) SDS) incubated for 20 minutes at 65°C then cooled on ice. 200µl of 5M KAc were added to the suspension, vortexed and incubated for 45 minutes on ice. The supernatant was mixed with 1.2ml EtOH and incubated at –20°C for 10 minutes. After centrifugation for 15 minutes at 4°C, the supernatant was precipitated with 430µl isopropanol and spun again for another 15 minutes at 4°C. The pellet was then resuspended in 200µl in TE and the DNA treated with 1µl of RNase A (20mg/ml in water) and incubated at 37°C for 15 minutes. The solution was phenol/chloroform extracted twice and 3M sodium acetate was added to the final aqueous phase at a concentration of 1 in 10µl. After addition of 3 volumes EtOH the DNA was pelleted and resuspended in 20µl - 50µl TE.

II M. RNA techniques

II M.1. RNA extraction

RNA extraction was essentially carried out as described in (Tollervey and Mattaj, 1987). 20 OD_{600} of exponentially growing cells were harvested. 500µl GTC (4M Guanadinium Thiocyanate, 0.1M Tris-HCl pH 7.5), 500µl of phenol and 500µl glass beads (Ø 0.45mm) were added and the mixture vortexed for 5 minutes at 4°C. 7.5ml of GTC and 7.5ml of phenol were added and the tube incubated at 65°C for 5 minutes. 7.5ml chloroform and 4ml sodium acetate (100mM sodium acetate in 1x TE) were added. The tube was centrifuged and the supernatant extracted twice with phenol/chloroform before the RNA was precipitated with 3 volumes of 100% EtOH. The pellet was washed with 70% EtOH, briefly air-dried and resuspended in H_2O. This procedure was scaled down as appropriate to the experimental requirements.

II M.2. RNA gel electrophoresis and Northern blotting

Polyacrylamide and agarose electrophoresis was performed as has previously been described (Sambrook et al., 1989). Low molecular weight RNAs (shorter than 500nt) were separated on 6% polyacrylamide urea gels (1x TBE: 0.9M Tris-borate pH 8.3, 20mM EDTA). The gels were stained with Ethidium bromide in 0.5x TBE and transferred to Hybond N^+ (Amersham) membranes by electroblotting in 0.5x TBE buffer at 50V for 4 hours.

High molecular weight RNAs (longer than 500nt) were separated on an agarose formaldehyde gel (1.2% agarose, 6 % formaldehyde, 1x Hepes). The gels were run in 1x Hepes buffer (50mM Hepes, 1mM EDTA, pH 7.8) overnight (16 hours at 60V) with circulation of the buffer. After migration was completed the gel was rinsed with water and washed with 75mM sodium hydroxide for 20 minutes followed by two washes with 0.5M Tris/ 1.5M sodium chloride to neutralise the gel and one wash with 10x SSC (1.5M sodium chloride, 150mM tri-sodium citrate pH7.0) for 20 minutes. The RNA was transferred in 10x SSC by capillary force to a Hybond N^+ membrane (Amersham). The

loading buffer for polyacrylamide gels was 47% formamide, 10mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol and for the agarose gels 50% formamide, 6% formaldehyde, 0.1x Hepes, 0.025% bromophenol blue, 0.025% xylene-cyanol and 0.2mg/ml ethidium bromide.

II M.3. Hybridisation of Northern blots

The membranes were hybridised with 5'end-labelled oligonucleotides. The oligonucleotides used are listed in section 2.5. 10pmol of oligonucleotide was labelled in a 15µl reaction containing 70mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM DTT, 4pmol {γ-^{32}P}ATP, 10U polynucleotide kinase (Stratagene) at 37°C for 30 minutes. The filter was pre-hybridised for 30 minutes at 37°C in 6x SSPE (0.9MNaCl, 60mM NaH2PO4, 6mM EDTA, pH7.4), 5x Denhardt's solution (0.1%ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.5% SDS, 200µg/ml herring sperm DNA (Boehringer). The labelled probe was added to the hybridisation mix and hybridised, shaking, overnight at 37°C. Following hybridisation, the filter was washed twice in 6x SSPE at temperatures ranging from 37°C to 42°C, according to the T_m of the oligonucleotide. The filter was wrapped in saran-wrap and exposed to X-ray film.

II M.4. Primer extension analysis

Primer extension analysis was performed as previously described (Beltrame and Tollervey, 1992). 4pmol of oligonucleotide primer was incubated in a volume of 10µl in 70mM Tris-HCl pH 7.6, 10mM magnesium chloride, 10 mM DTT, 12pmol {γ-^{32}P}ATP, 2U polynucleotide kinase (Stratagene) for 30 minutes at 37°C. The oligonucleotide was precipitated by the addition of 20µg glycogen, ammonium acetate to a concentration to 2M and 2 volumes of EtOH. The precipitated oligonucleotide was washed with 70% EtOH, briefly air-dried and resuspended in 50µl of water. 2µl of labelled oligonucleotide were incubated with 5µg of template RNA in 300 mM NaCl, 10 mM Tris-HCl pH 7.5, 2 mM EDTA pH 8.0 in a volume of 10µl, denatured at 80°C for 5 minutes and annealed at

46°C for 90 minutes. 40µl of pre-warmed buffer (1.25mM dATP, 1.25mM dCTP, 1.25mM dGTP, 1.25mM dTTP, 12.5mM DTT, 12.5mM Tris-HCl pH 8.4, 7.5mM magnesium chloride), 30U RNasin (Promega) and 7.5U AMV reverse transcriptase (Promega) were added and the reaction incubated for 45 minutes at 46°C. The reaction was stopped by addition of 50mM EDTA, 100mM sodium hydroxide and incubation at 55°C for 1 hour. The sodium hydroxide was neutralised by adding hydrochloric acid to a final concentration of 100mM and the RNA precipitated with EtOH/glycogen/ammonium acetate. The pellet was washed once with 70% EtOH, air-dried and resuspended in formamide loading buffer, diluted with water (1:2). Half of this reaction was run on a 6% PAGE urea sequencing gel. DNA digested with Msp1 restriction enzyme was labelled with {α-^{32}P} dCTP and used as a sequencing ladder that was run alongside the primer extension sample to enable the position of primer extension stops to be determined.

II M.5. RNase H cleavage analysis

RNA from the desired strain was prepared according to the protocol described in section II.M.1. To 10µg of RNA, 1µl of 10xhybridisation Mix (0.25M Tris pH7.5, 10mM EDTA, 0.5M NaCl), 10pmol of hybridizing oligonucleotide or oligonucleotide dT were added and the volume of the mixture adjusted to 10ul with DEPC. The reaction was heated to 68°C for 10min then left to cool slowly to 30°C. The mixture was then spun down and 10µl of 2x RNase H Buffer (40mM Tris pH7.5, 20mM MgCl, 2mM DTT) and 1.5µl RNase H enzyme were added and the reaction incubated at 30°C for 1hour. 13 µl of Stop Mix were then added and RNA was extracted once by Phenol/CHCl3 then by CHCl3 only. The RNA was precipitated with 100% EtOH. The pellet was washed with 70% EtOH, air-dried and resuspended in formamide loading buffer. The reaction was run on a 6% PAGE urea sequencing gel. The RNA was transferred in 10x SSC by capillary force to a Hybond N^+ membrane (Amersham). The membranes were hybridised with 5'end-labelled oligonucleotides according to the protocol described in section II.M.3. The oligonucleotides used are listed in section II.E.

II N. Epitope tagging of proteins

II N.1. Construction of TAP – tagged proteins

To construct proteins expressing a C-terminal tandem affinity purification tag (TAP-tag) a TAP-TRP1 cassette was amplified from a plasmid, pRS1479-TAP, using two primers containing flanking sequences specific to the sequence just upstream of the stop codon of the target gene (Rigaut et al., 1999). The amplified cassette was transformed into a yeast wild-type strain (D270) carrying a deletion in the TRP1 locus, and integration was selected for on SD-TRP plates. Integration of the cassette was confirmed by PCR and expression of the TAP-tag was established by Western blot analysis using PAP antibodies. The tag contains a calmodulin-binding domain linked to the sequence of *Staphylococcus aureus* protein A by a cleavage site recognised by a proteinase from the tobacco etch virus (TEV). The TAP-tag can be detected in cells lysates using peroxidase-anti-peroxidase, anti-Protein A (Sigma) or anti-calmodulin (Invitrogen) antibodies (Rigaut et al., 1999).

II O. Protein and immunological techniques

II O.1. Preparation of Yeast extracts

Yeast cells were grown in 2 litres of YPD to an optical density of 1.0 to 2.0 at 600nm. Cells were harvested by centrifugation, transferred into a 50ml-Falcon tube, washed once with 50ml cold, sterile water and once with 50ml of lysis buffer (100mM sodium chloride, 0.05mM Tris-HCl pH 7.5, 1.5mM magnesium chloride, 0.15 % NP40) containing protease inhibitor cocktail (Sigma) and 5mM PMSF. The cell pellet was resuspended in 10ml of lysis buffer containing 5mM PMSF, 1mM DTT and Mini Protease Inhibitor Cocktail tablets (1 tablet per 10ml buffer) (Roche) and 5ml of acid-

washed, siliconised glass beads were added. The cells were lysed by vortexing ten times for 30 seconds with 30-second intervals on ice. The lysate was centrifuged for 5 minutes at 5krpm to pellet the glass beads and any unbroken cells. After ultracentrifugation at 14krpm for 1 hour at 4°C the cleared extract was removed from underneath the floating lipid layer and transferred to a 50ml-Falcon tube with glycerol to a final concentration of 5%. The extract was frozen at -80°C.

II O.2. Immuno - precipitation/Affinity purification

TAP – tagged yeast proteins were precipitated using total rabbit IgGs (Sigma) (Rigaut et al., 1999). 500µl of 50% suspension of rabbit IgG agarose beads were washed three times in lysis buffer and then added to 3000-4000 OD_{600} equivalents of extract. After incubation for 1 hour at 4°C on a rotating-wheel, the IgG agarose beads were washed once with lysis buffer. The beads were taken up in 1ml lysis buffer containing 0.5mM DTT and transferred to Econo-column (Bio-Rad). 5µl of recombinant TEV protease were added and incubated at 18°C for 1 to 2 hours. The eluate was taken off and after precipitation with 10% TCA, the proteins were analysed by Western blot and Coomassie staining.

II O.3. Sucrose density gradient centrifugation

For sucrose density gradient analysis centrifugation 500 ml of a yeast culture was grown in YPD medium to an OD_{600} of 0.8–1.2. Cells were harvested by centrifugation and washed in 10ml ice-cold lysis buffer (100mM sodium chloride, 0.05mM Tris-HCl pH 7.5, 1.5mM magnesium chloride, 0.15 % NP40) containing protease inhibitor cocktail (Sigma), 5mM PMSF, 1mM DTT and Protease Inhibitor Cocktail (Roche). 1.4g glass beads (Ø 0.45mm) were added to 0.5ml of cell suspension. Cells were broken by vortexing for 5 minutes at 4°C. The suspension was centrifuged for 5 minutes at 14krpm. The supernatant (300µl) was loaded onto a 10%–50% sucrose gradient in lysis buffer without DTT and cycloheximide and centrifuged for 15 hours at 27krpm in an Ultracentrifuge SW40-Ti rotor (Beckman). 0.5ml-fractions were collected, of which

250µl were precipitated with TCA and analysed by SDS-PAGE and Western blot analysis. 250µl were used for RNA extraction and analysis by Northern hybridisation.

II O.4. Glycerol density gradient centrifugation

For the separation of low molecular weight complexes containing Rrp6p by glycerol gradient centrifugation, 3litres of a yeast culture was grown in YPD medium to an OD_{600} of 1-2. Cells were harvested by centrifugation and washed in 10ml ice-cold lysis buffer (100mM sodium chloride, 0.05mM Tris-HCl pH 7.5, 1.5mM magnesium chloride, 0.15 % NP40) containing Mini Protease Inhibitor Cocktail tablets (1 tablet per 10ml buffer) (Roche), 5mM PMSF, 1mM DTT. Glass beads (Ø 0.45mm, Sigma) were added to 10ml of cell suspension and cells were broken by vortexing for 10 minutes at 4°C. The suspension was then cleared by ultracentrifugation for 1hr at 32,000rpm. 500µl of the supernatant was loaded on a 10%-30% glycerol gradient in lysis buffer without DTT and centrifuged for 24 hours at 36,000 rpm in an Unltracentrifuge SW40-ti rotor (Beckman). Alternatively, the recovered supernatant was subjected to TAP-purification before loading on the gradient. In both cases, 0.5ml-fractions were collected, analysed by SDS-PAGE and Western blot analysis. 250µl were used for RNA extraction and analysis by Northern hybridisation.

II O.5. SDS-Polyacrylamide electrophoresis

Proteins were separated on polyacrylamide gels containing SDS as described by (Laemmli, 1970). Samples were denatured by adding an equal volume of protein loading buffer (125mM Tris-HCl pH 6.8, 200mM DTT, 4% SDS, 40% glycerol, 0.025% bromophenol blue), heating to 100°C for 5 minutes and centrifugation for 30 seconds.

II O.6. Western blotting

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

The gel was assembled on the nitro-cellulose membrane between 3 sheets of Whatman paper on each side, soaked in transfer buffer (25mM Tris base, 40 mMglycine, 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 5% low-fat dried milk (w/v) in PBS for 30 minutes to 1 hour shaking at room temperature.

Depending on the protein to be detected the blot was decorated with different immunological reagents. For detection of Protein A or TAP–tagged proteins the membrane was incubated with a 1:1000 dilution of rabbit IgG antibody coupled to horseradish peroxidase-anti-peroxidase (PAP, Sigma) for 1 hour at RT. Then the blot was washed four times 15 minutes with 1x phosphate buffered saline (PBS; 137mM sodium chloride, 3mM potassium chloride, 10mM di-sodium phosphate, 2 mM potassium di-phosphate). For detection of Rrp6p, Rrp4p and Npl3p, the membrane was incubated with anti-Rrp6p, anti-Rrp4p or anti-Npl3p with a 1:2000 dilution for 1 hour at RT. The blot was washed four times 15 minutes with PBS and incubated with the HRP-conjugated-secondary antibody for 1hour with a 1:10,000 dilution then washed again four times 15 minutes with PBS. The proteins were detected using an enhanced chemiluminescence (ECL) kit (Amersham), following the manufacturers instructions.

Chapter Three

Rrp6p is associated with complexes distinct from the exosome

III.A. Introduction

The exosome is a conserved complex of 3'-5' exoribonucleases that functions in both RNA processing and degradation pathways (van Hoof et al., 2000c;Allmang et al., 1999a). Two forms of the exosome, nuclear and cytoplasmic, exist and share ten core components (Rrp4p, Rrp40p, Rrp41p, Rrp42p, Rrp43, Rrp44p, Rrp45, Rrp46p, Mtr3p and Csl4p). In the cytoplasm, the exosome is involved in the minor 3'-5' pathway of mRNA turnover and requires the GTPase Ski7p and the Ski complex including the putative helicase Ski2p (Brown et al., 2000). In the nucleus, the exosome participates in the 3' end maturation of 5.8S rRNA, snRNAs and snoRNAs and the degradation of a 5' external transcribed spacer fragment (5'-A_0) (Allmang et al., 1999a). In addition, the nuclear exosome plays an important role in mRNA surveillance degrading aberrant RNAs in strains with defects in splicing, polyadenylation and export (Bousquet-Antonelli et al., 2000; Hilleren et al., 2001;Torchet et al., 2002).

The nuclear and cytoplasmic exosomes can be distinguished by the presence of the nuclear-specific component Rrp6p (Allmang et al., 1999b). Each of the core exosome subunits is essential for cell viability, whereas the deletion of *RRP6* results in temperature sensitive lethality and impaired growth at all temperatures (Briggs et al., 1998;Allmang et al., 1999a). In various aspects of RNA processing and degradation the deletion of Rrp6p results in a phenotype different from those observed in other exosome mutants (Allmang et al., 1999a;Bousquet-Antonelli et al., 2000;Torchet et al., 2002). Rrp6p mutants exhibit a unique accumulation of 5.8S rRNA species that are 3'-extended by 30 nucleotides. Rrp6p is also specifically required for the final 3' trimming step in snoRNAs maturation. Furthermore, defects in cleavage, polyadenylation and, termination generates 3'-extended readthrough transcripts that were shown to be initially processed by the core exosome independently from Rrp6p. These transcripts are then either polyadenylated or degraded by an Rrp6p-dependent mRNA decay pathway (Torchet et al., 2002). This is consistent with the isolation of Rrp6p as a suppressor of a *pap1-1* mutation that results in lower levels of poly(A)$^+$ mRNAs. Similar to the

degradation of transcripts with 3' end formation defects, normal mRNAs retained in the nucleus of export mutants, were also shown to be degraded in an Rrp6p-dependent pathway (Das et al., 2003).

These previous studies suggest a role for Rrp6p in nuclear RNA processing that is distinct from those of the core exosome. These observations led us to speculate that Rrp6p might either act in isolation or associate with (a) complex (es) other than the exosome.

III.B. Rrp6p associates with complexes distinct from the exosome

Because of the useful difference in their separation range, two types of density gradient were used in the work presented in this chapter. Sucrose gradients were selected for the analysis of whole cell lysates looking for the potential association of Rrp6p with high-molecular weight complexes. Glycerol gradients were used for the analysis of ultracentrifuged cleared lysates looking for the association of Rrp6p with small-molecular weight complexes.

III.B.1. Construction of TAP-tagged Rrp6p

The localisation and exosome association of Rrp6p was previously assessed using a protein A tagged construct (Allmang et al., 1999b). However, this underwent substantial degradation making its association with the exosome difficult to assess. This prompted us to repeat this study using the TAP-tag construct. Rrp6p was epitope-tagged by insertion of a C-terminal tandem affinity (TAP) tag (Figure 3.1A) in frame with the *RRP6* open reading frame in the chromosome using a one step PCR technique (described in chapter2). The expression of the fusion protein was confirmed by western blotting using peroxidase-anti-peroxidase antibodies (PAP) (Figure 3.1B). The Rrp6p-TAP

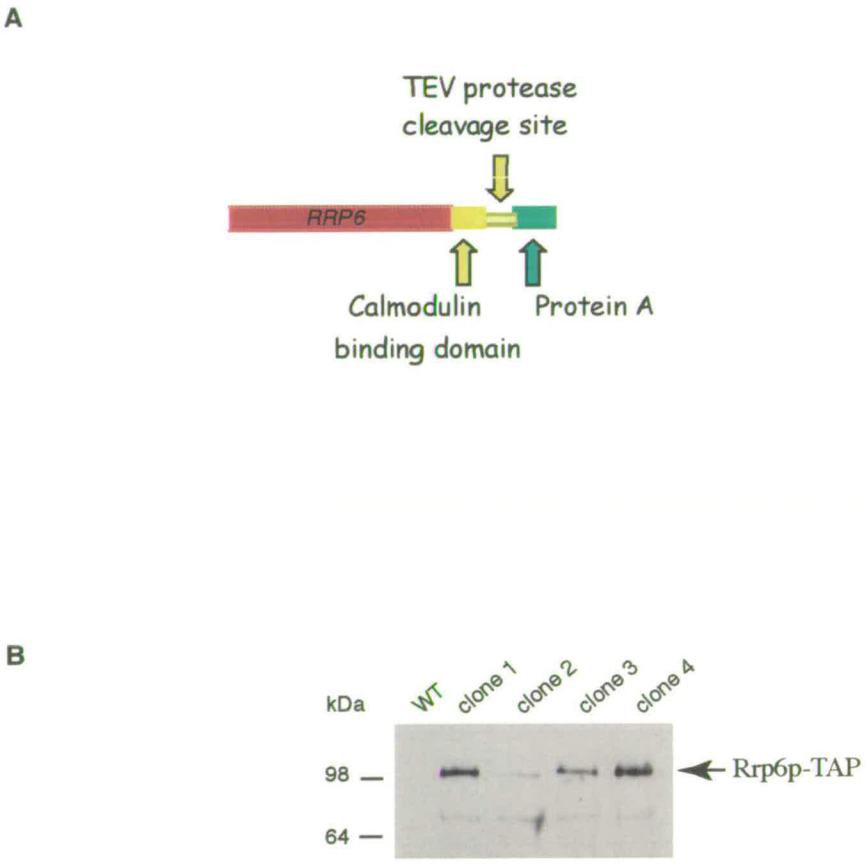


Figure 3.1. Rrp6-TAP.

A. Design of Rrp6p-TAP. Rrp6p was epitope-tagged by insertion of a tandem-affinity purification (TAP) tag in frame with the *RRP6* open reading frame in the chromosome. The TAP tag was fused to the C-terminal domain of Rrp6p (see chapter 2, section II.N.1).

B. Western blot showing Rrp6p-TAP consructs. Immunoblot analysis of strains expressing a Rrp6p-TAP (affinity purification tag). The blot has been decorated with anti-Protein A antibodies bound to horseradish peroxidase to visualise the TAP-tag. Rrp6p is a 84 kDa protein; the tag contributes 20 kDa to the size of the fusion protein.

fusion protein was the only source of Rrp6p in this strain. The strain exhibited no growth defects at 23°C and 37°C temperatures indicating that the fusion protein is functional (Figure 3.2).

Rrp6p mutants exhibit a unique accumulation of a 5.8S rRNA species that are 3'-extended by approximately 30 nucleotides (5.8S+30) (Briggs et al., 1998). To confirm further that the fusion protein construct is fully functional, we carried out Northern blot analysis on RNA extracted from the Rrp6p-TAP strain grown at 23°C and after transfer to 37°C (Figure 3.3). The strain expressing only Rrp6p-TAP showed a slight accumulation of the 5.8S+30 pre-rRNA, which became more significant when the strain was shifted to 37°C. However, the defect in 5.8S rRNA processing observed in the Rrp6p-TAP strain (lanes 3 and 4) was not significant when compared with that seen in the *rrp6*Δ strain (lanes 7 and 8). The slight defect in Rrp6p-TAP function is most probably due to the tag affecting the folding of the protein into the correct conformation. Western analysis indicated that the fusion protein was largely intact in extracts from cells grown at 23°C (Figure 3.1B). More degradation was seen in lysates from cells grown at 30°C or 37°C (data not shown) and analyses of the sedimentation behaviour of Rrp6p were therefore performed using extracts from cells grown at 23°C.

III.B.2. Only a small fraction of the Rrp6p population is associated with the exosome

To determine whether Rrp6p is present in (a) complex(es) other than the exosome, the sedimentation of Rrp6p-TAP was compared with that of Rrp4p, a core exosome component. Lysate from the Rrp6p-TAP strain grown at 23°C was prepared as described in Chapter 2, section II.O.4. 500 µl of lysate was loaded onto a 12 ml 10%-30% glycerol density gradient and fractionated by centrifugation for 24 hours at 36000 rpm. 24 fractions of 500 µl each were collected and analysed by Western blotting analysis using anti-Rrp4p or PAP antibodies.

Initial analysis of the exosome complex showed that it sediments in fractions 14-17 with a sedimentation coefficient of 14S (Mitchell et al., 1997). Western blot analysis of

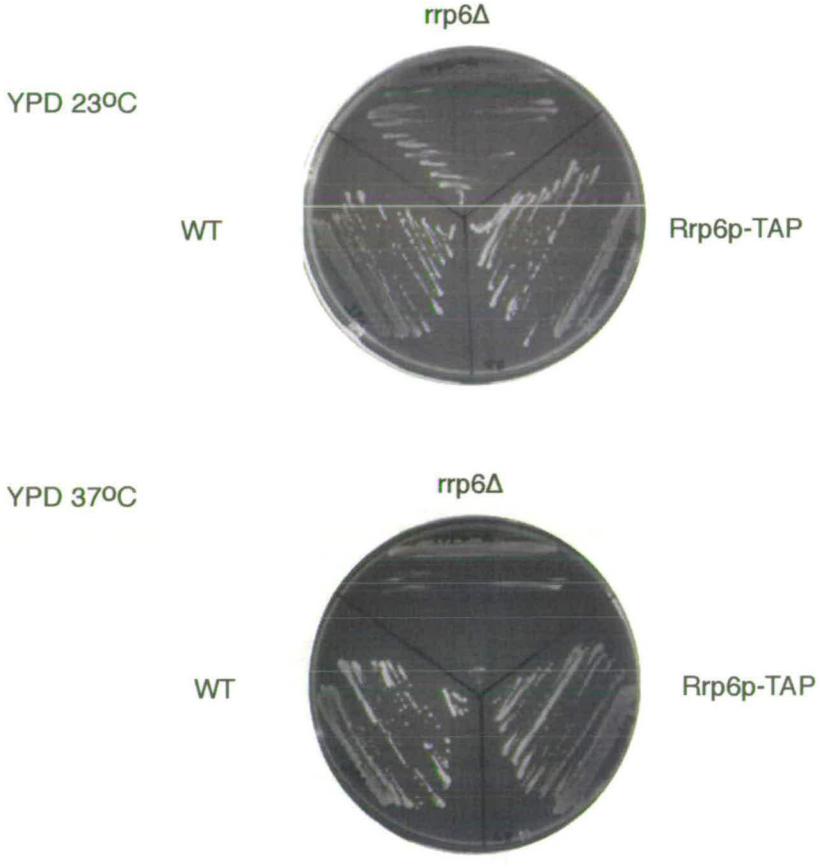


Figure 3.2 Rrp6p-TAP strains show no growth defects.
Plate growth assay of the Rrp6p-TAP on YPD (yeast extract peptone-dextrose rich medium) at 23ºC and 37ºC. As controls wild type (WT) and *rrp6*Δ strains were grown also. *rrp6*Δ shows growth defects at both temperatures. Plates were incubated for 2 days.

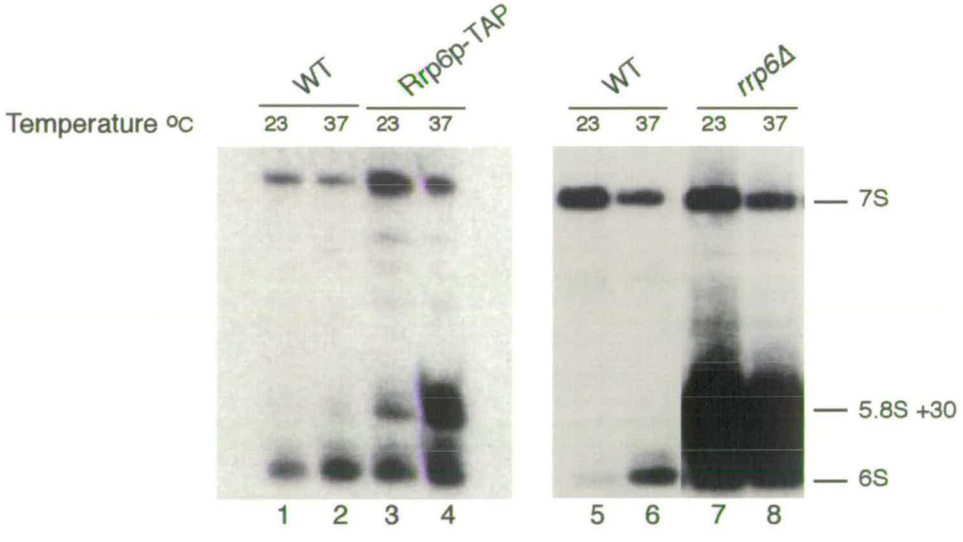


Figure 3.3. Analysing the function of Rrp6p-TAP.
Northern hybridisation analysis of wild-type (WT), Rrp6p-TAP and *rrp6*Δ strains was performed. RNA was extracted from the WT, Rrp6p-TAP and *rrp6*Δ strains at 23°C and following transfer to 37°C. RNA was separated on a 6% polyacrylamide gel and hybridised with probe number 20 (Chapter 2, section II.E).

glycerol gradient fraction with anti-Rrp4p antibodies detected the core exosome component Rrp4p in fractions 9-19 with a peak in fraction 17 and 18 (Figure 3.4). However, while Rrp6p-TAP overlapped the distribution of Rrp4p the peak was observed in fraction 13, clearly distinct from the peak of Rrp4p in fraction 18. Therefore, it appears that most of the Rrp6p-TAP protein does not cosediment with the exosome suggesting its association with another unknown complex which was designated complex A. This complex sedimented slower than the exosome indicating that it is smaller in size.

III.B.3 Rrp6p is associated with high-molecular weight complexes

To investigate whether Rrp6p is also associated with higher molecular weight complexes, lysate extracted from the Rrp6p-TAP strain was fractionated by sucrose gradient centrifugation (Chapter 2, section II.O.3). 500 µl of lysate was loaded onto a 12 ml 10%-50% sucrose density and centrifuged for 15 hours at 27000 rpm. 500 µl fractions were collected and assayed by Western blotting for Rrp6p and Rrp4p using PAP and anti-Rrp4p antibodies respectively (Figure 3.5). Three different peaks for the Rrp6p-TAP could be observed in fractions 5, 14 and 18, whereas the core exosome component Rrp4p was detected with a peak in fraction 5. Thus, it appears that only a small amount of Rrp6p cosediments with Rrp4p and the exosome.

The Rrp6p peak observed in fraction 6 is probably the same Rrp6p-associated complex (complex A) seen on glycerol gradient analysis, where it was better resolved. This better resolution is due to the densities of both sucrose and glycerol which affect their resolving capacities. In addition, Rrp6p could be detected in two other higher molecular weight complexes designated B and C (peaks 14 and 18). To determine whether these high molecular weight complexes represent 40S or 60S ribosomal particles, comparison of the sedimentation of Rrp6p-TAP to that of the rRNA species was carried out. Lysates were fractionated by sucrose gradient centrifugation as described above and fractions were analysed by Western blotting using the PAP antibody to detect Rrp6p-TAP (Figure 3.6*a*). In addition, RNA was extracted from each gradient fraction and analysed by Northern hybridisation analysis using probes against the 18S rRNA and 25S components

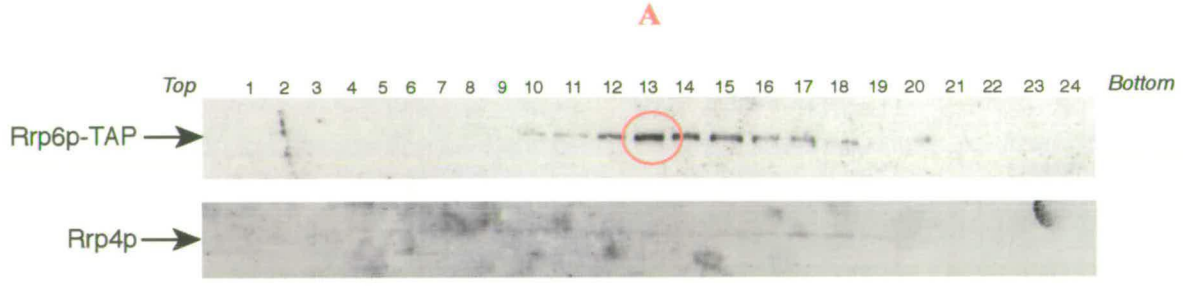


Figure 3.4 Rrp6p-TAP and Rrp4p do not cosediment on glycerol gradients. Lysate extracted from Rrp6p-TAP strain was fractionated through 10% - 30% glycerol density gradient. Aliquots of each fraction were resolved by SDS-PAGE; Rrp6p-TAP and Rrp4p were detected by Western blot analysis with PAP and anti-Rrp4p antibodies (Chapter 2, section II.H). Rrp6p-asoociated complex is labeled **A**.

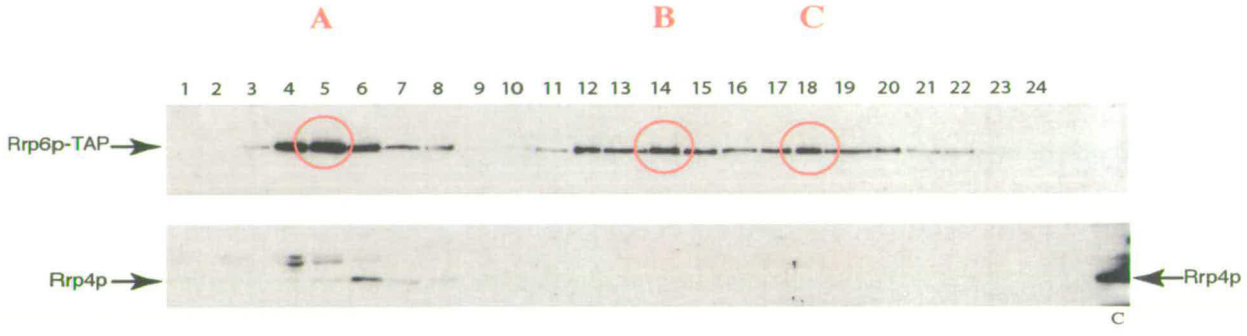


Figure 3.5. Rrp6p-TAP associates with complexes other than the exosome.

Sucrose density gradients analysis of Rrp6p-TAP and Rrp4p. Lysate extracted from Rrp6-TAP strain was fractionated through 10%-50% sucrose density. Aliquots of each fraction were resolved by SDS-PAGE; Rrp6p-TAP and Rrp4p were detected by western blot analysis using peroxidase anti-peroxidase and anti-Rrp4p antibodies (Chapter 2, section II.H). Whole cell lysate extracted from the Rrp6p-TAP strain was loaded in lane C as a control for the anti-Rrp4p antibodies. Three different Rrp6p-associated complexes could be detected and these were labelled A, B and C.

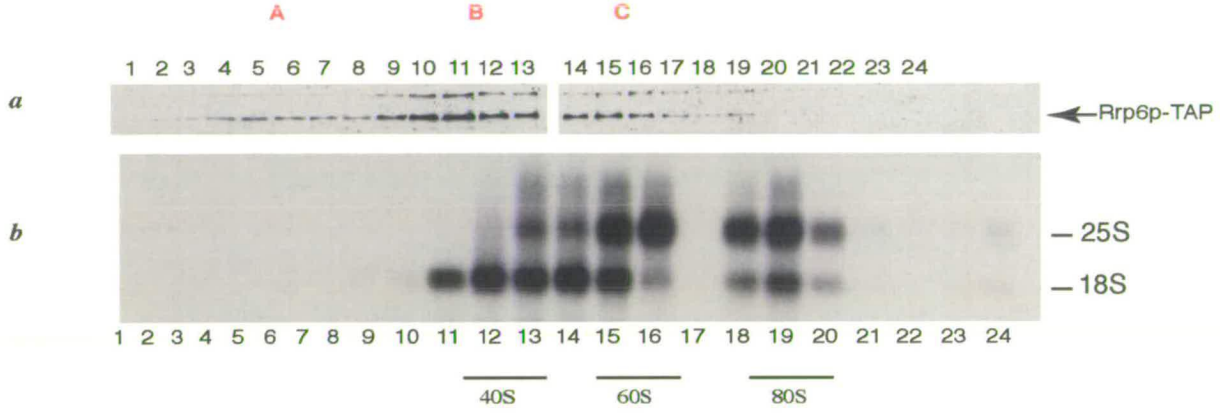


Figure 3.6. Rrp6p-associated complexes B and C cosediment with pre-40S and pre-60S ribosomes.

(a) Behaviour of TAP-tagged Rrp6p on a 10%-50% sucrose gradient. Rrp6p-TAP was detected by Western blot analysis using PAP antibodies (Chapter 2, section II.H).

(b) Northern analysis of RNA extracted from each gardient fraction. probe number against 18S rRNA component of the 40S ribosome and the 25S rRNA component of the 60S ribosome was used.

Position of the Rrp6p-asscoiated complexes and 40S and 60S ribosomal subunits are indicated.

of the 40S ribosomal and 60S ribosomal particles. The majority of Rrp6p was found to be enriched in the 40S region (lanes 11 to 13) with smaller amounts in the 60S region (lanes 14 to 16) (Figure 3.6*b*). This suggests that the association of Rrp6p with complexes B and C may reflect its association with pre-40S and pre-60S size particles respectively. Rrp6p plays a crucial role in converting 7S rRNA to 5.8S rRNA, which is an essential component of the large ribosomal subunit 60S (Briggs et al., 1998). However, Rrp6p has not been previously reported to play a direct role in the biogenesis of the 40S ribosomal subunit.

Notably, the 60S complex was not consistently observed on sucrose gradients in different experiments (Compare figure 3.5 with figure 3.6*a*). This could be due to the dissociation of the complex during lysate preparation or centrifugation. It can be concluded that the approximately 40S and 60S sized Rrp6p-associated complexes could be due to association with the pre-40S and pre-60S ribosomal particles.

III.B.4 Generation of a polyclonal antibody against Rrp6p

We were concerned that the sedimentation of Rrp6p-TAP on density gradients might be an artifact of the presence of the TAP-tag. To confirm the sedimentation data from Rrp6p-TAP gradients, antibodies were therefore raised in rabbit against Rrp6p expressed in *Eschericia.coli.* However, a his-tagged fusion protein comprising the full-length Rrp6p (84kDa) could not be expressed in *Escherichia coli.* This could be due to either the large size of the protein or the toxic effects of its exonuclease activity on the bacterial cells. However, a his-tagged fusion protein comprising the 222 N-terminal residues of Rrp6p was designed and expressed successfully in *E.coli.* The Rrp6p fragment was then purified on Ni-NTA agarose column and 1mg of the purified protein was used for rabbit immunisation.

The presence of anti-Rrp6p antibodies in the serum was confirmed by western blot analyses using lysates extracted from wild type strain and the *rrp6*Δ strain that does not express the protein (Figure 3.7). A band corresponding to Rrp6p could be detected in the wild type lysate but not in the *rrp6*Δ (lanes 1 and 2). Cleaved Rrp6p-TAP (app 90kDa)

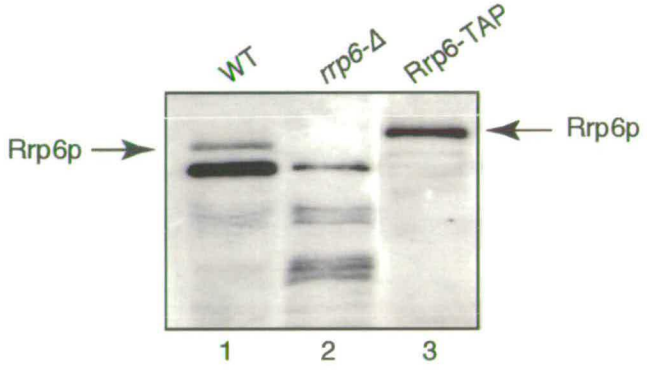


Figure 3.7. Analysis of the polyclonal antibody against Rrp6p. Western blot analysis of WT, *rrp6*Δ and Rrp6p-TAP strains. The blot has been decorated with anti-Rrp6p antibody that was raised in rabbit against the N-terminal 222 residues of *RRP6*. Cleaved Rrp6p-TAP was included as a positive control for the Rrp6p Western analysis (lane 3). This protein is predicted to migarte slower than the endogenous Rrp6p in SDS-PAGE gels due to the presence of the calmodulin-binding domain.

protein was used as a control in lane 3. This protein is predicted to migrate slower than the endogenous Rrp6p in SDS-PAGE gels due to the presence of the calmodulin-binding domain.

The generated anti-Rrp6p antibodies were then used to confirm the sedimentation data that were performed with the epitope-tagged version of the protein. Initial analyses of anti-Rrp6p antibodies used immunoprecipitates from strains expressing TAP-Rrp6p that were digested with tobacco etch virus protease (TEV), which cleaves the protein A. The purified lysate was then fractionated by glycerol gradient analysis. Fractions were analysed by Western blotting with antibodies raised against Rrp6p or antibodies raised against Rrp4p (Figure 3.8).

Rrp6p was successfully detected by the anti-Rrp6p antibodies with a peak in fraction 11. However, consistent with the data presented earlier in this chapter, the peak of Rrp4p (fraction 16) did not cosediment with that of Rrp4p. Anti-Rrp6p antibodies were also used to determine the sedimentation of wild-type Rrp6p on sucrose density gradients (Figure 3.9*a*). Again, 3 discrete peaks were observed in close agreement with the sedimentation of Rrp6p-TAP.

Thus, were able to confirm the results obtained from gradient analysis data done with the epitope-tagged version of Rrp6p. We can conclude that only a small amount of the Rrp6p population is associated with the exosome and that the major fraction of Rrp6p is not free but rather associated with complexes that remain to be characterised.

III.B.5 Rrp6p is associated with a ribonucleoprotein complex

To determine whether the observed Rrp6p complexes are associated with RNA, lysate extracted from wild type cells was treated with RNase A at 2μg ml^{-1} and incubated for 60 min on ice prior to fractionation on a sucrose gradient. Following centrifugation, fractions from treated and untreated lysates were analysed by Western blotting using the anti-Rrp6p antibodies (Figure 3.9). The distribution of the Rrp6p-associated complexes (A, B and C) appeared similar to what was previously observed in studies with Rrp6p-TAP. Complexes B and C were detected in the fractions of the untreated lysate (Figure 3.9*a*) but they were lost following treatment with RNase A (Figure 3.9*b*). In contrast, the

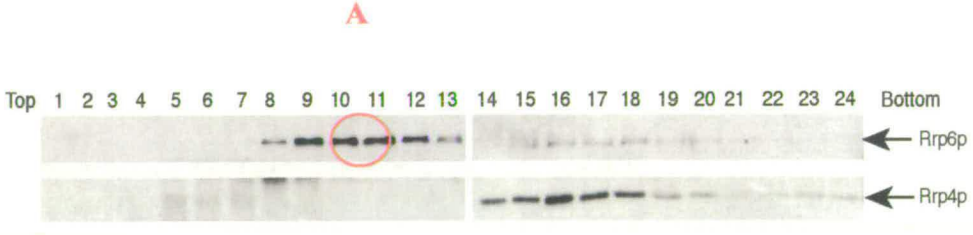


Figure 3.8. Rrp6p and Rrp4p do not cosediment on Glycerol Gradients. Tap purified Rrp6p was fractionated through 10%-30% glycerol gradient. Aliquots of each fraction were resolved by SDS-PAGE; Rrp6p and Rrp4p were detected by Western blot analysis with anti-Rrp6p antibodies and anti-Rrp4p antibodies (Chapter 2, section II.H).

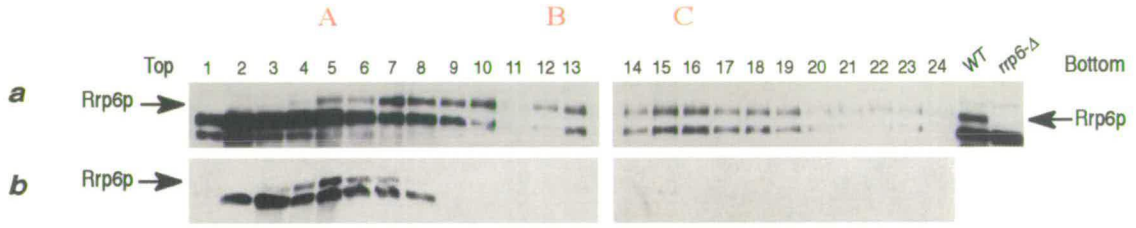


Figure 3.9. Rrp6p associates with RNP complexes.
Sucrose density gradient analysis of wild-type strain. Lysates extracted from wild-type strain were fractionated without (***a***) or with (***b***) prior digestion with RNaseA through 10%-50% sucrose gradient. Aliquots of each fraction were resolved by SDS-PAGE; Rrp6p was detected by Western blot analysis with anti-Rrp6p (see chapter 2, table).

A complex was unaffected by RNase treatment, suggesting that it does not associates with any RNA species. This demonstrates that in contrast to complex A the high molecular complexes B and C represent ribonucleoprotein particles. Previous studies have failed to detect any RNA species associated with the exosome complex (Fatica and Tollervey, 2002). This is consistent with the above results since these potential high molecular weight Rrp6p complexes that appear to be distinct from the exosome.

III.6. Discussion

The results show that Rrp6p is associated with at least two complexes other than the exosome, a low molecular weight complex of app 10S (A), a high-molecular weight complex that sediments with the 40S pre-ribosome (B). Some Rrp6p could also be detected and was shown to sediment with the 60S pre-ribosome (C) however whether this is a distinct complex is still not clear. Both high molecular weight complexes appeared to be sensitive to RNase treatment suggesting that they are RNP complexes.

III.6.1. Rrp6p functions independently from the exosome

In both processing of stable RNAs and degradation of aberrant mRNAs, Rrp6p appears to have functions distinct from the core exosome. The gradient analysis presented in this chapter showed that there is no free Rrp6p, showing that it does not function alone but rather as part of (a) complex (es). A combination of glycerol and sucrose gradient fractionation revealed that in addition to the exosome Rrp6p is associated with three other complexes.

The earliest Rrp6p complex to be observed is complex (A) that sedimented slower than the exosome and was not sensitive to RNase A treatment. The composition of this complex was analysed by mass spectrometry and will be discussed in the next chapter.

As predicted, Rrp6p was seen associating with the exosome complex at substoichiometric levels. Previous studies have shown that 10 to 20% of the exosome is associated with Rrp6p however the fraction of Rrp6p that associated with the exosome was not addressed (Allmang et al, 1999b). The results presented in this chapter reveal that only a small fraction of the Rrp6p population that was observed cosedimenting with the core exosome component Rrp4p on glycerol gradients.

Complex B, which sedimented with the pre-40S ribosome was consistently seen on the gradients. This complex is believed to be a pre-ribosomal particle since this complex was lost upon treatment with RNase A. However, no exonuclease has not been reported previously to be involved in the biogenesis of the small (40S) ribosomal subunit.

Depletion of any exosome components is known to affect the early cleavages in the 35S precursors at sites A_0, A_1 and A_2 (figure 1.3, chapter1) (Allmang et al., 2000). Defective cleavage at A_2 leads to the depletion of the 20S and the 27SB pre-rRNA and as a consequence the levels of the 18S and the 25S rRNA were reduced. However these early cleavages require endonucleases and no direct role for an exonuclease can be readily envisaged. Many mutations that affect the synthesis of the 5.8S and 25S rRNA and the 60S ribosomal subunit also affect the synthesis of the 18S rRNA. This is apparently achieved via indirect effects and this is also likely to be the case for the exosome mutations (Allmang et al., 2000).

The assembly of the 60S synthesis factors is likely to be monitored to ensure that only correctly processed and assembled pre-rRNAs are matured to ribosomal subunits. In wild-type cells this control mechanism probably functions only to transiently delay processing until the missing factor has bound whereas in strains genetically depleted of processing factors results in the partial or complete inhibition of processing (Allmang et al., 2000).

Moreover, complex B could also represent a non-ribosomal particle. Scp160p, a protein with homology to yeast hnRNPs was recently shown to associate with an mRNP

complex (Lang and Fridovich-Keil, 2000). From the time they leave the transcription complex, pre-mRNAs also referred to as heterogeneous nuclear RNAs (hnRNAs) are associated with proteins (hnRNPs), which have been proposed to function in various steps of RNA maturation and in RNA export (Kiledjian and Dreyfuss, 1992;Lee and Silver, 1997). This Scp160p mRNP complex detected on sucrose gradients is a 1300kDa sized complex (around 40S), which was shown to be sensitive to RNase treatment and to contain the poly(A) polymerase Pab1p. The presence of Pab1p in RNase sensitive Scp160p complexes indicates that Scp160p is primarly bound to polyadenylated RNAs (Lang and Fridovich-Keil, 2000). Whether or not the Rrp6p that associates with complex B binds to only specific sets of RNAs will be the subject of future studies.

Rrp6p was found to associate with the RNP complex C that sedimented with the pre-60S ribosome, however Rrp6p association with pre-60S particles *in vivo* has not yet been detected in proteomic analysis of pre-ribosomes. The presence of Rrp6p in this complex is predicted because of the role played by the protein in the biogenesis of the 60S subunit. 5.8S rRNA is an essential component of the 60S ribosomal subunit and loss of Rrp6p activity inhibits 60S subunit biogenesis (Briggs et al., 1998). Several pre-60S particles have been recently identified in the pre-60S assemble pathway and synthesis factors associated with each of the particles have been identified (Nissan et al., 2002). However, it is likely that several further distinct pre-60S particles remain to be characterised since several known 60S synthesis factors have not yet been detected in pre-ribosomal particles.

Other processing enzymes, including the core exosome complex and 5'-3' exonucleases Rat1p/Xrn1p were absent from these pre-60S particles, suggesting that they may associate only transiently or weakly with these particles (Fatica and Tollervey, 2002). This could explain the variation in the yield and distribution of the high molecular weight complexes upon fractionation on sucrose gradients. The RNA and protein composition of complexes B and C remains to be determined and would potentially give more insights into the role of Rrp6p within these complexes.

Chapter Four

Identification and analysis of Rrp6p-associated proteins

IV.A. Introduction

Initial biochemical purification of the exosome has shown that Rrp6p associates with 10-20% of the purified exosome and that this subfraction is confined to the nucleus (Allmang et al., 1999b). Furthermore, previous studies of nuclear RNA turnover provided evidence for the separable activities of Rrp6p and the core exosome components (Burkard et al., 2000;Libri et al., 2002;Torchet et al., 2002). This raises the question as to whether Rrp6p interacts with other proteins in each of these functions.

Rrp6p was first isolated as a suppressor of the poly(A) polymerase temperature sensitive mutation (*pap1-1*) in *S. cerevisiae*. Deletion of Rrp6p was able to alleviate the transcription site accumulation of both poly(A)$^-$ and poly(A)$^+$ mRNA seen in *pap1-1* and export mutants respectively. In addition, Rrp6p was shown to interact physically with the nuclear poly(A) mRNA binding protein Npl3p and to co-purify with the cap-binding protein Cbp1p (Burkard and Butler, 2000; Das et al., 2000). These findings suggest possible interactions between Rrp6p and components of RNA processing machineries.

The results presented in chapter 3 provided support for the association of Rrp6p with at least two complexes distinct from the exosome. To demonstrate possible physical associations between Rrp6p and other proteins, immunoprecipitation experiments with Rrp6p-TAP were carried out.

IV.B. Proteomic analysis of Rrp6p-associated proteins

IV.B.1. Rrp6p is associated with Upf2p within the Rrp6p complex A

To identify proteins associated with Rrp6p in the designated A complex, fractions 9, 10, 11 and 12 from the glycerol gradient (see chapter 3, figure 3.8) were pooled and precipitated with TCA. The proteins were separated by SDS-PAGE, and detected by Coomassie blue staining (Figure 4.1). The observed bands were then excised and the

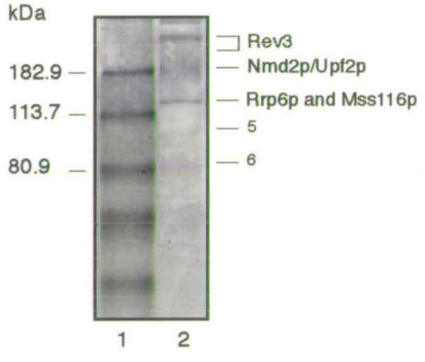


Figure 4.1. Proteomic analysis of Rrp6p complex A.
Fractions 11, 12, 13 and 14 from the glycerol gradient analysis shown in figure 3. were pooled and the proteins were precipitated by TCA and resolved on an 8% SDS-PAGE gel (lane2). A molecular weight marker was run in lane 1.Bands were visualised by Coomassie staining.

proteins digested into peptides by the sequence specific protease trypsin (cleaves after arginine or lysine). The eluted peptides are then subjected to mass spectrometric analysis using matrix-assisted laser desorption/ionisation (MALDI). The mass spectrum of the eluted peptides is acquired, which results in a "peptide-mass fingerprint" of the protein being studied. The mass spectrum is obtained by MALDI from a time-of-flight distribution of the peptides comprising the mixture. The obtained mass spectra are then searched against databases of theoretical tryptic digests of all proteins (Jensen et al., 1997;Berndt et al., 1999). In addition to Rrp6p, mass spectrometry identified the zeta subunit DNA polymerase Rev3p (*YPL167c*), Upf2p (Nmd2p, *YHR077c*) and Mss116p (*YDR194c*). Bands 5 and 6 could not be identified, possibly due to the small amounts of the proteins.

Rev3p is involved in double-strand break repair via homologous recombination. Interestingly, Rrp47p, a novel exosome-associated cofactor, which will be the subject of the following chapter, was also shown to be involved in DNA double strand break repair (Erdemir et al., 2002). Mss116p is a DEAD box protein involved in the splicing of mitochondrial introns located on nuclear genes (Niemer et al., 1995). Upf2p is a component of the nonsense-mediated decay pathway involved in the degradation of transcripts harbouring premature translation termination codons (Czaplinski et al., 1999).

The identification of two proteins involved in RNA metabolism gives grounds for optimism that biologically relevant proteins are being identified.

IV.B.2 Rrp6p is associated with the proteins Srp1p and Gbp2p

In a further attempt to identify novel proteins associated with TAP-Rrp6p, a two-step affinity purification was performed and purified proteins were separated by SDS-PAGE using different gel conditions (Chapter 2, section II.K.6). Rrp6p is an 84kDa protein and to better detect the protein by SDS-PAGE it is necessary to use an 8% gel. However, to separate small sized proteins that might co-purify with TAP-Rrp6p, a higher percentage gel was used. The proteins were detected by Coomassie blue staining. Bands were excised and subjected to analysis by mass spectrometry using MALDI.

A relatively strong band was observed at around 90kDa and was later identified as Rrp6p. (Figure 4.2). This protein is predicted to migrate slower that the endogenous Rrp6p in SDS-PAGE gels due to the presence of the calmodulin-binding domain (5kDa). As expected components of the exosome co-purified with Rrp6p-TAP and were identified as Rrp44p, Rrp4p and Csl4p. The other exosome components (Rrp41p, Rrp46, Mtr3p and Rrp40) could not be detected on the 8 and 10% SDS-PAGE gels run due to their low molecular weights (28-29 kDa).

In addition to exosome components, two novel proteins were co-purified with Rrp6p-TAP and identified as Srp1p (*YNL189w*) and Gbp2p (*YCL011c*). Srp1p, is the yeast homologue of Importin α, which functions as the nuclear localisation signal receptor *in S.cerevisiae*. Proteins containing nuclear localisation signals (NLSs) are transported to the nucleus by a heterodimer composed of Srp1p (Kap60p) and Kap95p (Tabb et al., 2000). Srp1p (suppressor of RNA *p*olymerase I) was initially isolated as a suppressor of certain temperature-sensitive (ts) mutations in RNA polymerase I (pol I), which suggests other functions in addition to protein import (Yano et al., 1992). Gbp2p (G-strand binding protein) binds single stranded G-telomere sequence and may play a role in telomere maintenance, possibly in RNA telomerase biogenesis (Ferrezuelo et al., 2002). Gbp2p was recently characterised as a poly(A)$^+$ RNA-binding protein involved in the export of mRNAs to the cytoplasm and shown to associate with the TREX (transcription –export) complex (Windgassen and Krebber, 2003; Strasser et al., 2002). Gbp2p is highly homologous to Hrb1p and is related to Npl3p, a well characterised shuttling RNA-binding protein (Windgassen and Krebber, 2003). Both Gbp2p and Hrb1p were shown to localise to the nucleus and to contain three RNA recognition motifs (RRM) in addition to the Arg-Gly-Gly (RGG) repeats motifs at their N-terminus (Windgassen and Krebber, 2003). However, the *gbp2* null and *gbp2/hrb1* double null mutant do not display a defect in telomere length maintenance relative to the wild type (Ferrezuelo et al., 2002).

The possible association of Rrp6p with Srp1p and Gbp2p suggest possible interactions between Rrp6p and the RNA export machinery.

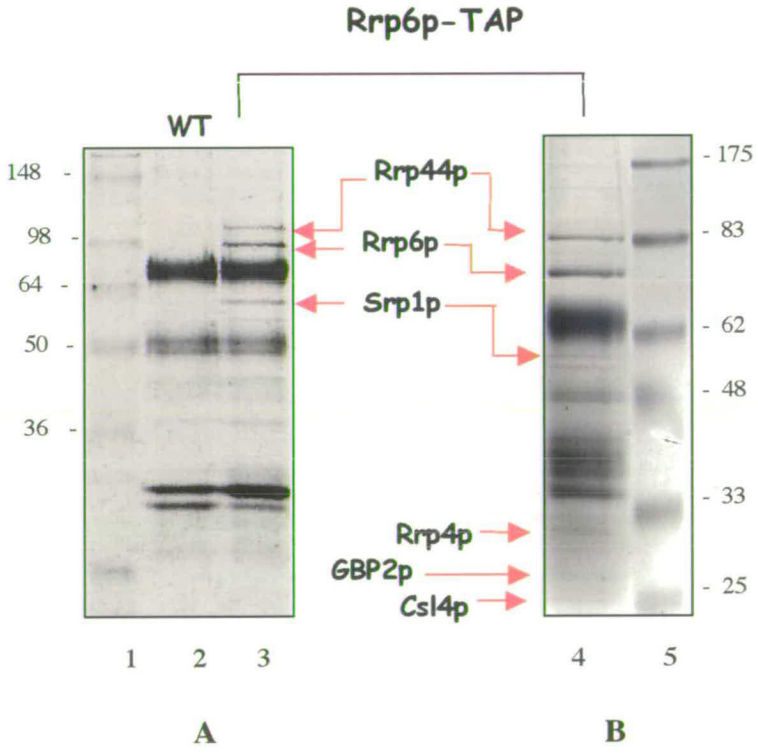


Figure 4.2. Proteomic analysis of Rrp6p-TAP. Purified proteins obtained from immunoprecipitation with Rrp6p-TAP. The proteins were resolved on different SDS-PAGE gels according to size (lanes 3 and 5). (**A**) 8% polyacrylamide gel. (**B**) 10% polyacrylamide gel. Molecular weight markers were loaded in lanes 1 and 4. Bands were visualised by Coomassie staining.

IV.B. Rrp6p mutants accumulate nonsense-containing *CYH2* RNAs

The non-sense mediated decay pathway (NMD, see chapter 1) degrades mRNAs that contain a premature stop codon and requires the trans-acting factors Upf1p, Upf2p and Upf3p. Recently, the medical importance of NMD in several human disorders has been well reported. For example, cystic fibrosis and Duchenne muscular dystrophy can be caused by mutations that generate premature termination codons (Sun and Maquat, 2000). Furthermore, unspliced pre-mRNAs that leak to the cytoplasm are also targeted for degradation by NMD. In yeast, one example of this phenomenon in the unspliced *CYH2* pre-mRNA that can be inappropriately exported to the cytoplasm (Hilleren and Parker, 1999). A 25 fold accumulation of *CYH2* pre-mRNA was observed in Upf mutants (He and Jacobson, 2001). Mass spectrometric analyses of Rrp6p-associated complex identified Upf2p. Due to the characterised role of Upf2p in NMD and our interest in RNA surveillance, we chose to study the interaction of this protein with Rrp6p

To investigate the involvement of Rrp6p in NMD, we performed Northern hybridisation analysis and looked for accumulation of *CYH2* pre-mRNAs in *rrp6Δ* and wild type strains (Figure 4.3). Accumulation of the *CYH2* pre-mRNA could be clearly observed in the *rrp6Δ st*rain grown at 23°C (compare lane 1 with lanes 3 and 4). Phosphoimager quantification of the bands revealed that the *CYH2* pre-mRNA accumulates 3 fold in *rrp6*Δ and that this accumulation was accompanied by a smaller increase in the levels of the mature *CYH2* mRNA (Figure 4.4, panels A, B). This suggests that the loss of Rrp6p is not inhibiting splicing of the pre-*CYH2* but rather increases the stability or the transcription of the pre-*CYH2* mRNA.

We also studied the accumulation of the *CYH2* pre-mRNA in strains carrying a ts mutation in the core exosome component Ski6p (Rrp41p) and lacking the novel exosome-associated cofactor Rrp47p. Rrp47p will be the subject of the following chapter. In *rrp47*Δ and *ski6-100* mutants the levels of pre-*CYH2* were not significantly

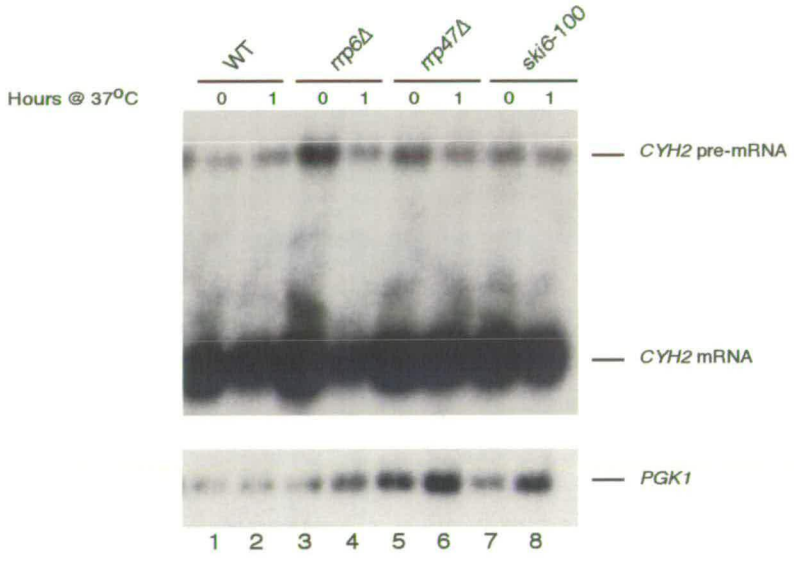


Figure 4.3. *rrp6Δ* accumulates CYH2 precursor. Northern analysis of wild-type (WT), *rrp6*Δ, *rrp47*Δ and *ski6-100* mutant strains. RNA was extracted from each strain at 23°C and following transfer to 37°C for 1 hr. 7μg of RNA was loaded in each lane and resolved on 1.2% agarose gel. *CYH2* mRNA was detected by anti-CYH2 probe number 405 (Chapter 2, section II.E).

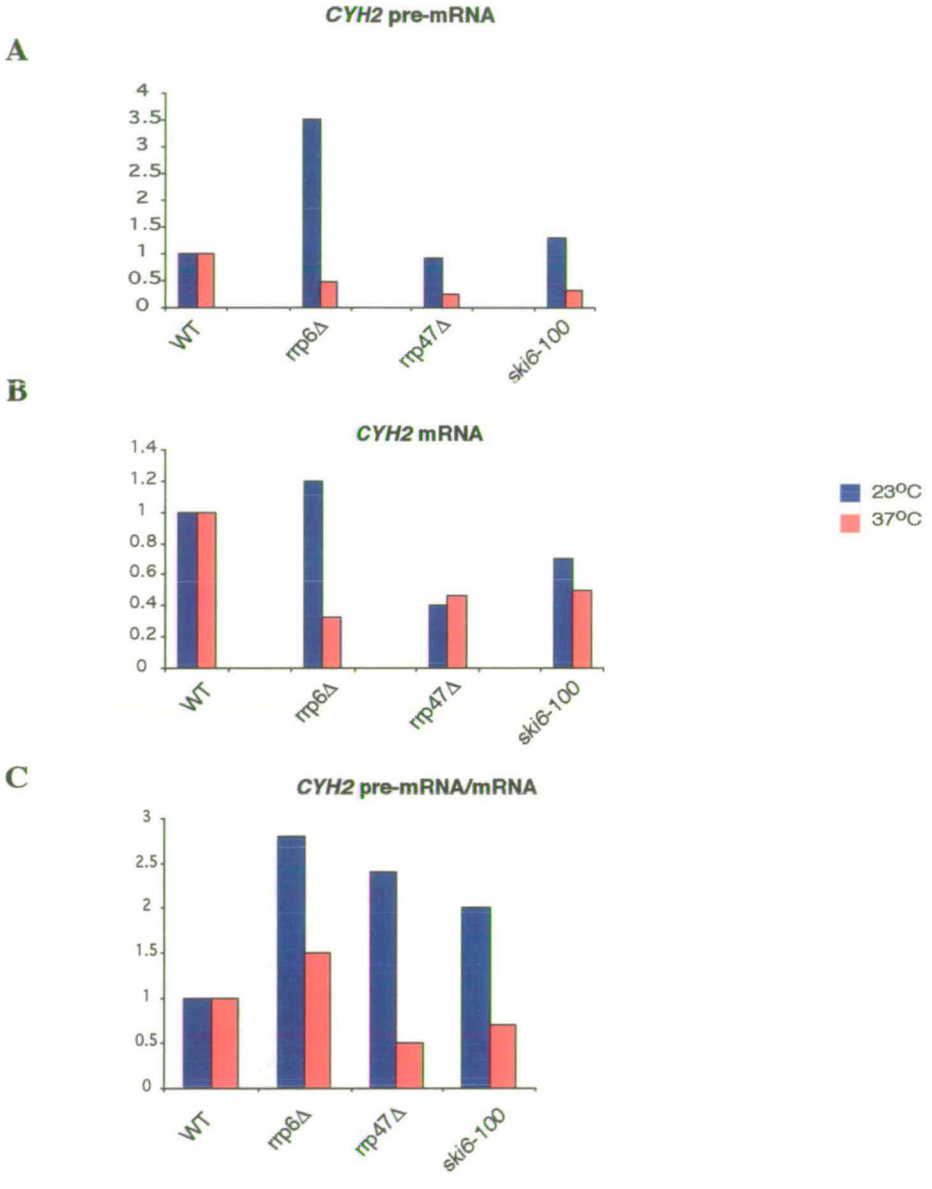


Figure 4.4. Effect of depletion of Rrp6p, Rrp47p and mutation in Ski6p on the accumulation of *CYH2* pre-mRNA and mRNA. Levels of *CYH2* RNA from the Northern hybridisation analysis were quantified by Phosphoimager and normalised to a loading control. The wild type was arbitrarly set to a value of 1.
(A) levels of *CYH2* pre-mRNA. (B) levels of *CYH2* mRNA. (C) ratio of *CYH2* pre-mRNA to *CYH2* mRNA.

altered whereas *CYH2* mRNA levels were decreased at 23°C and this decrease became more significant when the strains were transferred to 37°C (Figure 4.3 and 4.4, panels A and B). The CYH2 gene encodes a ribosomal protein Rpl28p and its expression will be down-regulated in cells with a reduced growth rate (Kaufer et al., 1983).

IV.D. Srp1p and Gbp2p are not required for the processing of stable RNAs

To determine if Srp1p or Gbp2p participates with Rrp6p in the processing of the 5.8S rRNA, northern blot analysis was performed on RNAs extracted from *srp1-31*, *srp1-49* *gbp2Δ* and *hrb1Δ* mutant strains.

Since both *srp1-31* and *srp1-49* mutants were previously reported to be temperature sensitive (Tabb et al., 2000). RNAs were extracted from these mutants at the permissive temperature of 23°C and after 1.5hrs shift to 37°C (Figure 4.5). Neither of these mutants displayed a defect in the processing of the 5.8S rRNA when compared with *rrp6Δ* strains. Thus, unlike mutations in Rrp6p and the exosome, these alleles of Srp1p do not inhibit the processing of the 5.8S rRNA. Rrp6p is also involved in the final trimming of the 3'end of a group of snoRNAs and deletion of Rrp6p results in the accumulation of snoRNAs with short 3' extensions. Analysis of U18 snoRNA processing in *srp1-31* and *srp1-49* did not reveal any defect in the final processing of U18.

Similar analyses were carried out with *gbp2Δ* and *hrb1Δ* strains (Figure 4.6). Both Gbp2p and Hrb1p are nonessential and do not show growth defects at any tested temperature (23°C, 30°C, 37°C) (data not shown). RNAs were extracted from *gbp2Δ*

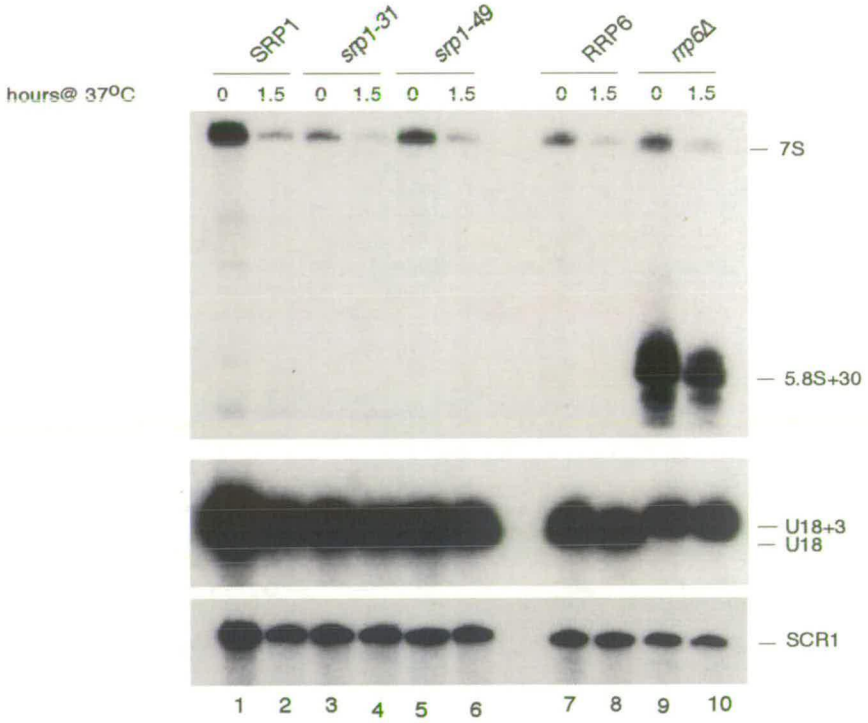


Figure 4.5. Srp1p does not play a role in 5.8S pre-rRNA and snoRNA processing. Northern blot analysis of *srp1-31*, *srp1-49* and *rrp6*Δ and their isogenic wild type (SRP1 and RRP6) strains. Strains were grown at 23°C and then shifted to 37°C for 1.5 hrs. For each panel 7μg of RNA was separated on a 6% polyacrylamide gel and analysed by Northern hybridisation with a probe specific for the pre-5.8S species extended into ITS2 and a probe specific for U18 snoRNA probes number 20 and 405 (Chapter 2, section II.E).

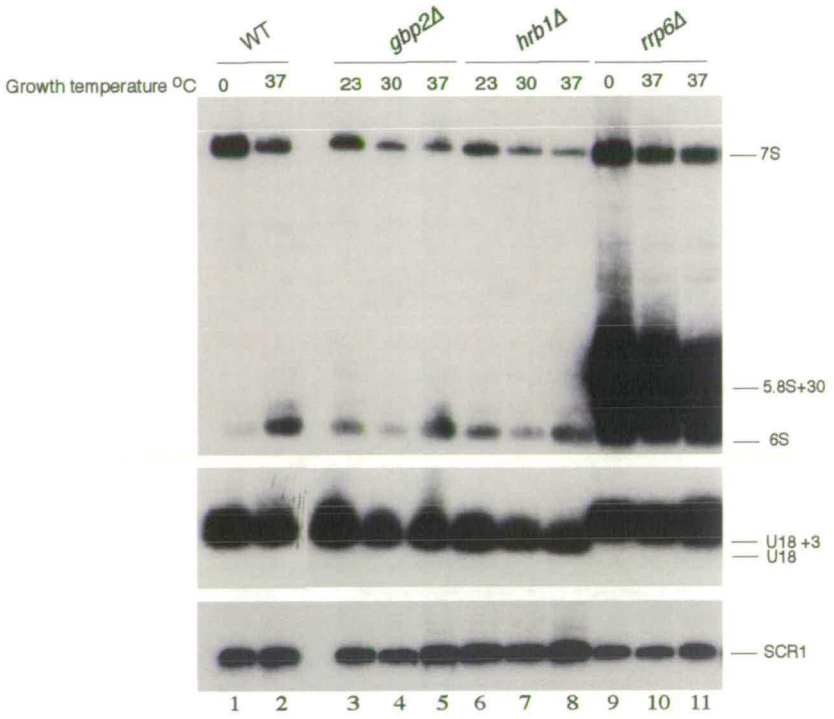


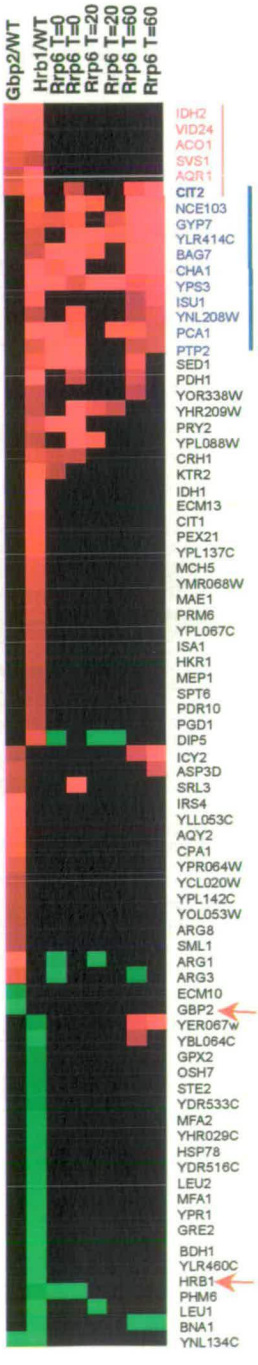
Figure 4.6. Gbp2p and Hrb1p are not required for 5.8S pre-rRNA or snoRNA processing.

Northern analysis of wild-type (WT), *gbp2*Δ, *hrb1*Δ and *rrp6*Δ strains. RNA was extracted from *gbp2*Δ, *hrb1*Δ grown at 23°C, 30°C and 37°C. 7µg of RNA was loaded in each lane and resolved on 6% polyacrylamide gel and analysed by Northern hybridisation using a probe specific for the pre-5.8S rRNA and for U18 probes number 20 and 205 (Chapter 2, section II.E).

and *hrb1Δ* strains following growth at 23°C, 30°C, and 37°C and analysed by Northern blotting for 5.8S and U18 processing. The *gbp2Δ* and *hrb1Δ* strains resembled the isogenic wild-type with no defects in the processing of 5.8S or U18 being observed.
It can be concluded from these results that *srp1-31*, *srp1-49*, *gbp2Δ* and *hrb1Δ* mutants do not show any of the processing defects that are characteristic of Rrp6p for stable RNA synthesis. However, Rrp6p plays an important role in nuclear RNA surveillance and Srp1p, Gbp2p or Hrb1p might be involved in these functions of Rrp6p.

IV.E. Microarray analysis identifies a set of genes specifically regulated in Rrp6p and Hrb1p

To gain more insight on the interaction between Gbp2p and Rrp6p, we decided to use microarray analysis to identify potential genes that are regulated by Rrp6p, Gbp2p and its homologue Hrb1p. Microarray analysis was done in collaboration with Dr. Frederic Devaux in France. The advantage of this technique is that it allows the measure of levels of gene expression (mRNA abundance) for thousands of genes simultaneously (discussed in more detail in chapter 6). mRNA was purified from the *rrp6Δ*, *gbp2Δ*, *hrb1Δ* and the wild type strains and labelled nucleotides were incorporated into cDNA during the reverse transcription reaction. A two-colour hybridisation strategy was used in which the cDNA from two different samples (isogenic wild type and mutant strains) was labelled with two different fluorescent dyes, Cy3 and Cy5. The ratio of the two differentially labelled target signals on each microspot directly reveals whether the targets are present in different or similar concentration in the isogenic wild type and mutant strains. The microarray slides carried probes corresponding to 5800 yeast open reading frames. The microarray data were represented in a clustergram where green and red colours are indicative of mRNAs with reduced or increased abundance relative to the wild-type respectively (Figure 4.7). Only genes that showed significant alteration (> 2 fold) are listed.



Gene	Function	Cellular component
IDH2	Isocitrate dehydrogenase (NAD)activity, involved in TCA cycle	mitochondrion
VID24	Involved in negative regulation of gluconeogenesis and vesicle mediated transport	Cytoplasmic vesicle
ACO1	Aconitate hydratase activity involved in TCA cycle	Cytosol, mitochondrial matrix
SVS1	Molecular function unknown	vacuole
AQR1	Acid Quinine Resistance, involved in drug transport	Plasma membrane
CIT2	Citrate synthase , involved in TCA cycle and serine-isocitrate lyase pathway	Peroxisome
NCE103	Carbonic anhydrase- like protein, unknown function	Cytoplasm- nucleus
GYP7	Rab GTPase-activating protein, involved in vesicle mediated protein trafficking	Cytoplasm
YLR414C	Unknown protein, unknown function	Bud- cytoplasm
BAG7	GTPase activating ptotein, involved in small GTPase signal transduction activity	intracellular
CHA1	Serine and threonine ammonia lyase activity, involved in serine and threonine amino acid catabolism	mitochondrion
YPS3	Gpi-anchored aspartic protease, involved in protein metabolism	Plasma membrane
ISU1	Iron-sulfur cluster nifU-like protein, involved in iron ion homoestasis and iron-sulfur cluster assemble	mitochondrion
YNL208w	Unknown protein	unknown
PCA1	P-type cation-transporting ATPase, involved in resistance to copper ion toxicity	membrane
PTP2	Protein tyrosine phosphatase, involved in inactivation of MAPK, protein amino acid dephosphorylation…	nucleus

Figure 4.7. Clustergram analysis of *gbp2Δ*, *hrb1Δ* and *rrp6-Δ*. Genes that were upregulated in *hrb1*Δ and *gbp2*Δ are shown in pink. Genes that are upregulated in *rrp6*Δ and *hrb1*Δ are shown in blue. The table summarises the function and localisation of the proteins encoded by theses genes.

As expected, both *GBP2* and *HRB1* mRNA were reduced in abundance in the *gbp2*Δ and *hrb1*Δ mutant strains (indicated by arrows). Strains lacking Gbp2p or Hrb1p over-express mRNAs for enzymes that function in the tricarboxylic acid cycle, TCA (*IDH2*, *ACO1* and *CIT2*). The TCA cycle is a central pathway of oxidative metabolism. Enzymes of the TCA cycle are important for biosynthetic processes including gluconeogenesis and amino acid and heme biosynthesis (Przybyla-Zawislak et al., 1999). Many of the TCA cycle enzymes are not required for growth on fermentable carbon sources such as glucose. In the presence of rapidly fermentable sugars, glycolysis is activated and the synthesis of these enzymes and gluconeogenesis are repressed (Rolland et al., 2002). Previous studies have shown the abundance of *HRB1* mRNA increases during glucose repression (Planta et al., 1999). Thus, it is possible that Gbp2p and Hrb1p are involved in the regulation of the TCA cycle enzymes in response to the available carbon source.

In addition, analysis of the microarray data revealed a small number of genes that are over-expressed in both *hrb1*Δ and *rrp6*Δ (Figure 4.7, table). These genes appear to encode a variety of proteins, which include a GTPase, an ATPase, a citrate synthase, a tyrosine phosphatase and others (Figure 4.7, table). The citrate synthase protein functions in the tricarboxylic acid cycle (TCA) that is activated during aerobic respiration when fermentable carbon sources are unavailable. *CIT2* mRNA was the only mRNA found to be over-expressed in all three mutants *gbp2*Δ, *hrb1*Δ and *rrp6*Δ.

IV.F. Discussion

IV.F.1. A role for Rrp6p in NMD?

The NMD factor Upf2p was identified by mass spectrometry as part of the 10S Rrp6p-associated complex (complex A) seen on glycerol gradients. Upf1p, Upf2p and Upf3p

form "the surveillance complex" required for the degradation of transcripts containing premature stop codons (PTCs) (see chapter 1, section I.C.4). Although Upf1 is cytoplasmic and associated with polysomes, Upf2p localises to the perinuclear region and Upf3p shuttles between the nucleus and the cytoplasm (Wilusz et al., 2001). In yeast, NMD is triggered by the binding of the hnRNP-like protein Hrp1p to the mRNA at a downstream sequence element (DSE). A model was proposed in which the translating ribosomes halt at a premature termination codon and therefore fail to displace Hrp1p from the DSE. The surveillance complex recognises the bound Hrp1p and triggers degradation of the transcript (Gonzalez et al., 2001).

Recent studies have provided evidence for the existence of an NMD pathway in yeast involving accelerated deadenylation followed by 3'-5' degradation by the exosome (Mitchell et al., 2003b). Furthermore, in mammalian cells 3'-5' NMD was shown to take place in both the nucleus and the cytoplasm and to involve the human homologue of Rrp6p (PM-Scl100) (Lejeune et al., 2003). Upf NMD factors coimmunopurified not only with PM-Scl100 but also with the decapping enzyme Dcp2p, the deadenylase PARN and the 5'-3' exoribonucleases Rat1p and Xrn1p. NMD was shown to degrade mRNA from both 5' and 3' ends by recruiting decapping and 5'-3' exonuclease activities as well as deadenylating and 3'-5' exonuclease activities (Lejeune et al., 2003). This same study demonstrated the localisation of human Rrp6p to nuclear and cytoplasmic compartments of the mammalian cell in contrast to its yeast counterpart, which is confined to the nucleus.

Aberrant mRNAs, including unspliced pre-mRNAs that escape to the cytoplasm, are rapidly degraded by NMD. The accumulation of unspliced *CYH2* pre-mRNA in strains that lack Rrp6p suggests that this PTC-containing RNA is degraded in both cytoplasm and nucleus. The degradation of the *CYH2* RNAs in the nucleus could either involve a novel Rrp6p decay pathway or a nuclear NMD pathway. Rrp6p is involved in nuclear NMD in the nuclei of mammalian cells, but, whether NMD occurs in the nucleus of yeast cells is still not known (Lejeune et al., 2003). Furthermore, the levels of the mature *CYH2* mRNAs were mildly elevated in *rrp6*Δ indicating that there is no defect in splicing. The existence of a nuclear surveillance pathway that degrades unspliced pre-

mRNAs was first demonstrated from studies of strains carrying a mutation in the splicing factor Prp2p (Bousquet-Antonelli et al., 2000). Mutations in the exosome components Rrp41p, Mtr3p, Rrp44p and Rrp6p inhibited pre-mRNA degradation in the splicing mutant *prp2-1* resulting in the accumulation of unspliced pre-mRNAs. In some cases, the inhibition of pre-mRNA degradation increased the accumulation of spliced mRNA suggesting that it competes with the splicing machinery. Competition between splicing and pre-mRNA degradation may be a normal feature of gene expression (Bousquet-Antonelli et al., 2000). Thus, in the case of *CYH2* mRNA, degradation of the pre-mRNA by an Rrp6p-dependent pathway could be competing with the synthesis of the mature RNA.

However, the absence of the exosome cofactor Rrp47p or the core exosome component Ski6p (Rrp41p) did not have the same effect on levels of *CYH2* pre- and mRNAs. Rrp47p is a non-essential cofactor that associates specifically with the nuclear exosome, this protein will be the subject of discussion in the following chapter. Studies have shown that Rrp47p is not required for nuclear mRNA surveillance. This is in agreement with the non-significant alteration in the level of *CYH2* pre/mRNA observed in the absence of this Rrp47p. However, inhibition of *CYH2* pre-mRNA degradation was not observed in the *ski6-100* mutant suggesting that this is another function of Rrp6p that is independent of the exosome. Thus, it could be that Rrp6p through its interaction with Upf2p ensures that mRNAs do not contain PTCs before being exported to the cytoplasm.

The Mss116p that copurified with the Rrp6p-associated complex, is a mitochondrial protein involved in the splicing of mitochondrial introns. The protein is encoded by a nuclear gene and presumably imported into the mitochondria where it is involved in splicing (Niemer et al., 1995). The sequence of the *MSS116* shows a high degree of sequence homology to the DEAD box family of proteins, which share the highly conserved motif Asp-Glu-Asp, together with six other conserved elements. Members of the DEAD box family were shown to participate in a variety of RNA-associated functions including translation initiation, splicing and ribosome assembly. In addition, some members of this family were shown to have an ATP-dependent RNA unwinding

activity (Niemer et al., 1995). Thus, it is possible that Mss116p functions as a cofactor assisting Rrp6p in the degradation of aberrant transcripts by unwinding RNA structure similar to the Mtr4p component of the nuclear exosome, which is a putative RNA helicase (de la Cruz et al., 1999). Interestingly, systematic mass spectrometry revealed the association of Mss116p with many pre-60S ribosome factors including Nog1p, Ebp2p, Nop2p, Nop4p and others. This suggests that Mss116p could be a component of the Rrp6p complex C, which is predicted to be part of the pre-60S ribosomal particle (discussed in the previous chapter).

In addition to Nmd2p and Mss16p, Rev3p was also identified by mass spectrometry in the Rrp6p-associated complex (complex A). Rev3p is involved in translesion synthesis in damage tolerance pathways and in double-strand break repair via homologous recombination (Baynton et al., 1998). The reason for the association of this protein with Rrp6p is not very clear however a recently characterized exosome cofactor Rrp47p (discussed in chapter 5) was also found to function in the repair of DNA double-strand breaks. Thus, it is possible that the Rev3p protein possesses a novel uncharacterised function in RNA metabolism. Alternatively, Rrp6p may also play a role in DNA repair. It may be relevant that the exonuclease domain of Rrp6p has clear homology to the 3'-5' exonuclease, proofreading domain of DNA polymerase I (Mian, 1997).

IV.F.2 Srp1p and Gbp2p are two novel Rrp6p-associated proteins

The identification of two further putative Rrp6p-associated proteins, Srp1p and Gbp2p, is reported in this chapter. As expected Rrp6p co-purified with the exosome components Rrp44p, Rrp4p and Csl4p.

Srp1p is the yeast homologue of importin α. Many nuclear (karyophilic) proteins contain short stretches of amino acids known as nuclear localisation signals (NLSs) that mediate its transport through the nuclear pore complex and into the nucleus. Srp1p was shown to bind to NLSs and to form a heterodimer with Kap95p (Importin β), which interacts with the nuclear pore complex facilitating the transport (Tabb et al., 2000). In

addition to proteins, yeast export substrates include tRNAs, rRNAs and mRNAs. However, signals for the export of the RNA (NES, nuclear export signal) are thought to be present in proteins interacting with the RNA rather than within the RNA itself (Jensen et al., 2001). Srp1p was also shown to participate in a variety of nuclear function including mitosis and protein degradation through the ubiquitin-proteosome system (Tabb et al., 2000). The association of Srp1p and Kap95p with Rrp6p and other exosome subunits (Csl4p, Rrp45p and Rrp46p) was recently independently reported (Gavin et al., 2002;Peng et al., 2003). This, therefore, appears to be a genuine interaction. However, analyses of *srp1* temperature-sensitive alleles did not reveal a phenotype in stable RNA synthesis resembling that of *rrp6*Δ mutants. It is, however, notable that the cause of lethality in *rrp6*Δ strains remains unclear, indicating that Rrp6p has substrates that remain to be identified. Moreover, Srp1p might be involved in the import of Rrp6p into the nucleus.

Gbp2p belong to a family of proteins referred to as heterogeneous nuclear ribonucleoproteins hnRNPs that bind pre-mRNA during the series of processing events that take pace in the nucleus before its travelling to the cytoplasm (Lee and Silver, 1997). Furthermore, Gbp2p has recently been shown to associate with the THO/TREX complex, a conserved multimeric complex that consists of several transcription and export factors (Chavez et al., 2000;Strasser et al., 2002). This complex includes the hnRNP protein Hrp1p, the splicing factor Sub2p and the mRNA export factor Yra1p, which binds Mex67p, a major exporter for mRNA. The existence of this complex is thought to evidence for the coupling of transcription, splicing and export processes. Gbp2p was recently characterised as a novel RNA-binding protein involved in the export of poly(A)$^+$ mRNA to the cytoplasm (Windgassen and Krebber, 2003). The recycling of the protein back into the nucleus was found to be mediated by the receptor Mtr10p and depends upon the phosphorylation of the Gbp2p by Sky1 kinase (Windgassen and Krebber, 2003).

Furthermore, Rrp6p was previously reported to interact with another shuttling RNA-binding protein Npl3p implicated in pre-mRNA processing and mRNA nuclear export although Npl3p was not identified in association with Rrp6p in our analysis (Burkard

and Butler, 2000). Npl3p shares 27% identity with Gbp2p with the first two RRMs in Gbp2p being conserved in Npl3p, whereas the third RRM is less conserved. In addition the N-terminus of Gbp2p shows similarity to the C-terminus of Npl3p in that it contains RGG repeat motifs. The export of both Npl3p and Gbp2p appears to depend on ongoing transcription providing further evidence for the coupling of these nuclear events (Windgassen and Krebber, 2003;Burkard and Butler, 2000). Also similar to Gbp2p, Npl3p is phosphorylated by Sky1p and is imported into the nucleus bound to the import receptor Mtr10p. Mutations in Npl3p and the over-expression of Gbp2p result in the accumulation of poly(A)$^+$ mRNA in the nucleus. Recent studies have revealed that Npl3p interacts with Yra1p that acts at a later stage in mRNA export. In turn Yra1 interacts with Sub2p, yet another member of the THO/TREX complex. Yra1p is an RNA annealing protein that associates with chromatin in a transcription-dependent manner (lei et al., 2001). Mutations in the components of the THO complex (*yra1, sub2* or *hpr1*) result in nuclear retention and decay of transcripts. However, deletion of Rrp6p in any of these mutants alleviated the nuclear accumulation of pre-mRNAs (Libri et al., 2002;Zenklusen et al, 2002). These findings provide further evidence for the interaction of Rrp6p with the export and transcription machineries.

Hrb1p is a novel shuttling hnRNP protein, that was shown to require ongoing RNA polymerase II for its export but did not bind poly(A)$^+$ mRNA in a cross-linking experiment (Shen et al., 1998). Hrb1p shares 39% identity to Gbp2p and similar to Gbp2p interacts with Mtr10p. Furthermore, a systematic mass spectrometric analysis showed that Hrb1p, Gbp2p, Npl3p and Ctk1p (the catalytic subunit of a kinase that phosphorylates the CTD) form a complex known as Ctk1-associated complex (Gavin et al., 2002).

In the nucleus, hnRNPs and other RNA-binding proteins package mRNAs into ribonucleoprotein particles. Some of these proteins remain in the nucleus whereas others are co-transported to the cytoplasm with the mRNP where they dissociate from the mRNP and move back to the nucleus for further rounds of export. By interacting with the hnRNPs Gbp2p and Npl3p and possibly Hrb1p, which are involved in the export of poly(A)$^+$ mRNAs, Rrp6p in conjunction with other proteins, monitors the quality of the

mRNP such that only export competent mRNPs are transported to the cytoplasm. Aberrant mRNPs are retained in the nucleus and targeted for degradation by the nuclear exosome in a 3'-5' direction. The role of Srp1p would probably be in the transport of hnRNPs or other proteins back into the nucleus.

A recent study in *Drosophila* has revealed that the nuclear exosome associates with the elongation factor Spt6p and with elongating RNA polymerase II (Andrulis et al., 2002). This is consistent with the requirement of ongoing RNA polymerase II transcription for the export of Gbp2p, Hrb1p and Npl3p (Windgassen and Krebber, 2003;Burkard and Butler, 2000). Thus, the nuclear events of transcription, processing and export appear to be also coupled to a surveillance mechanism that ensures that the quality of the transcript is monitored at each step.

The abundance of mRNAs encoding TCA cycle enzymes (*IDH2, ACO1* and *CIT2*) was shown to be increased in *hrb1*Δ and *gbp2*Δ strains and *CIT2* was also elevated in *rrp6*Δ. High levels of TCA enzymes are not required during growth on fermentable carbon sources such as glucose but become more important when non-fermentable carbon sources such as ethanol, acetate (or others) must be metabolised (Rolland et al., 2002). Previous studies have shown that the degradation of nuclear pre-mRNA is regulated by the availability of glucose, the preferred carbon source for yeast (Torchet et al., 2002). Degradation of defective pre-mRNAs is favoured during growth on glucose-containing media whereas processing to functional mRNAs is favoured during growth on other carbon sources. Evidence for such regulation comes from studies of *rna14.1* mutants that result in the accumulation of 3'-extended pre-mRNAs due to a defect in cleavage and transcription termination. On glucose medium, these extended species are targeted for complete degradation by the exosome. However, on galactose medium or in the absence of Rrp6p, the products of the initial exosome degradation are stabilised and undergo polyadenylation (Torchet et al., 2002).

We propose that in wild type cells and during growth on glucose increased nuclear degradation limits the level of mRNAs encoding TCA cycle enzymes. However, when cells are grown on non-fermentable carbon sources, nuclear degradation is slowed-down allowing the expression of enzymes required in the TCA cycle.

All steps in gene expression are utilised for regulation for at least some genes. It therefore seems possible that a class of mRNAs would exist for which the expression levels would be potentially regulated by the activity of Rrp6p and nuclear degradation.

Chapter Five

Rrp47p is a novel exosome-associated cofactor

V.A. Introduction

How the different exonucleases are organised within the exosome and how they are activated is still not fully understood. A related issue is how the variety of RNA substrates are recognised by the exosome and targeted to either full degradation or precise 3' end processing. Genetic studies indicate that exosome function in vivo requires the activity of additional cofactors present only at substoichiometric levels and/or weakly associated with the exosome. In the nucleus, the activity of the exosome was shown to require the putative RNA helicase Mtr4p/Dob1p (Liang et al., 1996;de la Cruz et al., 1998). In the cytoplasm, the exosome mediated 3'-5' mRNA turnover pathway depends upon Ski7p and the Ski complex comprising the putative RNA helicase Ski2p and the proteins Ski3p and Ski8p (Anderson and Parker, 1998;Brown et al., 2000;van Hoof et al., 2000c).

To identify substoichiometric proteins associated with the exosome, purification of exosome complexes from whole-cell lysates was carried out by Dr Philip Mitchell. This led to the identification of Yhr081p (designated Rrp47p), which showed sub-stoichiometric Mg^{++}-labile association with the exosome (Mitchell et al., 2003a, attached paper). The localisation of Rrp47p to the nucleus and its association with the exosome prompted us to study its possible involvement in the processing and degradation activities of the nuclear exosome.

V.B. Immunoprecipitation of epitope-tagged Rrp47p confirms its association with the exosome

To identify novel exosome cofactors, affinity chromatography analysis was performed on cell lysate from a strain expressing an epitope-tagged form of the exosome component Rrp44p (zz-Rrp44p), which contains two copies of the "z" domain of protein A fused to its N terminus. Cell lysate from the zz-Rrp44p strain was passed over an IgG-Sepharose column and associated proteins were eluted in a gradient of 0 to 2 M $MgCl_2$, resolved by SDS-PAGE and identified by mass spectrometry. A protein of ~25kDa that dissociated from zz-Rrp4p at ~0.2 to 0.4 M $MgCl_2$ was recovered in the eluate fraction at substoichiometric levels as judged by visual inspection of Coomassie-stained gels (Mitchell et al., 2003a, Figure 1A). Mass spectrometric analysis identified this protein as the product of the *YHR081w* gene. This protein was designated Rrp47p on the basis of its role in rRNA processing (see below) and its copurification with the exosome complex. The dissociation of Rrp47p from the exosome in the presence of relatively low $MgCl_2$ concentration and its stoichiometry indicated that it is not a core exosome component.

To confirm the interaction between Rrp47p and the exosome, a strain was constructed that expressed a C-terminal fusion protein linked by a TEV protease cleavage site (Rrp47p-zz). This protein was expressed from the *RRP47* locus in the chromosome under the control of the endogenous promoter. Following immunoprecipitation, proteins that co-purified with Rrp47p-zz were analysed by Western blotting using an anti-Rrp4p antiserum (Figure 5.1, lanes 3 and 4). Lysate from a strain expressing TAP-tagged Rrp44p was also analysed as a positive control (Figure 5.1, lane 2). Rrp4p was recovered from both the Rrp47p-zz and Rrp44p-TAP columns but was not detected in a mock precipitate from the wild type strain (Figure 5.1, lane 1), confirming the interaction between Rrp47p and Rrp4p.

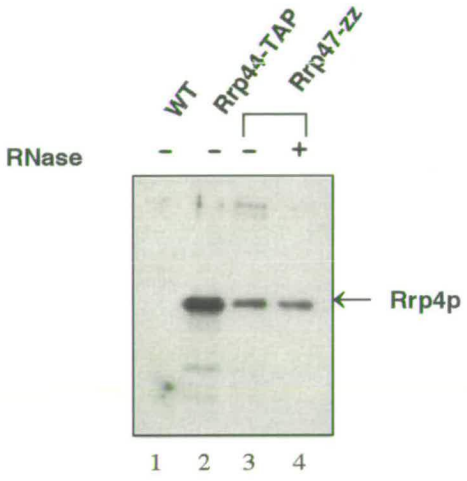


Figure 5.1. Rp47p is a novel exosome-associated cofactor.
Rrp47p is associated with Rrp4p. Western blot analyses of immunoprecipitates from wild-type (lane 1), Rrp44p-TAP (lane 2) and Rrp47-zz (lane 3 to 4) strains, with antisera specific to Rrp4p was done. Rrp47-zz immunoprecipitates were eluted with or without prior digestion with RNase A.

To determine whether Rrp47p is associated indirectly with the exosome via a ribonucleoprotein complex, lysate from Rrp47-zz strain was treated with RNase A at 2μg ml^{-1} and incubated on ice for 1 hour prior to passing over an IgG-Sepharose column. However, Rrp4p was detected at comparable levels with or without treatment indicating that no RNA species is required for its co-purification with Rrp47p (Figure 5.1, lanes 3 and 4). The immunoprecipitation analyses shown were performed in buffer containing 0.5M NaCl and the coimmunoprecipitation of Rrp47p with Rrp4p therefore reflects a stable interaction.

V.C. Rrp47p and Rrp6p play related roles in the processing of stable RNAs

The nuclear exosome plays an essential role in the 3' processing of 5.8S rRNA, snRNAs and snoRNAs and in the degradation of the 5' external transcribed spacer (5'ETS).
The work presented in this chapter and in the attached paper aimed at investigating the role of Rrp47p in rRNA, snRNA and snoRNA processing and degradation of 5'ETS. In addition, the role played by Rrp47p in each of these processes was compared to that of the nuclear exosome component Rrp6p.

V.C.1. Depletion of Rrp47p results in the accumulation of the 5.8S+30 rRNA intermediate seen in rrp6Δ

To determine whether Rrp47p is involved in the processing in 5.8S rRNA, Northern hybridisation analysis was carried out on RNA isolated from the isogenic wild type, from *rrp47Δ* and *rrp6Δ* during growth at 23°C and after 1 hour shift to 37°C (Figure 5.2). The 1hr shift to 37°C was chosen based on previous studies of exosome mutants (Mitchell et al., 1996;Allmang et al., 2000). Northern blot hybridisation with probes

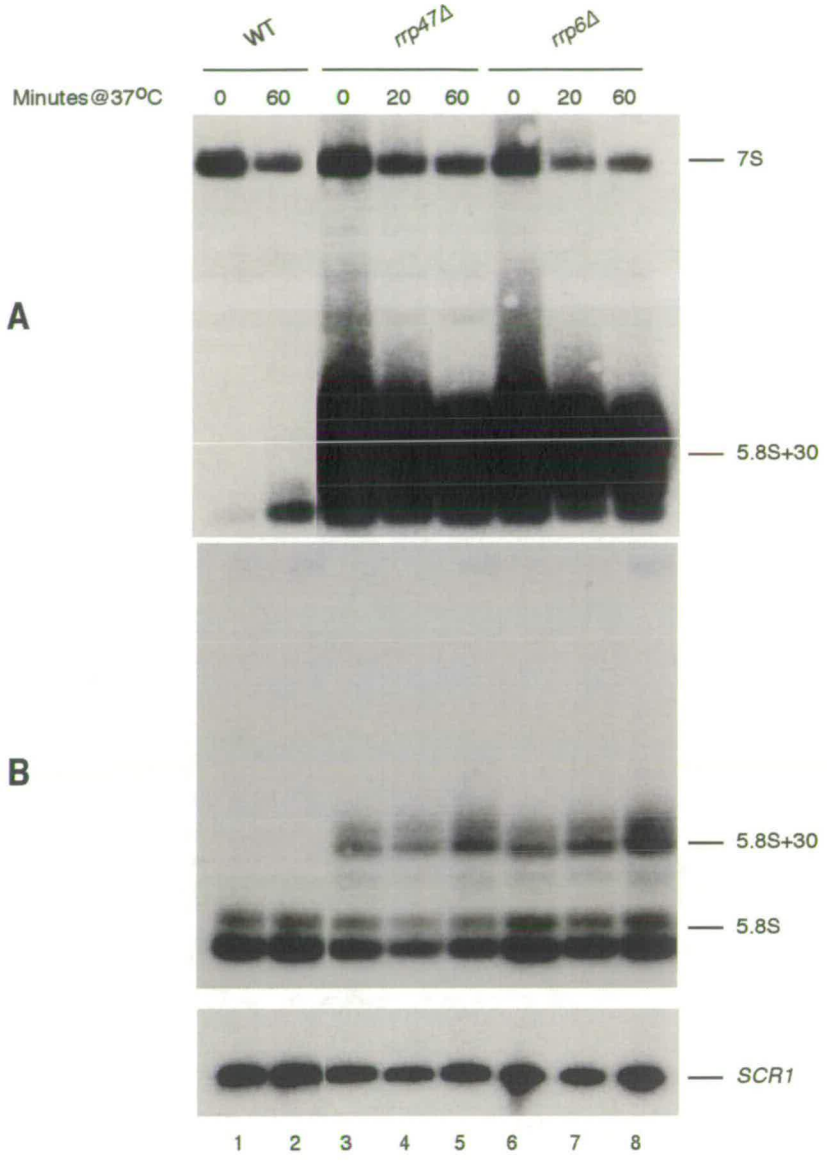


Figure 5.2. Rrp47p and Rrp6p play similar roles in the processing of 7S rRNA. Northern analysis of wild-type (WT), *rrp6*Δ and *rrp47*Δ strains. RNA was extracted from the WT, *rrp6*Δ and *rrp47*Δ strains on glucose medium at 23°C and following transfer to 37°C for 20 and 60 minutes. for each panel 7ug of RNA was seperated on a 6% polyacrylamide gel.(A) Hybridisation with a probe specific to the 5.8S species extended into ITS2 (probe number). (B) Hybridisation with a probe specific for the mature 5.8S rRNA,probe number 17.

complementary to the mature 5.8S rRNA and to the 5.8S-ITS2 boundary (Chapter1, Figure 1.3) hybridised to the same 5.8S+30 species in the *rrp47Δ* and *rrp6Δ* mutants (Figure 5.2A and B, lanes 3 to 8). This defect is different from that seen in core exosome mutants like Rrp4p, which characterised by the accumulation of longer 3'extended 5.8S species (Mitchell et al., 2003a; attached paper).

The role of Rrp47p in the processing of 5.8S therefore appears to resemble closely that of Rrp6p rather than the core exosome components.

V.C.2. rrp47Δ strains show a defect in the final 3'-end trimming of snoRNAs

Mutations in the exosome complex cause the accumulation of 3'-extended and polyadenylated snoRNA precursors (Allmang et al., 1999). In addition the *rrp6* mutation, but not mutations for core exosome components, lead to most box C/D snoRNA (e.g. U18) retaining 3 untrimmed nucleotides at their 3'-ends.

To address the role of Rrp47p in the 3' end formation of snoRNAs, Northern hybridisation analysis was performed on RNA isolated from the isogenic wild type and from *rrp47Δ* and *rrp6Δ* mutants (Figure 5.3). As previously reported, increased levels of precursors to U18 snoRNA, which have mature 5' ends but are 3' unprocessed were observed in the *rrp6Δ* strain (labelled U18-3', lanes 1 to 3). A very similar pattern of 3'-extended U18 precursors was observed in the *rrp47Δ* mutant strain (lanes 4 to 6). These 3'-extended species were shown to be polyadenylated in the *rrp6Δ* mutant yielding diffuse hybridisation signals in the region of some bands (van Hoof et al., 2000b). The similar hybridisation pattern observed in the *rrp47Δ* and *rrp6Δ* mutants strongly indicate that the extended species observed in the *rrp47Δ* are also polyadenylated. In the *rrp6Δ* mutant, mature U18 snoRNAs retained discrete 3' extensions of approximately 3 nt (indicated as U18+3) (lanes 1 to 3). However, in the *rrp47Δ* mutant the U18+3 species appeared to be slightly shorter after transfer to 37°C (lanes 4 to 6).

The increased levels of 3'-extended snoRNAs precursors was observed in the *rrp47Δ* mutant for all tested snoRNAs. In addition, other tested box C/D snoRNAs (snR13, U14, and snR38), were shown to retain 3'-extensions of ~ 3 nt (Mitchell et al., 2003a).

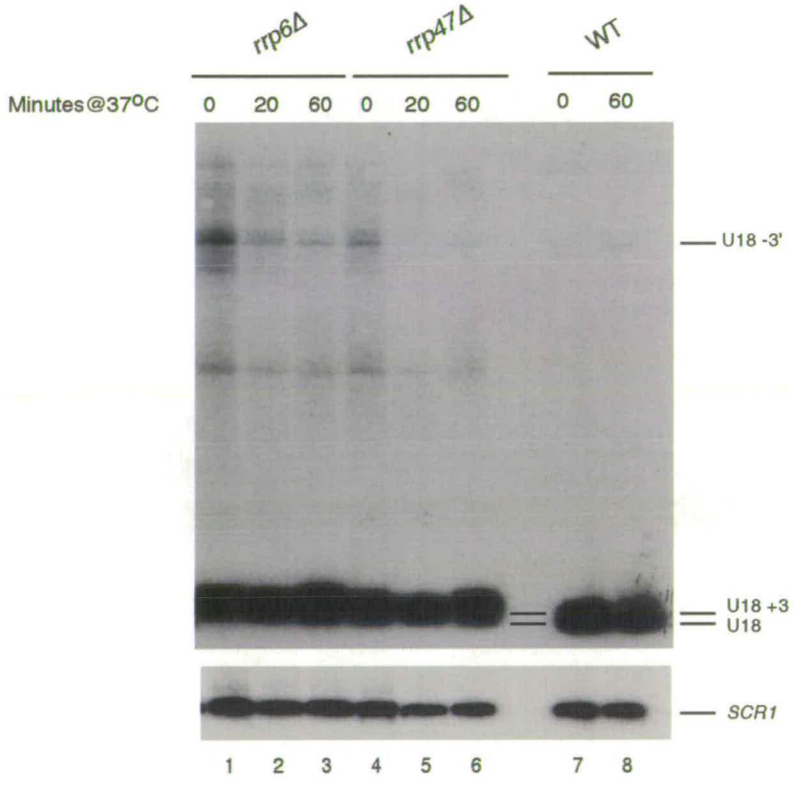


Figure 5.3. snoRNA processing defects in *rrp47Δ* resemble those observed in rrp*6Δ*

Northern analysis of wild-type (WT), *rrp6*Δ and *rrp47*Δ strains. RNA was extracted from the WT, *rrp6*Δ and *rrp47*Δ strains on glucose medium at 23°C and following transfer to 37°C for 20 and 60 minutes. for each panel 7ug of RNA was seperated on a 6% polyacrylamide gel and hybridised with U18 probe, number (see chapter 2).

Thus it can be concluded that Rrp6p and Rrp47p play similar roles in the initial 3'processing of pre-snoRNAs. The lack of either Rrp6p or Rrp47p causes delayed 3' processing from the initial cleavage but a complete block to the final trimming of the last few nucleotides. However, the lack of Rrp47p appears to have milder effects on the trimming of the last few nucleotides than the absence of Rrp6p.

V.D. Rrp47p is not required for the expression of Rrp6p or its association with the exosome

The *RRP47* and the *RRP6* genes are known to be nonessential for cell viability. Furthermore, similar to *rrp6*Δ, *rrp47*Δ mutants were shown to be defective in growth at all temperatures and temperature-sensitive lethal. The functional similarities between these two proteins led us to investigate the interdependence of their association with the exosome and whether Rrp47p associates with Rrp6p in a complex distinct from the exosome.

V.D.1. Rrp47p and Rrp6p associate with the same exosome fraction

The precipitation data showed that Rrp47p and Rrp6p were each associated with subpopulations of the exosome but did not reveal whether they are both associated with the same complex. To assess whether Rrp47p and Rrp6p are associated with the same exosome fraction, immunoprecipitation from Rrp47-zz lysates was performed and assayed by Western blotting with anti-Rrp6p antiserum (Figure 5.4). Cleaved Rrp6p-TAP was used as a positive control in the western analysis. A single band of the size of Rrp6p was detected in the Rrp47-zz immunoprecipitates indicating that Rrp47p and Rrp6p associate with the same exosome fraction.

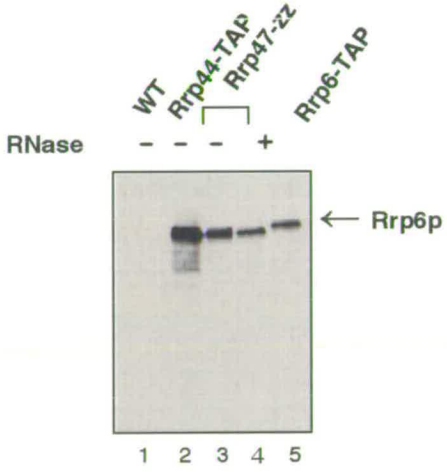


Figure 5.4. Rp47p and Rrp6p associate with the same exosome fraction.

Western blot analyses of immunoprecipitates from wild-type (lane 1), Rrp44p-TAP (lane 2) and Rrp47-zz (lane 3 to 4) strains, with antisera specific to Rrp6p was done. Rrp47-zz immunoprecipitates were eluted with or without prior digestion with RNase A. Cleaved Rrp6p-TAP was included as a positive control for the Rrp6p Western analysis (lane 5). This protein is predicted to migrate slower than the endogenous Rrp6p in SDS-PAGE gels due to the presence of the calmodulin-binding domain.

Rrp6p was previously shown to copurify with the exosome at substoichiometric levels as judged by visual inspection of Coomassie-stained gels (Allmang et al, 1999b). To compare the relative abundances of Rrp6p, Rrp47p, Rrp4p and Rrp44p, Western blot analysis was performed on whole lysates from the strains carrying protein A epitope-tagged versions of the proteins (Figure 5.5). The blot was decorated with horseradish peroxidase anti-peroxidase antibodies. As a loading control, the blot was stripped and reprobed with anti-Npl3p antibodies. Phosphoimager quantification of the bands relative to the loading control revealed that there is 2-fold more Rrp44p and Rrp4p than there is Rrp6p. These results are in agreement with a recent systematic, high-throughput study that evaluated the abundance of each of the exosome subunits by measuring the number of molecules per cell of each of the exosome components (Ghaemmaghami et al., 2003). Earlier studies have shown that Rrp6p associates with 10-20% of the purified exosome. Thus if total Rrp6p represents 50% of total Rrp4p, 60-80% of total Rrp6p does not appear to be associated with the exosome. This is consistent with the results presented in chapter 3, which suggested that Rrp6p associates with complexes distinct from the exosome. Moreover, the levels of Rrp47p were ~ 5-fold lower than any of the other tested exosome components and appeared to be substoichiometric to Rrp6p. By visual inspection, the band intensity of Rrp47p is similar to that previously seen in exosome purification where Rrp47p was predicted to associate with 10-20% of the purified exosome (Mitchell et al., 2003). A recent study performed with TAP purified Rrp6p showed that Rrp47p is less abundant than Rrp6p (Peng et al., 2003). In addition, the number of Rrp47p molecules per cell could not be detected in a systematic high-throughput study indicating its very low abundance relative to other exosome components (Ghaemmaghami et al., 2003).

Thus, from the stoichiometric studies, it can be concluded that the Rrp47p population is largely exosome-associated.

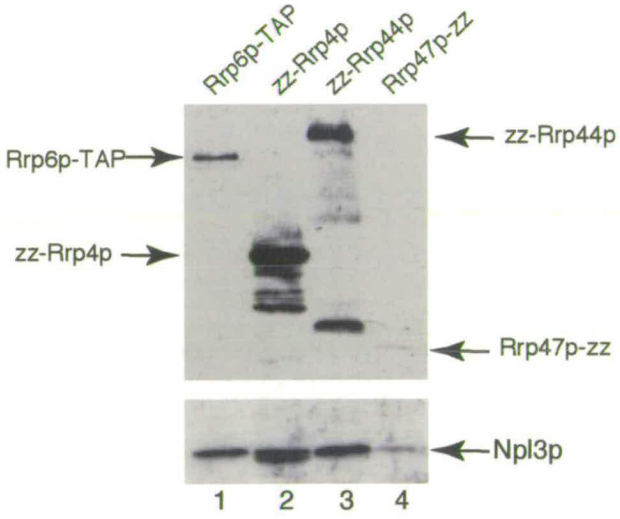


Figure 5.5. Comparison of the relative abundances of Rrp6p-TAP, zz-Rrp4p, zz-Rrp44p and Rrp47p-zz within the exosome. Lysates from Rrp6p-TAP, zz-Rrp4p, zz-Rrp44p and zz-Rrp47p were resolved on a 8% polyacrylamide gel. Proteins were detected by Western blot analysis using the peroxidase anti-peroxidase antibody. As a loading control the blot was stripped and rehybridised with anti-Npl3p antibodies (Chapter 2, section II.H).

V.D.2. Rrp47p is not required for the expression of Rrp6p or its association with the exosome

To determine if the expression of Rrp6p is affected by the absence of Rrp47p, western blot analysis was performed on lysates extracted from wild-type, *rrp6*Δ and *rrp7*Δ strains. The blot was decorated with the anti-Rrp6p antibodies (Figure 5.6). Rrp6p were not significantly affected in the absence of Rrp47p indicating that Rrp47p is not required for the expression of Rrp6p (lanes 1 and 3).

To analyse the requirement for Rrp47p in the association of Rrp6p with the exosome, immunoprecipitation was performed on lysates from strains expressing zz-Rrp4p combined with a deletion of Rrp47p. The proteins that co-purified with the zz-Rrp4p/*rrp47*Δ or zz-Rrp4p strains were analysed by Western blotting with an anti-Rrp6p antiserum (Figure 5.7). The coimunoprecipitation of Rrp6p with zz-Rrp4p was unaffected by the absence of Rrp47p (lanes 5 and 6). It should be noted that comparisons of lanes 1 to 2 with lanes 3 to 4 shows that Rrp6p is more efficiently depleted with zz-Rrp4p. However, this difference is not reflected in the levels of Rrp6p detected in eluates from zz-Rrp4p and zz-Rrp4p/rrp47Δ strains. This may be explained by protein degradation of the unbound fraction during the incubation of the lysate, and was not seen in a duplicate experiment.

We conclude that Rrp47p is not required for the expression of Rrp6p or its association with the exosome.

V.E. Rrp47p does not clearly cosediment with either Rrp6p or Rrp4p

To investigate the possibility that Rrp47p is associated with Rrp6p in a complex distinct from the exosome, lysates isolated from Rrp47p-zz strain were fractionated by glycerol

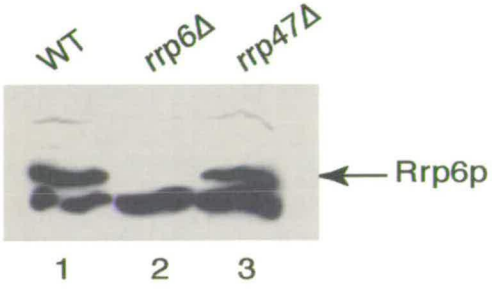


Figure 5.6. Rrp47p is not required for the expression of Rrp6p. Western blot analysis of cell extracts from wild type, *rrp6*Δ and *rrp47*Δ strains with anti-Rrp6p antibodies. Equal amount of protein were loaded in each lanes as estimated from the Bradford protein assay.

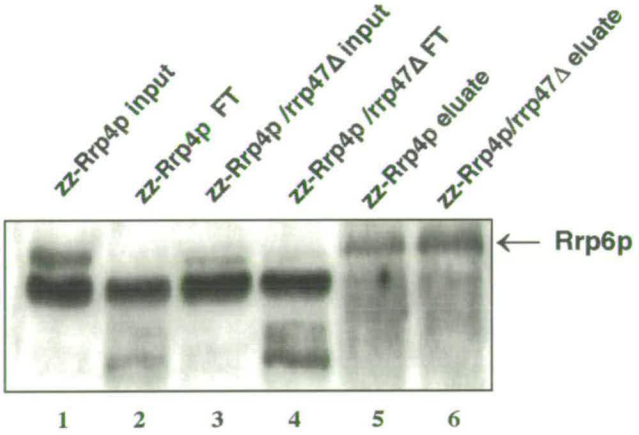


Figure 5.7. Rrp47p is not required for the association of Rrp6p with the exosome. immunoprecipitates from zz-Rrp4p (lane 5) and zz-Rrp4p/*rrp47*Δ (lane 6) strains were analysed by Western blotting with the Rrp6p antiserum. Input and flow-through (FT) lysates from both strains were also analysed (lanes 1 to 4).

gradient centrifugation (Chapter 2, section II.O.4). Fractions were collected and Rrp47p-zz, Rrp4p and Rrp6p were detected by Western blotting using PAP, anti-Rrp4p and anti-Rrp6p antibodies. Rrp47p was detected with a broad distribution and a peak at fraction 3 or 4 (Figure 5.8). This pattern of sedimentation suggests that it corresponds to free Rrp47p with the majority being concentrated in fractions 3 and 4. Although there is some Rrp47p cosedimenting with Rrp6p, the peak of Rrp47p did not correspond to that of Rrp6p. It should be noted that this gradient was centrifuged for less time than glycerol gradients shown in chapter 3. This explains the shift in the position of the Rrp6p and Rrp4p peaks on the gradient relative to what was seen in previous gradients.

Furthermore, little Rrp47p is seen sedimenting with the core exosome component Rrp4p (panel B, lane 14), although Rrp47p was purified with the exosome (Mitchell et al., 2003a). This result could be attributed to free Rrp47-zz dissociating from complexes during purification. In the Western blot analysis of the pellet fraction of the Rrp47-zz glycerol gradient, a significant amount of Rrp47p could be detected. Thus it is also possible that the Rrp47p-associated complex is itself bound to larger structures.

V.F. Discussion

An investigation into the role of the newly identified exosome-associated protein Rrp47p is reported in this chapter. The results show that Rrp47p can be isolated in association with the exosome complex and is required for the processing of stable RNAs. The RNA processing defects in the *rrp47Δ* resembled those previously observed in strains lacking Rrp6p. However, Rrp47p was not required for the association of Rrp6p with the exosome.

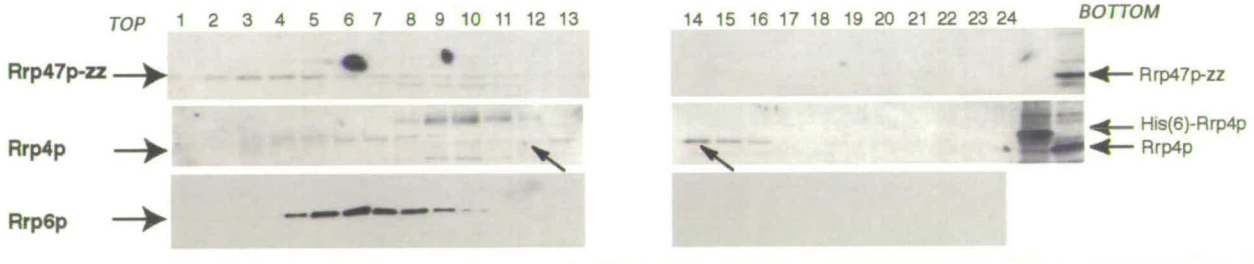


Figure 5.8 Rrp47p shows a broad distribution on Glycerol gradients. Glycerol gradient analysis of Rrp47p-zz. Lysate extracted from Rrp47-zz strain was fractionated through 10%- 30% glycerol density. Aliquots of each fraction were resolved by SDS-PAGE; Rrp47p-zz, Rrp4p and Rrp6p were detected by Western blot analysis with PAP, anti-Rrp4p and anti-Rrp6p antibodies. Cell extract from the Rrp47p strain was loaded as a control (Lane A).

As controls for the specificity of the Rrp4p antiserum, cell extracts were loaded from strains expressing wild-type Rrp4p (lane B) or epitope-tagged His(6)-Rrp4p (lane C).

V.F.1. Rrp47p is a substrate specific exosome cofactor

The exosome complex plays a key role in RNA 3' end maturation and degradation pathways. This dual nature of the exosome raises the important issue of how the exosome distinguishes between the RNA substrates destined for accurate 3' end processing and those targeted for complete degradation. It was suggested that the exosome differentiates between the various substrates based on cofactors that are predicted to associate with the exosome at substoichiometric levels. Purification of exosome complexes allowed the identification of a substoichiometric component, which was identified as the nuclear protein Yhr081p (Rrp47p). Rrp47p is the yeast homologue of human C1D, which was reported to be a nuclear matrix protein belonging to the family of non-histone polypeptides involved in higher order chromatin folding (Yavuzer et al., 1998;Erdemir et al., 2002). Recent evidence suggests that the nuclear matrix proteins are involved in important cellular processes, such as recombination, DNA repair, transcription regulation, translocation and apoptosis (Bode et al., 2000). Human C1D was shown to play an essential role in DNA double-strand repair and in V(D)J recombination, a process specific to thymocytes that is required for development of the immune system (Smith and Jackson, 1999). In addition, the mRNA and protein levels of C1D are induced in response to DNA damaging agents particularly those causing double-strand breaks (Yavuzer et al., 1998). C1D is highly conserved amongst various species and these include *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Drosophila melanogaster*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Analysis of the functions of yeast Rrp47p revealed that the protein is involved in nonhomologous end joining and homologous recombination, the two main pathways used to repair DNA double-strand breaks (Erdemir et al., 2002). Both nonhomologous end joining and homologous recombination events involve nucleotide removal from the free ends by exonucleases. Rrp6p belongs to the RNase D family of 3'-5' exonucleases, which is closely related to the proofreading domain of RNA polymerases (Moser et al., 1997).

Rrp47p may therefore play a role in the exonucleolytic activities required for both stable RNA processing and DNA repair.

Immunoprecipitation of epitope-tagged Rrp47p confirmed its association with both the exosome and the nuclear-specific exosome component Rrp6p. In addition, Rrp47p is likely to interact directly with the exosome, since this interaction was unaffected by RNase treatment. Although the majority of the Rrp47p population was predicted to be associated with the exosome, glycerol gradient analysis revealed a different distribution of the Rrp47p population. A large amount of free Rrp47p was observed and little Rrp47p could be detected sedimenting with the exosome. However, the copurification of Rrp47p-zz with Rrp4p was performed in buffer containing 0.5 M NaCl reflecting a stable interaction between Rrp47p and the exosome. The presence of the tag on the Rrp47p could be influencing the stability of the protein, not allowing it to fold into the proper conformation and thus affecting its association with the exosome complex. Moreover, the role played by Rrp47p in DNA repair suggests the presence of Rrp47p in complexes distinct from the exosome.

Northern analysis revealed that deletion of *RRP47* results in the accumulation of the 5.8S+30 pre-rRNA species observed in strains lacking Rrp6p but not other core exosome components. Furthermore, the snoRNA processing defects in the *rrp47Δ* mutant also resembled those previously observed in strains specifically lacking Rrp6p. The functional relationship between Rrp47p and Rrp6p was further analysed in the *rrp47Δ/rrp6Δ* double mutant. The double mutant was viable indicating that the proteins do not have redundant functions, and the RNA processing defects seen in the double mutant were similar to those observed in either single mutant (Mitchell et a., 2003a, refer to paper). These results and the fact that Rrp47p was not predicted to have exonuclease activity suggested that Rrp47p functions to promote Rrp6p function. Support for this Rrp47p function comes from studies that showed that the absence of Rrp47p did not significantly affect either the expression level of Rrp6p or its assembly into the exosome. The human C1D protein binds strongly to nucleic acids and therefore, Rrp47p might be playing a role in substrate recruitment and targeting to Rrp6p (Nehls et al., 1998).

Not all nuclear functions of Rrp6p required Rrp47p. In particular, Rrp47p was not required for Rrp6p-dependent degradation of 3'-extended nuclear pre-mRNAs seen in the *rna14.1* mutant (Torchet et al., 2002;Mitchell et al., 2003a). However, we cannot rule out the possibility of Rrp47p being associated with Rrp6p-associated complex A (discussed in Chapter 3), which was predicted to comprise proteins involved in the pre-mRNA surveillance activity of Rrp6p. Rrp47p copurified with the exosome but could not be clearly detected associating with the exosome on glycerol density gradients. This suggests that Rrp47p could be associating with Rrp6p complex A although this could not be observed in the sedimentation pattern of Rrp47p on density gradients.

In a two-hybrid assay analysis carried out in collaboration with Hybergenics SA (Paris), Rrp47p was shown to interact with the proteins Naf1p, Nrd1p, Tif4631p, Tif4632p. Naf1p is a nuclear protein that is recruited to the CTD of RNA polymerase II and promotes snoRNP assembly by binding to nascent H/ACA snoRNAs (Fatica et al., 2002). Nrd1p is also a nuclear RNA-binding protein that was shown to bind to nascent snoRNA and snRNA transcripts and promote transcription termination via interactions with the C-terminal domain of RNA polymerase II (Steinmetz et al., 2001;Yuryev et al., 1996;Conrad et al., 2000). The function of Nrd1p will be further discussed in Chapter 6. The interaction of Rrp47p with Naf1p and Nrd1p is in agreement with Rrp47p being also participating in an aspect of snoRNA synthesis. Tif463p and Tif4362p are the yeast homologs of the translation initiation factor eIF4G (Goyer et al., 1993). eIF4G is a member of the translation initiation complex eIF4F which also comprises the cap-binding protein eIF4E and the DEAD box helicase eIF4A (Gingras et al., 1999). Although the translation initiation complex is localised to the cytoplasm, recent studies have revealed the presence of a nuclear pool of eIF4G (Yiota Kafasla, unpublished data). Experiments are being carried out by other members of the Tollervey lab, to gain more insights on the role played by eIF4G in the nucleus.

Chapter Six

The control of gene expression at the level of nuclear turnover

VI.A Introduction

The nuclear degradation machinery has been best characterised in budding yeast where the predominant nuclear turnover pathway is 3'-5' and requires the activity of the exosome (Bousquet-Antonelli et al., 2000). The first evidence for a nuclear turnover pathway came from the identification of *rrp6* mutations as suppressors of a conditional defect in poly(A) polymerase, *pap1-1* that results in decreased level of poly(A)$^+$ mRNAs. Inactivation of Rrp6p partially restores the levels of poly(A)$^+$ mRNAs suggesting that Rrp6p as a component of the nuclear exosome, destroys slowly or improperly processed pre-mRNAs (Burkard and Butler, 2000).

Recent analyses have identified a number of related but distinct RNA decay pathways that degrade aberrant nuclear pre-mRNA. These pathways are part of a surveillance mechanism for monitoring proper mRNA synthesis, eliminating RNAs with defects in splicing, 3'end formation or export. Thus it appears that there is a dynamic competition between the processing of nascent transcripts and their degradation by the nuclear exosome. Previous studies have shown that the degradation of nuclear pre-mRNA is regulated by the availability of glucose, the normal and preferred carbon source for yeast. Degradation of defective pre-mRNAs appeared to be favoured during growth on glucose-containing media whereas processing to functional mRNAs is favoured with growth on other carbon sources. It may be that quality control is very stringent when energy is abundant but is less stringent during growth on poor carbon sources when energy is limiting.

To identify potential endogenous targets for nuclear turnover, microarray analyses were performed on strains lacking the nuclear-specific exosome components Rrp6p, the exosome cofactor Rrp47 or carrying a ts mutation in Rrp41p (Ski6p).

VI.B. Using Microarray to identify potential endogenous targets for nuclear turnover

The advantage of using the microarray technology is that it allows the simultaneous analysis of thousands of genes within a single experiment and in a relatively short period of time. This technique is based on hybridisation experiments involving comparisons of relative amounts of cellular mRNA from two different samples (e.g. different mutants). Arrays containing immobilised DNA probes are hybridised to cDNAs to poly(A)$^+$ RNA extracted from the cells and labelled with a fluorescent marker. The intensity of the fluorescent signal for each probe reflects the abundance of the target RNA in the RNA sample.

In collaboration with Frederic Devaux (Pierre and Marie Curie, Paris, France) we made use of this technique to analyse mRNAs that are up-regulated or down-regulated in *rrp6*Δ, *ski6-100* and *rrp47*Δ mutants. The aim was to identify candidate genes whose expression levels may be regulated at the level of nuclear turnover.

There are different methods for preparing of labelled material for measurements of gene expression. In our case, poly(A)$^+$ mRNA was purified from the *rrp6*Δ, *rrp47*Δ, *ski6-100* and the wild type strains and labelled nucleotides were incorporated into cDNA during the reverse transcription reaction. A two-color hybridisation strategy was used in which the cDNA from two different samples (isogenic wild type and mutant strains) was labelled with two different fluorescent dyes, Cy3 and Cy5. The fluorophore Cy3 emits green light at a wavelength of 532nm whereas Cy5 emits red light at a wavelength 635nm. Subsequently, equal samples amounts are mixed and co-hybridised to the array. After a series of washes to eliminate unbound nucleotides and unspecific binding, the array is scanned at the two different wavelengths. The ratio of the two differentially labelled target signals on each microspot directly reveals whether the targets are present in different or similar concentration in each of the isogenic wild type and mutant strains. The arrays used in this study were purchased from the Japanese company Hitachi and

contained probes for 5800 open reading frames of the yeast genome. The probes are PCR products that have been designed not to be longer than 800 base pairs. On average, about 4000-4500 significant signals can be analysed from a total of 5800 present on the slides.

Once the signal for Cy3 and Cy5 are calculated and artifacts are eliminated, it is possible to define for every probe a ratio of Cy3 to Cy5, which ideally should corresponds to the abundance of the mRNA in each of the samples (mutant strain/ wild type). However, this ratio is affected by the emission quality of Cy3 and Cy5 since the Cy5 emission is 1.7 times more intense. Thus normalisation is required in order for the Cy3/Cy5 ratio to be indicative of the abundance of each probe in the two samples to be compared. Normalisation was done based on the total signal. This method assumes that the majority of the probes are identical between the two samples and normalisation is performed relative to the average of the total signal (sum of the signals).

VI.C. Microarray results require validation by the conventional methods

To confirm the results obtained from the microarray data, the levels of several mRNAs chosen from the microarray data were analysed by Northern analyses of RNA extracted from *rrp6*Δ, *rrp47*Δ, *ski6-100* and wild type strains (Tables 6.1, 6.2). The levels of RNA observed were quantified by the phosphoimager and normalised to a loading control.

In the microarray analyses *NRD1* was found to be increased ~3 fold in each of the exosome mutants (Table 6.1). Northern hybridisation confirmed this accumulation, however the accumulation of the *NRD1* mRNA appeared to be greater than predicted by the microarray (Figure 6.1). The mRNA levels measured by Microarray appeared to be more significant at 23°C than at 37°C and this was also observed by Northern hybridisation (Table 6.1 and Figure 6.1, lanes 3, 5, 7). *NRD1* encodes an RNA-binding

Gene	Accumulation of mRNA					
	rrp6Δ		rrp47Δ		ski6-100	
	23°C	37°C	23°C	37°C	23°C	37°C
NRD1	2.28	1.83	3.3	2.1	3.7	0.51
MRPL17	-	-	-	-	0.45	-
BAG7	4.16	3	2.1	-	2.6	1.8
YPL245w	2.04	2.61	3	2.2	3.3	-
NOG2	-	8.61	-	5.47	-	-

Table 6.1. Microarray analysis of *rrp6Δ, rrp47Δ and ski6-100* strains.
mRNA was purified from the *rrp6Δ*, *rrp47*, *ski6-100* and the wild type strains and labelled nucleotides were incorporated into cDNA during the reverse transcription reaction. A two-color hybridisation strategy was used in which the cDNA from two different samples (isogenic wild type and mutant strains) was labelled with two different fluorescent dyes, Cy3 and Cy5.The ratio of the two differentially labelled target signals on each microspot directly reveals whether the targets are present in different or similar concentration in each of the isogenic wild type and mutant strains. (-) refers to a sample present in similar concentration in wild type and mutant strains.

Gene	Cellular compartment	Function
NRD1	nucleus	RNA-binding protein, directs transcription termination on non polyadenylated transcripts (snoRNAs and snRNAs)
MRPL17	mitochondrial large ribosomal subunit	structural constituent of ribosome, involved in protein biosynthesis
BAG7	intracellular	Rho GTP-ase activator, involved in small GTP-ase mediated signal transduction
YPL245w	cytoplasm, nucleus	molecular function and biological process unknown
NOG2	nucleus, nucleolus	Involved in ribosomal large subunit export, ribosome biogenesis and RNA splicing

Table 6.2. The function and the localisation of Nrd1p, MRPL17p, Bag7p, Ypl245w and Nog2p.

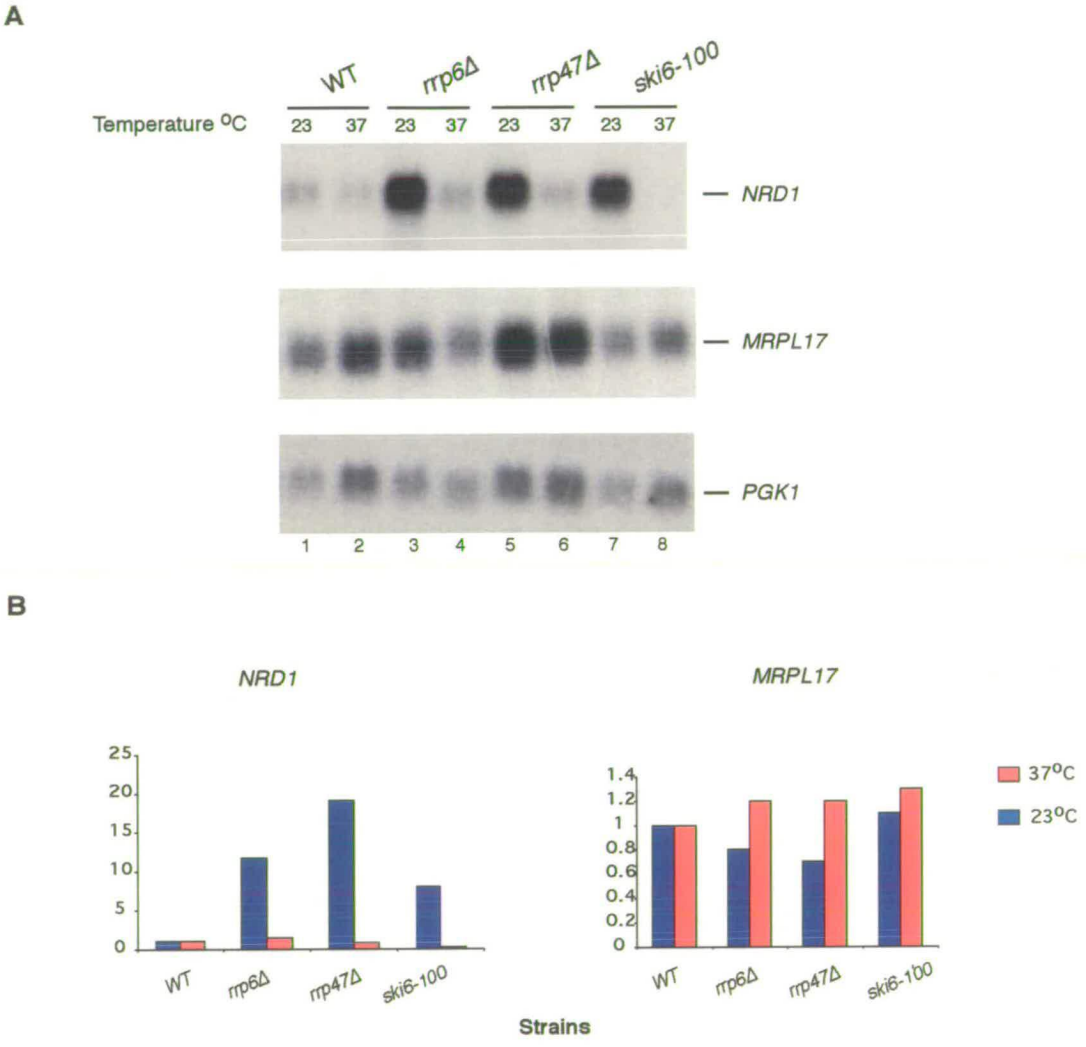


Figure 6.1. Confirmation of the Microarray results by Northern hybridisation

A. Northern blot analysis of *rrp6Δ*, *rrp47Δ* and *ski6-100* strains.
Strains were grown at 23°C and then shifted to 37°C for 1 hr. For each panel 7µg of total RNA was separated on a 1.2% agarose gel and analysed by northern hybridisation using the probes indicated on the right (Chapter 2, section II.E).

B. Quantitative analyses of mRNA levels from the data shown in (A). Phosphoimager data normalised to the corresponding *PGK1* mRNA are shown.
The wild type was arbitrarly set to 1.

protein that was recently shown to direct termination by RNA polymerase II and 3'-end formation of snoRNAs. Nrd1p was also reported to autoregulate its own mRNA synthesis possibly by promoting premature transcription termination (Steinmetz and Brow, 1996; Steinmetz et al., 2001).

The levels of the *MRPL17* mRNA were found to be unaffected by the absence of either Rrp6p, Rrp47p or by a mutation in Ski6p which was confirmed by the Northern hybridisation experiment (Figure 6.1). *MRPL17* is located downstream of the *NRD1* gene and encodes a structural subunit of the mitochondrial ribosome involved in protein biosynthesis (Graack and Wittmann-Liebold, 1998).

However, the results obtained form Northern analyses for *YPL245w*, *BAG7* and *NOG2* genes showed discrepancies with the microarray data (Figure 6.2). The function and localisation of each of these proteins are listed in Table 6.2. *BAG7* was found to be increased by ~ 3 fold in each of the *rrp6*Δ, *rrp47*Δ and *ski6-100* in the microarray analyses. Similar to the *NRD1* mRNA the accumulation of the *BAG7* mRNA was greater at 23°C than at 37°C in all three mutants. In Northern analyses no increase in *BAG7* mRNA could be observed for the mutant strains when compared with the wild type strain (Figure 6.2). In the case of *YPL245w*, comparison of the mRNA levels did reveal an accumulation in the mutants relative to the wild type strain but not as significant as that predicted by the microarray data (Table 6.1 and Figure 6.2A and B). In addition, *NOG2* was predicted by microarray analyses to be unaffected in *ski6-100* but increased ~ 8 and 6 fold in both *rrp6*Δ and *rrp47*Δ at 37°C (Table 6.1). Results from Northern analyses revealed only a slight accumulation of *NOG2* mRNA in each of the *rrp6*Δ and *rrp47*Δ mutants (Figure 6.3).

These results indicate that confirmation of the microarray data by the conventional method of Northern hybridisation is required.

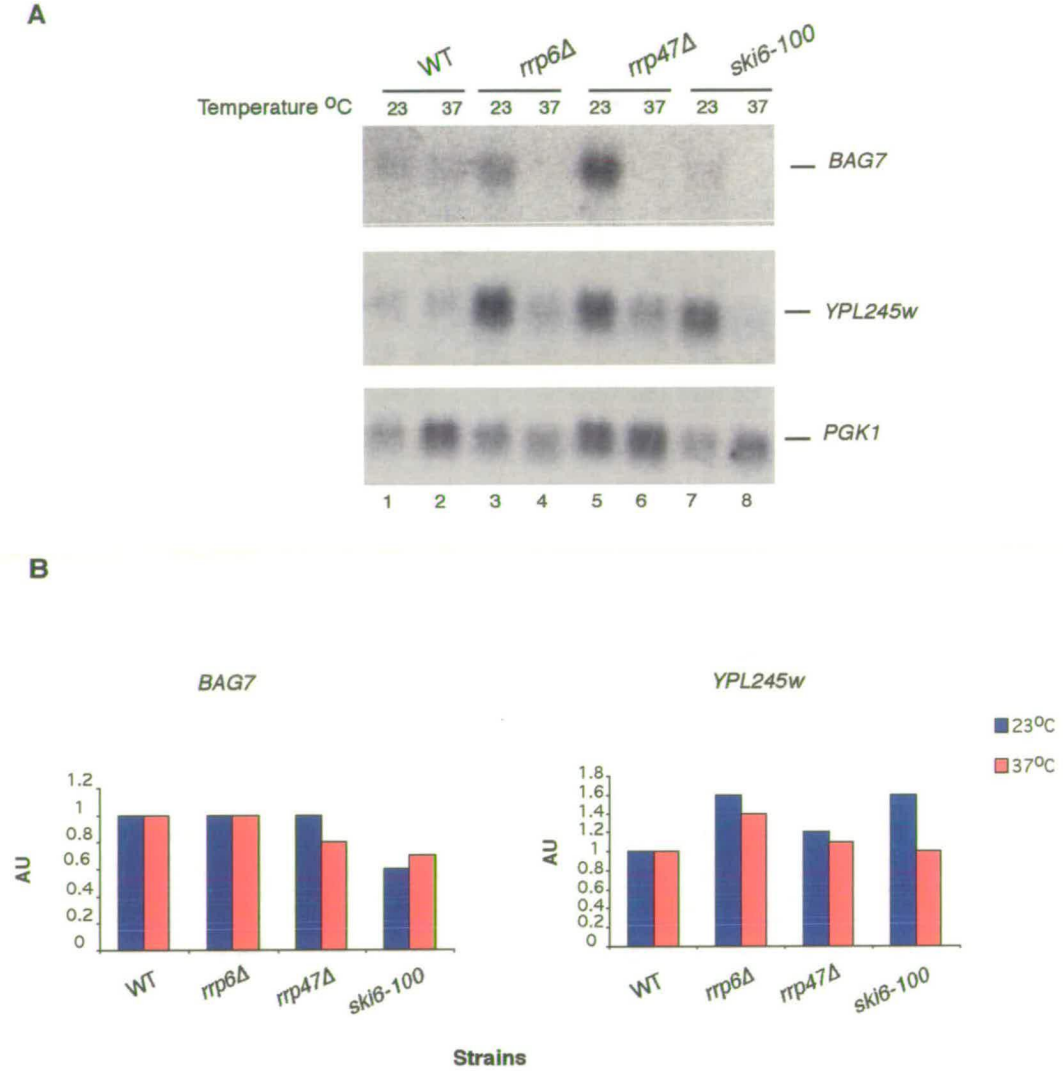


Figure 6.2. Discrepancies between Microarray and Northern hybridisation in the analyses of *BAG7* and *YPL245w* genes.

A. Northern blot analysis of *rrp6Δ*, *rrp47Δ* and *ski6-100* strains.
Strains were grown at 23°C and then shifted to 37°C for 1 hr. For each panel 7µg of total RNA was separated on a 1.2% agarose gel and analysed by northern hybridisation using the probes indicated on the right (Chapter 2, section II.E).

B. Quantitative analyses of mRNA levels from the data shown in (A). Phosphoimager

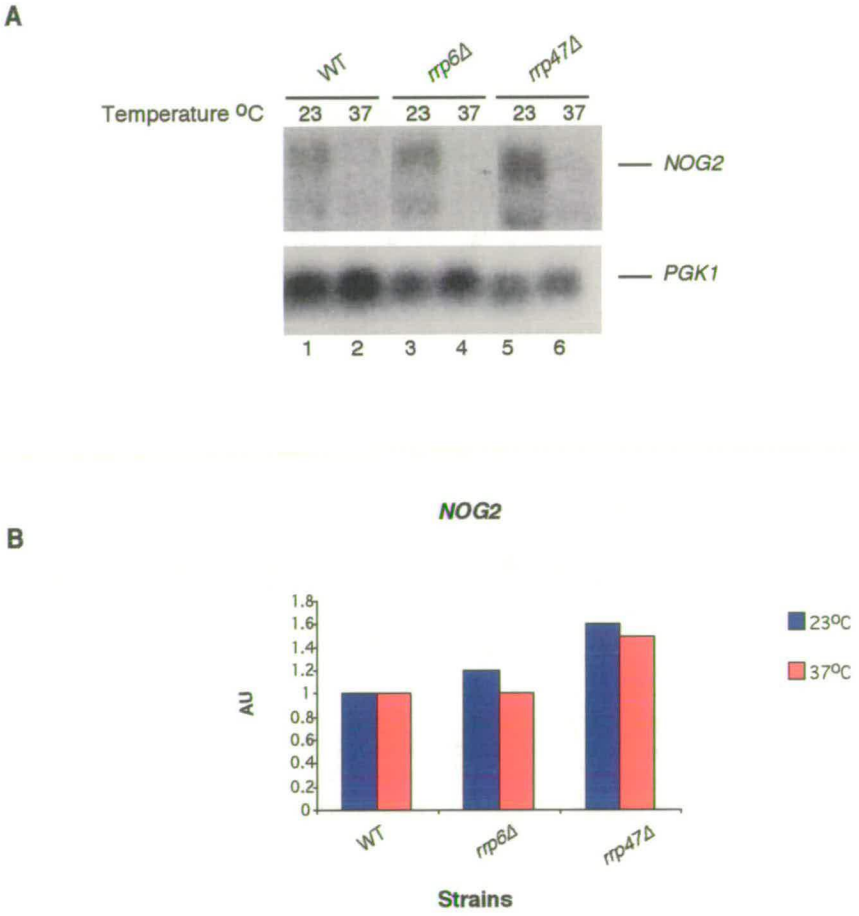


Figure 6.3. Discrepancies between Microarray and Northern hybridisation in the analysis of *NOG2*.

A. Northern blot analysis of *rrp6*Δ and *rrp47*Δ strains.

Strains were grown at 23°C and then shifted to 37°C for 1 hr. For each panel 7µg of total RNA was separated on a 1.2% agarose gel and analysed by northern hybridisation using a probe against *NOG2* mRNA (Chapter 2, section II.E).

B. Quantitative analyses of mRNA levels from the data shown in (A). Phosphoimager data normalised to the loading control are shown. The wild type was arbitrarly set to 1.

VI.C. Binding of Nrd1p to its mRNA promotes recruitment of the nuclear exosome and mRNA degradation

Yeast *NRD1* (for nuclear pre-mRNA down regulation) is an essential gene encoding a protein with homology to mammalian SCAF8 and SCAF4 (Yuryev et al., 1996; Steinmetz and Brow, 1996). Nrd1p contains an N-terminal pol II CTD-interacting domain, a single RNA recognition motif (RRM) and a short segment containing arginine-glutamate and arginine-serine (RE/RS) dipeptides (Steimnetz and Brow, 1996). *NRD1* was originally identified as a gene encoding an hnRNP that mediates the severe reduction in expression of a reporter gene containing an exogenous sequence element in its intron. This down-regulation results in lower levels of reporter pre-mRNAs and in the production of 3'-truncated pre-mRNA fragments. This effect was suppressed by mutations in either the intronic element or by mutation in *NRD1*. It was later shown that Nrd1p binds to an Nrd1-binding sequence in this artificial element resulting in truncation of the reporter pre-mRNA. Efficient response to this element also requires the RNA helicase Sen1p, the RNA-binding protein Nab3p and the interaction of Nrd1p with the CTD of RNA polymerase II (Steinmetz and Brow, 1996, 1998; Conrad et al., 2000). Recent studies have revealed a role for Nrd1p in the 3'-end formation of snRNAs and snoRNAs. Mutations in *NRD1* lead to the production of read-through transcripts from snRNAs and snoRNAs extending into the downstream open reading frame. The binding of Nrd1p to a sequence element in these nascent transcripts was found to be required for proper termination by RNA polymerase II and for 3'-end maturation (Steinmetz et al., 2001). In addition, Nrd1p was found to auto-regulate the levels of the *NRD1* transcripts through binding to an Nrd1p-responsive element near the 5'-end of the *NRD1* mRNA. The binding of Nrd1p to its own transcript would lead to the synthesis of truncated transcripts with reduced full-length *NRD1* coding mRNA (Steinmetz et al., 2001).

Microarray analyses found *NRD1* to be increased ~3 fold in each of the exosome mutants, an observation, which was confirmed by Northern hybridisation. This prompted us to investigate a potential role for the exosome in regulating the expression of *NRD1*. We envisaged three possible models by which this regulation might be achieved. First, it could be that the *NRD1* mRNA is simply unstable and its degradation by the exosome is unrelated to its autoregulation. Second, Nrd1p autoregulation might be achieved via activated degradation of the *NRD1* mRNA by the exosome. This could be mediated by interactions between the Nrd1p complex (Nrd1p, Nab3p and Sen1p) and a component of the exosome. Recent studies have revealed that Nab3p coimmunoprecipitates with Mtr4p/Dob1p, a cofactor of the nuclear exosome suggesting a possible mechanism for the recruitment of the exosome to the *NRD1* mRNA. Thirdly, the binding of the Nrd1p complex to the *NRD1* mRNA leads to premature termination generating 3'-truncated transcripts. Stabilisation of these truncated fragments in exosome mutants might lead to sequestration of the Nrd1p complex causing the upregulation of the full-length *NRD1* mRNA. We decided to test these hypothetical models to gain more insight on the involvement of the exosome in the regulation of the *NRD1* mRNA levels at the level of nuclear turnover.

VI.C.1. Nrd1mRNA levels are not regulated by the cytoplasmic 3'-5' turnover pathway

To confirm that the regulation of *NRD1* expression occurs at the level of nuclear turnover and involves the nuclear exosome, northern blot analysis was performed on RNA extracted from mutants in the cytoplasmic Ski complex (*ski2*Δ, *ski3*Δ and *ski8*Δ) and the GTPase Ski7p (*ski7*Δ). Previous studies have shown that the Ski complex recruits mRNAs to the exosome via Ski7p, which then activates the exosome to degrade the mRNAs in a 3'-5' direction (van Hoof et al., 2000). Comparison between the wild type strain and the *ski* mutants did not reveal any clear accumulation of the *NRD1* mRNA (Figure 6.4). The autoregulation of the *NRD1* pre-mRNA levels does not therefore require the cytoplasmic 3'-5' mRNA turnover pathway.

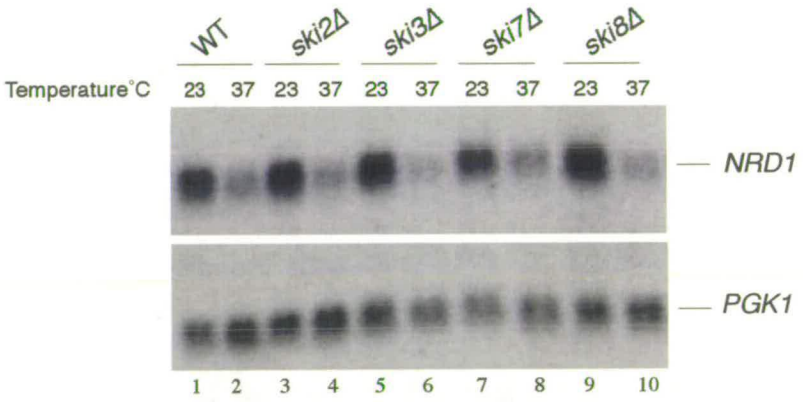


Figure 6.4 *NRD1* levels are not regulated at the level of cytoplasmic mRNA turnover.

Northern analyses of *ski2Δ*, *ski3Δ*, *ski7Δ*, *ski8Δ* strains. Strains were grown at 23°C and then shifted to 37°C for 1 hr. For each panel 7μg of RNA was separated on a 1.2% agarose gel and analysed by northern hybridisation using the NRD1 probe number RH2 (Chapter 2, section II.E).

VI.C.2. Nrd1p, Rrp6p and Sen1p appear to function in the same pathway

To determine whether Nrd1p, Sen1p and the nuclear exosome function in the same pathway Northern hybridisation analysis was performed on total RNA extracted from the temperature-sensitive lethal *nrd1-102* and *sen1-1* strains and exosome mutant strains (*rrp6*Δ, *ski6-100* and *rrp47*Δ) (Figure 6.5). Strong accumulation of the *NRD1* mRNA was seen in the *nrd1-102* mutant and in the strain carrying a mutation in the RNA helicase *sen1-1* after shift to 37°C (lanes 4 and 8). In contrast, the accumulation observed in exosome mutants was mainly seen at 23°C (lanes 13, 15 and 17).

If Nrd1p, Sen1p and the nuclear exosome were involved in two distinct pathways we would expect that double mutants of Nrd1p or Sen1p with Rrp6p would result in substantial stabilisation of the *NRD1* mRNA. To determine if mutation of Rrp6p is synergistic with mutation of Nrd1p or Sen1p, the levels of the *NRD1* mRNA in the double mutants *nrd1-102/rrp6*Δ and *sen1-1/rrp6*Δ were compared to those observed in each of the single mutants. The levels seen in *sen1-1/rrp6*Δ were higher than those observed in the *sen1-1* whereas for the *nrd1-102/rrp6*Δ the change was not significant (lanes 7 to 10 and 19 to 22). These findings indicate that the effects of the Nrd1p, Sen1p and Rrp6p are additive but not synergistic.

The results of the Northern hybridisation were further confirmed by primer extension analysis. Two major transcription start sites were detected, which were estimated to be 250 and 150 nt upstream of the start codon (Figure 6.6). The primer extension stops in the *NRD1* mRNA were elevated by similar levels to those observed by Northern hybridisation in each of the tested mutants. It can be concluded that Rrp6p, Nrd1p and Sen1p are involved in regulating the expression of the *NRD1* mRNA and all three proteins appear to function in the same pathway.

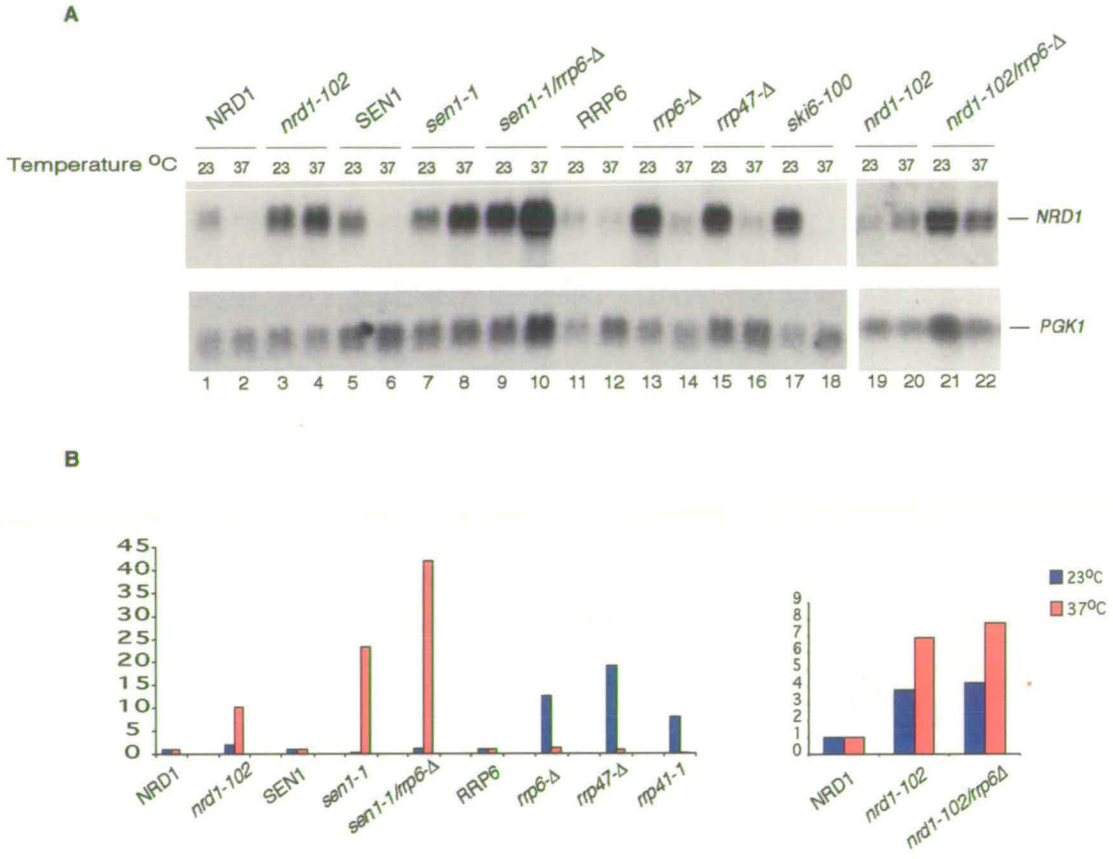


Figure 6.5. Regulation of NRD1 accumulation by Nrd1p, Sen1p, Rrp6p, Rrp47p and Ski6p. (A) Northern blot analysis of *nrd-102*, *sen1-1*, *rrp6*Δ, *rrp47*Δ and *ski6-100* strains. Strains were grown on glucose medium at 23°C and then shifted to 37°C for 1 hr. For each panel 7μg of RNA was seperated on 1.2% agarose gels and analysed by Northern hybridisation using the NRD1 probe RH1 (Chapter 2, section II.E). **(B)** Phosphoimager quantification of the Northern hybridisation data shown in **A**. mRNA levels were normalised to the corresponding *PGK1* mRNA. The isogenic wild type of each strain was arbitrarly set to 1.

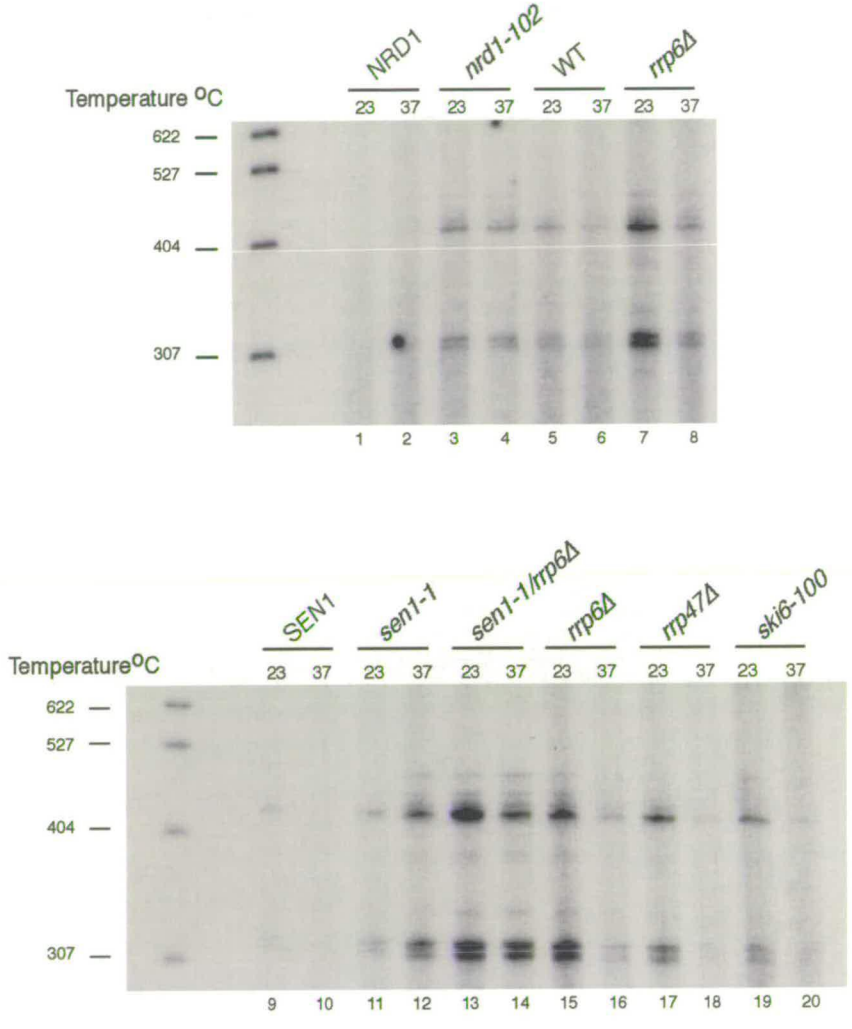


Figure 6.6. Primer extension analyses of *NRD1* levels confirms the microarray and Northern hybridisation results. Primer extension analysis of *nrd1-102*, *sen1-1*, *sen1-1*/*rrp6*Δ, *rrp6*Δ, *rrp47*Δ, *ski6-100* mutant strains and their isogenic wild type. Strains were grown on glucose medium at 23°C and then transfered to 37°C for 1hr. Primer extension was performed using RH2 oligo (Chapter 2, section II.E).

VI.C.3. NRD1 transcripts are polyadenylated in exosome mutants

To investigate potential activated degradation from the 3'-end of the *NRD1* mRNA, RNase H digestion was carried out on total RNA extracted from *nrd1-102*, *rrp6*Δ and *rrp47*Δ mutant strains (Figure 6.7).

In the isogenic wild type of the *nrd1-102* strain (NRD1, lanes 1 and 2), the *NRD1* mRNA is heterogenous in length due to the presence of the poly(A) tail. However, treatment with RNaseH and oligo(dT) removed this poly(A) tail and resulted in the production of three different deadenylated mRNA species (size 1, 2 and 3). Thus it appears that the *NRD1* transcript contains three different polyadenylation sites, generating a heterogenous population of poly(A)$^+$ mRNAs. As shown by the shift in mobility following treatment with oligo(dT) and RNase H, the major polyadenylated *NRD1* mRNA generates size 1 species after deadenylation (lanes 3 and 4). These heterogenous deadenylated species of *NRD1* mRNA were observed to similar levels in the *nrd1-102* mutant and the wild type strain (NRD1).

However, the polyadenylated species observed in *rrp6*Δ and *rrp47*Δ mutant strains and their isogenic wild type (WT, lanes 5 to 6) were different. The major polyadenylated species in these strains corresponded to deadenylated species of size 2. The differences in the polyadenylation pattern observed between NRD1 and WT strains indicates a heterogeneity between these two strains. Heterogeneity is frequently observed between different laboratory yeast strains, making it important to use the appropriate wild-type control strains.

VI.C.4. The presence of truncated NRD1 pre-mRNA species generated from the depletion of exosome components

Previous studies have shown that the binding of Nrd1p to the artificial sequence element leads to down regulation of the reporter pre-mRNA containing the segment. In addition this was accompanied by the appearance of 3'-truncated pre-mRNA fragments

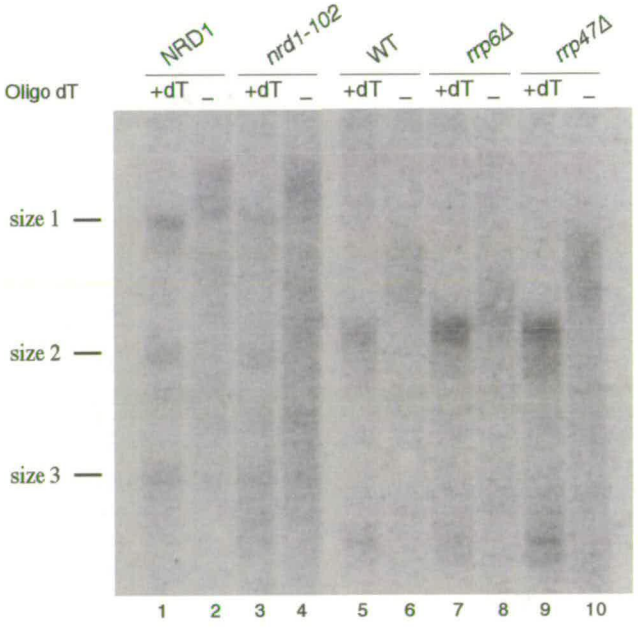


Figure 6.7. RNase H analysis of *NRD1* mRNA in *nrd1-102*, *rrp6Δ* and *rrp47Δ* mutants.

Total RNA was extracted from the NRD1 isogenic wild type (NRD1), *nrd1-102*, *rrp6*Δ, *rrp47*Δ and their isogenic Wild type strain (WT) grown in glucose medium at 23^{O}C. Samples were treated with RNase H plus oligo(dT) (+dT lanes) and compared with untreated samples, then separated on 6% acrylamide/8.3M urea gel, transferred to Nylon and hybridised with NRD1 probe number RH5 (Chapter 2, section II.E).

containing partial intron sequences (Steinmetz and Brow, 1996, 1998). However, no such evidence had been presented for endogenous *NRD1* mRNA. We speculated that if the nuclear exosome is involved in the degradation of these truncated species, they should be stabilised in the *rrp6Δ*, *rrp47Δ* and *ski6-100* mutant strains. Consistent with this hypothesis, 3'-truncated species of the *NRD1* pre-mRNA were detected by Northern hybridisation in each of the exosome mutants and in the double mutant *sen1-1/rrp6Δ* (Figure 6.8). Similar to the accumulation of the full-length *NRD1* pre-mRNA, the accumulation of the 3'-truncated species was more significant in the exosome mutant at the permissive temperature. Furthermore, two forms of truncated species were observed, one form of approximate size of 630 not and another smaller form of approximate size of 400 nt. The truncated species could not be detected in the *nrd1-102* or *sen1-1* mutants. This is consistent with a requirement for Nrd1p complex (Nrd1p, Sen1p and Nab3p) binding to its own mRNA, which would in turn lead to premature termination and generation of truncated species. The two different truncated *NRD1* pre-mRNA species could be explained by the presence of two distinct termination sites in the *NRD1* transcript. The presence of the truncated species doesn't seem to be due to the two different transcription start sites mapped by primer extension and this is because the difference in their sizes (~ 230 nt) does not match that between the two transcription start sites (~ 100 nt).

The stabilisation of these truncated species in the exosome mutants suggests that they are normally degraded by the exosome in a 3'-5' direction. In the *nrd1-102* and *sen1-1* mutants full-length *NRD1* mRNA accumulates due to failure of the Nrd1 complex to bind and down regulate the levels of the Nrd1p via 3'-activated exosome degradation.

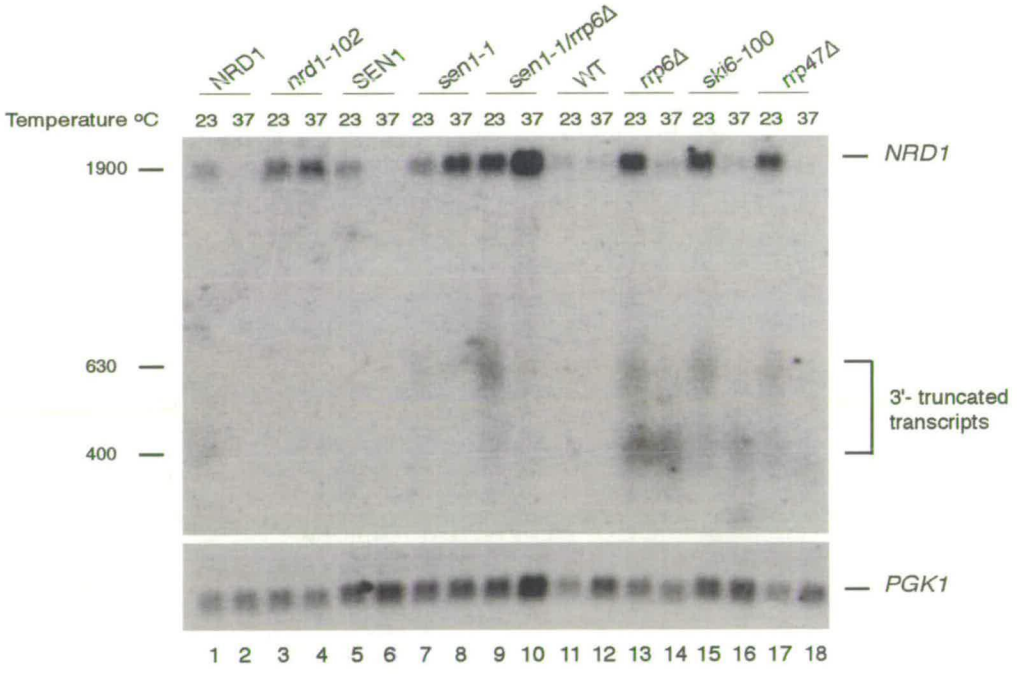


Figure 6.8. Exosome mutants accumulate 3'-truncated *NRD1* transcripts.

Northern blot analysis of *nrd1-102, sen1-1, sen1-1/rrp6Δ, rrp47Δ ,ski6-100* and the corresponding isogenic wild-type strains. Strains were grown on glucose medium at 23°C and then shiftted to 37°Cfor 1hr. For each panel, 7µg of total RNA was seperated on a 1.2% agarose gel and analysed by Northern hybridisation using NRD1 probe RH1 (chapter 2, section II.E).

VI.D. Discussion

The identification of genes potentially regulated at the level of nuclear turnover was the focus of this chapter. We made use of the Microarray technique to investigate the abundance of a wide number of mRNAs in each of the exosome mutants, *rrp6Δ*, *rrp47Δ* and *ski6-100*. The distinction of Rrp6p from other exosome components was confirmed by the microarray results and a group of genes specifically regulated by Rrp6p was observed. In addition, the accumulation of the previously characterised *NRD1* gene was analysed in detail. The autoregulation of the *NRD1* mRNA by Nrd1p appears to take place at the level of nuclear turnover and involves the activity of the nuclear exosome. The results present further evidence for the existence of a pathway for the control of gene expression that involves nuclear turnover.

VI.D.1. Microarray results need to be validated by conventional methods

To take advantage of the massive increase in the amounts of available DNA sequence information, new technologies are required. Among the most powerful tools of genomics are the high-density DNA arrays that allow complex mixtures of RNA or DNA to be analysed in a parallel and quantitative fashion. One of the most prominent uses of DNA arrays is to measure levels of gene expression (mRNA abundance) for thousands of genes simultaneously (Templin et al., 2002). This miniaturised technique enables the analysis of the whole of *S.cerevisiae* genome (6000 ORFs). We used this technique in collaboration with Frederic Devaux, in order to identify potential genes whose expression is regulated at the level of nuclear turnover. Before analysing the significance of the microarray data, we decided to confirm the results obtained by Northern hybridisation.

In the case of the *NRD1* and *MRPL17* genes, the accumulation observed in the microarray analysis was confirmed by the northern analysis. However, in the case of *NRD1*, the accumulation was more significant in the Northern hybridisation experiment. Nrd1p is an essential nuclear RNA-binding protein that was previously characterised and shown to direct 3' truncation of reporter mRNA transcripts in response to an artificial element containing a Nrd1p-binding sequence. The levels of the *NRD1* mRNA are autoregulated by Nrd1p and require the binding of the protein to a sequence element in the *NRD1* mRNA. In addition, Nrd1p was recently shown to prevent read-through transcription from yeast small nucleolar and small nuclear RNA genes into adjacent genes and to direct their 3' end formation. The *MRPL17* gene is found downstream of the *NRD1* gene encodes a mitochondrial ribosomal subunit (Graack and Wittmann-Liebold, 1998). However, read-through transcripts from the *NRD1* gene into the adjacent *MRPL17* gene were not detected in any of the exosome mutants suggesting a different regulatory mechanism from Nrd1p.

Discrepancies between the microarray data and the Northern hybridisation analyses were observed for *BAG7*, *NOG2* genes and the ORF YPL245w. In the case of *BAG7* and YPL245w, the predicted accumulation was several fold more than that observed by northern analyses. Moreover, *NOG2* appeared to be down-regulated in the northern hybridisation as opposed to the accumulation predicted by the microarray data. Bag7p is a GTPase involved in signal transduction whereas Nog2p is a nuclear protein and a putative GTPase. Nog2p was previously shown to associate with the pre-60S ribosomal subunits in the nucleolus and is required for their nuclear export and maturation (Bassler et al., 2001).

The source of most false positives is random noise, biological variation or the occasional array-specific defect. For measurements that cover such a large number of genes it is important to maintain high standards of data quality to keep false-positives to a minimum. The incidence of false-positives is lower in the cases where changes are observed for sets of genes rather that individual ones.

One of the most difficult and critical aspects of microarray analysis is making sense of the vast quantities of data and extracting conclusions and hypotheses that are

biologically meaningful. Thus, it is important to validate the data obtained from the gene expression analysis before moving to the next stage of understanding the underlying process. From the above results this new approach to measuring gene expression does not replace conventional methods. Standard methods such as Northern blots, Western blots, primer extension or RT-PCR need to be used to complement the broader measurements.

VI.D.2. A novel pathway for the regulation of gene expression involving Nrd1p and the nuclear exosome

Microarray analyses revealed the accumulation of *NRD1* mRNA in exosome mutants, a result that was confirmed by northern hybridisation. We used a variety of molecular techniques to investigate potential pathways and mechanisms suggested by the array result.

Nrd1p was first identified as a trans-acting factor that causes the under-accumulation of a reporter pre-mRNA harbouring an exogenous sequence element in its intron. The decrease in pre-mRNA abundance was accompanied by the appearance of 3' truncated pre-mRNA fragments containing partial intron sequences. The N-terminal of Nrd1p contains a CTD-binding motif, which facilitates its interaction with RNA polymerase II (Steinmetz and Brow, 1998). The CTD interacting domain of Nrd1p (CID) was previously demonstrated to be important for pre-mRNA down-regulation, although it was shown to be dispensable for cell viability. The CID may provide only one of multiple redundant mechanisms to ensure interaction between Nrd1p and RNA polymerase II. Nrd1p interacts with Nab3p and the CTD simultaneously, suggesting that Nab3p may provide one of the mechanism that facilitates the Nrd1-CTD interaction (Conrad et al., 2000). Nab3p in turn interacts with Ctk1p, which encodes the catalytic subunit of a kinase that phosphorylates the CTD (Lee and Greenleaf, 1991). The downregulation of the reporter gene by Nrd1p required an additional factor, Sen1p, a putative ATP-dependent helicase. Sen1p localised to the nucleus and contains an RNA

helicase motif similar to that of the NMD factor Upf1p (Rasmussen and Culbertson, 1998).

Nrd1p, Sen1p and Nab3p were recently shown to be involved in a pathway for 3'-end formation of snRNA and snoRNA. Mutations in this Nrd1p-dependent pathway result in the accumulation of 3' extended snoRNA transcripts that fail to terminate properly and read through the downstream gene. These transcripts include coding regions and presumably terminate at the 3'-processing signals of neighbouring genes, thus becoming polyadenylated.

The results presented in this chapter reveal the accumulation of the *NRD1* pre-RNA in *rrp6*Δ, *rrp47*Δ and *ski6-100* mutant implying a possible role of the nuclear exosome in regulating the expression of *NRD1*. The observed *NRD1* accumulation was independent of the cytoplasmic 3'-5' degradation pathway that involves the cytoplasmic exosome. This is in agreement with the nuclear localisation of the Nrd1p and the involvement of the nuclear specific exosome components Rrp6p and Rrp47p in the regulation of *NRD1* mRNA levels.

Northern blot analyses presented here show that the abundance of the *NRD1* mRNA is increased in the temperature-sensitive *nrd1-102* and *sen1-1* mutant strains which is in agreement with published data about the autoregulation of the *NRD1* mRNA by Nrd1p. In addition to Nrd1p this regulation involves the factors Sen1p and Nab3p (Steinmetz et al., 2001). However, the increased levels of the *NRD1* mRNA in the exosome mutants suggest the involvement of the nuclear exosome in this regulation. We speculated that Nrd1p binds to a specific sequence element in the *NRD1* mRNA and recruits the exosome to degrade the mRNA in a 3'-5' direction. The interaction between the exosome and Nrd1p could be mediated through the Nab3p-Mtr4p interaction. Mtr4p/Dob1p, an ATP-dependent helicase that function as a cofactor for the nuclear exosome was recently shown to coimmunoprecipitate with Nab3p (Gavin et al., 2002).

Alternatively, the binding of Nrd1p to the *NRD1* mRNA could lead to premature termination as previously shown for snoRNAs 3'-end formation pathway (Steinmetz et al., 2001). In this case, premature termination would be accompanied by the generation of 3'-truncated *NRD1* transcripts which would be targeted for degradation by the

exosome. Finally, Nrd1p and the exosome might function in two separate pathways that independently regulate *NRD1* mRNA abundance.

Rrp6p was not clearly synergistic with either Nrd1p or Sen1p suggesting that Rrp6p, Nrd1p and Sen1p function in the same pathway. In the case where Rrp6p would be functioning in a different pathway we would have expected a very strong accumulation upon depletion of Rrp6p in the *nrd1-102* mutant. The appearance of 3'-truncated *NRD1* mRNA in the exosome mutants would agree with the second proposed model.

The results presented in this chapter would fit into a model where the binding of Nrd1p to its own message leads to premature termination of transcription by RNA polymerase II generating 3'-truncated products that are targeted for degradation by the nuclear exosome. Nuclear pre-mRNA surveillance was previously implicated in the degradation of transcripts with defects in 3'-end formation, splicing or export (Chapter 1, section I.C.5). This provides further support for the degradation of the abnormal 3'-truncated transcripts by the nuclear exosome.

VI.D.4. A model for the regulation of gene expression by the exosome at the level of nuclear turnover

The model that we propose is based on the regulation of the *NRD1* mRNA but suggests that this pathway might be used for the controlled expression of other genes at the level of nuclear turnover (Figure 6.9).

The amount of synthesised Nrd1p exerts a down-regulating effect on the transcription of the *NRD1* mRNA. Thus, when sufficient Nrd1p is being synthesised, Nrd1p binds through its C-terminal domain to the Nrd1p binding element in the 5'-UTR of the *NRD1* transcript recruits Sen1p and Nab3p, leading to premature termination of transcription by the RNA polymerase II. The premature termination results in the accumulation of 3'-truncated transcripts that sequester Nrd1p, resulting in its down-regulation. In wild-type cells the Nrd1p-Sen1p-Nab3p complex may actively recruit the nuclear exosome to degrade the transcript in a 3'-5' direction. Recent 2-hybrid analyses (Hybergenics) have shown that Nab3p interacts with the exosome component Mtr4p while Rrp47p interacts with Nrd1p suggesting potential physical links between the exosome and the Nrd1p complex.

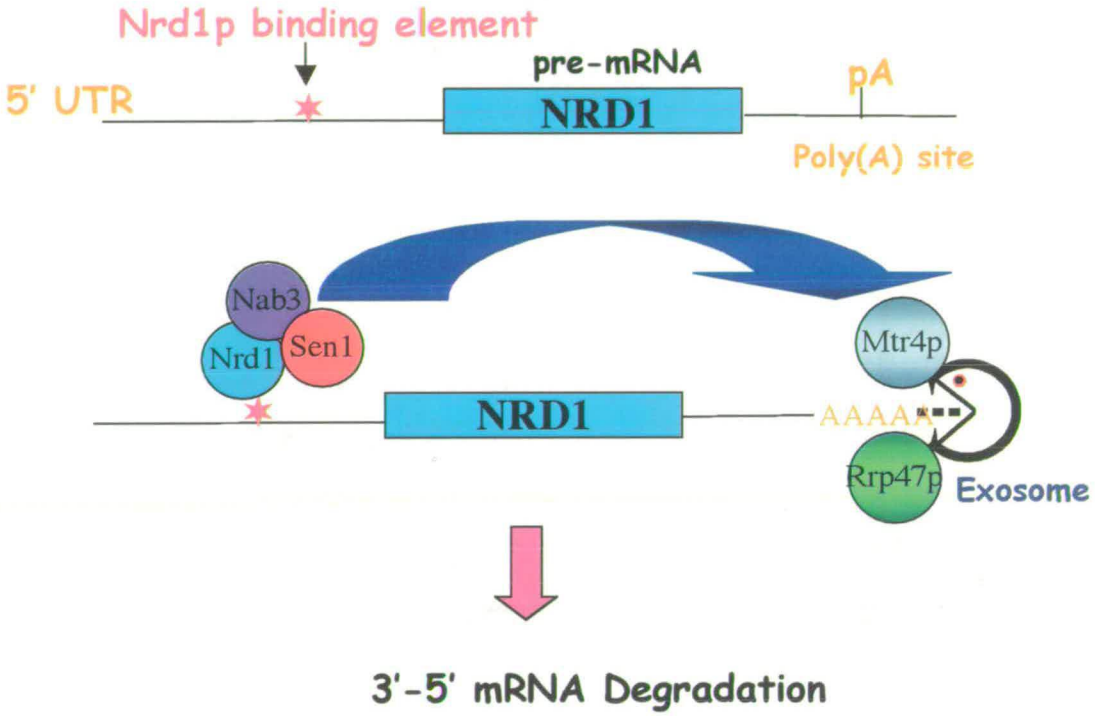


Figure 6.9. A model for the regulation of *NRD1* expression at the post-transcriptional level by pre-mRNA turnover. The binding of Nrd1p to the Nrd1p binding element leads to subsequent binding to Nab3p and the putative helicase Sen1p. This complex is believed to recruit the exosome through an interaction of Nab3p with Mtr4p or through an interaction between Nrd1p and the exosome cofactor Rrp47p. The exosome then degrades the body of the transcript in 3'-5' direction and results in the decreased expression of the *NRD1* mRNA.

Chapter Seven

Final Discussion

The aim of this thesis was to seek a deeper understanding of the function of the exosome component Rrp6p in nuclear RNA turnover. The involvement of Rrp6p in RNA processing and degradation was shown to be distinct from other exosome components. The implications of the results presented in this work for the understanding of the particular Rrp6p function in nuclear RNA turnover and other cellular processes are discussed in this chapter.

VII.A. The association of Rrp6p with complexes distinct from the exosome

VII.A.1 Identification of novel Rrp6p-associated complexes

The purification of the exosome component has added a new level of complexity to the understanding of RNA metabolism. This complex of 3'-5'exonucleases present in nuclear and cytoplasmic forms was shown to be involved in both the processing and the degradation of RNA. The two forms of the exosome share a core of ten exonucleases and differ by the presence of Rrp6p that was only detected in the nucleus. While the core subunits are all essential for growth, deletion of Rrp6p only causes temperature-sensitive lethality (Briggs et al., 1998). Cells lacking Rrp6p activity accumulate stable nuclear RNAs with incompletely processed 3' ends. Rrp6p has also been implicated in the turnover of nuclear pre-mRNAs and mRNAs and the retention near the transcription site of mRNAs containing aberrant 3'-ends (Burkard and Butler, 2000; Hilleren et al., 2001). These differences between Rrp6p and other exosome components suggested that Rrp6p could function independently from the exosome complex.

The first assays conducted were to assess whether all the Rrp6p population in the cell is found in association with the exosome. Density gradient analysis revealed that only a

fraction of the Rrp6p population is associated with the exosome. The majority of the protein appeared to associate with three different size complexes (A, B and C).

Complex A sedimented slower than the exosome and the peak of Rrp6p did not cosediment with the peak of the core exosome component Rrp4p. Previous results showed that Rrp6p is present at substoichiometric levels in 10-20% of the purified exosome.

Analysis of complexes B and C showed that they are approximately 40S and 60S sized RNP complexes. The association of Rrp6p with a pre-60S ribosomal particle is predicted based on its role in the processing of the 5.8S rRNA component of the 60S ribosomal subunit (Briggs et al., 1998). However, so far many of the enzymes known to be involved in 60S subunit biogenesis including Rrp6p, could not be detected in purified pre-ribosomal particles (Fatica and Tollervey, 2002). In addition the possible association of Rrp6p with a pre-40S ribosomal particle would be unexpected. Previous studies have showed that many of the factors known to be required for 40S subunit synthesis were associated with the 35S pre-rRNA but showed little or no co-precipitation of 20S pre-rRNA. These factors were thought to have dissociated from the pre-rRNA at or soon after following A_2 cleavage of the 35S pre-rRNA precursor (Grandi et al., 2002). Strains lacking Rrp6p or other exosome components do show defects in 40S subunit synthesis. This is, however, also the case for most 60S synthesis factors and is believed to rise from some yet uncharacterised surveillance system.

However, the possibility of complex B being an mRNP complex cannot be ruled out. The size of this complex is similar to a newly identified mRNP complex containing Scp160p, a protein that shares homology to yeast hnRNPs (Lang and Fridovich-Keil, 2000). The Scp160p-associated complex was shown to be an RNase sensitive complex of apparent molecular weight >1300kDa (slightly bigger than a 40S-sized complex). Furthermore, affinity purification of this complex revealed the presence of poly(A)-binding protein (Pab1p), a well characterised component of eukaryotic mRNPs (Lang and Fridovich-Keil, 2000).

In conclusion, the identification of Rrp6p-associated complexes distinct from the exosome provides further support for Rrp6p functioning independently from the exosome and in conjunction with novel proteins.

VII.A.2 Analysis of Rrp6p-associated complexes

Preliminary mass spectrometric analysis of complex A identified the NMD factor Upf2p in addition to the mitochondrial helicase Mss16p and Rev3p. Subsequent experiments to investigate the Rrp6p-Nmd2p interaction suggested that Rrp6p might be involved in NMD. The levels of a nonsense containing RNA, *CYH2*, were elevated in *rrp6*Δ strains but not to the extent seen in Upfp mutants. The cytoplasmic exosome was recently implicated in an NMD pathway involving accelerated deadenylation and 3'-5' degradation by the exosome. However, there is no evidence for NMD occurring in the nucleus of yeast cells, whereas studies in mammalian cells showed that NMD does occur in the nucleus and involves Rrp6p and the exosome (Lejeune et al., 2003).

To gain more insights about novel proteins interacting with Rrp6p, affinity purification of epitope-tagged Rrp6p from whole cell lysates was also performed. Srp1p and Gbp2p copurified with Rrp6p and were identified by mass spectrometric analysis. Srp1p is the yeast homologue of Importin α, which functions as the nuclear localisation signal adaptor. Gbp2p was recently characterised as a novel RNA-binding protein involved in the export of poly(A)$^+$ mRNA to the cytoplasm and shown to associate with the TREX (transcription –export) complex (Windgassen and Krebber, 2003; Straßer et al., 2002). Analyses of Gbp2p and Srp1p mutants did not reveal defects in 5.8S rRNA or snoRNA processing, suggesting their possible involvement in the surveillance functions of Rrp6p. Various studies have linked Rrp6p to a surveillance system that ensures that only properly processed mRNAs are released from the transcription site and exported to the cytoplasm (Bousquet-Antonelli et al., 2000;Hilleren et al., 2001;Torchet et al., 2002). However, these activities are not readily detected in otherwise wild-type cells.

In conclusion, Gbp2p and Srp1p could be part of a surveillance complex that monitors the quality of mRNA exported to be come functional in the cytoplasm. Whether Rrp6p and the nuclear exosome are involved in a nuclear NMD pathway remains to be elucidated.

VII.B Rrp47p: a novel-exosome associated cofactor

The exosome contains exonucleases that are able to carry out both RNA processing and RNA degradation reactions for which it is required. How the exosome recognises the variety of its substrates and why it processes some RNAs to shorter forms while it completely degrades other is still not clear. One possible explanation is that the exosome requires specific additional proteins to act on the various substrates.

The results presented in this work report the purification and the characterisation of a novel exosome cofactor that was designated Rrp47p (Mitchell et al., 2003a). Rrp47p copurified with the exosome at substoichiometric levels but was shown to stably associate with the exosome.

Rrp47p was previously shown to be localised to the nucleus suggesting that it only associates with the nuclear form of the exosome (Mitchell et al., 2003a). Consistent with this finding, Rrp47p was not required for cytoplasmic mRNA turnover (Mitchell et al., 2003a). The defects seen in *rrp47Δ* strains in the processing of 5.8S rRNAs resemble those seen in strains that lack Rrp6p. Furthermore, Rrp6p and Rrp47p played similar roles in the 3' processing of pre-snoRNAs. The functional similarities between these two proteins prompted us to investigate the interdependence of their association with the exosome. Although Rrp47p and Rrp6p associated with the same exosome fraction, the absence of Rrp47p did not affect either the expression level of Rrp6p or its assembly into the exosome.

Finally, to investigate the possible association of Rrp47p with an Rrp6p complex distinct from the exosome, density gradient analysis was performed. An epitope-tagged version of Rrp47p did not clearly cosediment with the peak of Rrp6p corresponding to complex A and the majority of the protein was also not concentrated in the exosome fractions. This sedimentation pattern of Rrp47p might be attributed to the tag on the protein, potentially destabilising its interaction with the exosome.

In summary, Rrp47p is an exosome associated-cofactor that may function in the recruitment of specific classes of exosome substrates and targeting to Rrp6p.

VII.C. The regulation of gene expression at the level of nuclear turnover

In budding yeast, the predominant nuclear decay pathway for aberrant mRNAs involves 3'-5' degradation by the exosome (Bousquet-Antonelli et al., 2000). To identify potential natural targets for nuclear turnover, microarray analysis were performed in collaboration of Frederic Devaux, Ecole Normale Superieure, France. The advantage of using microarray technology is that it allows the simultaneous analysis of thousands of genes within a single experiment. These analyses were carried out on strains lacking the nuclear-specific exosome component Rrp6p, the exosome cofactor Rrp47p or carrying a temperature sensitive mutation in the core exosome component Rrp41p.

At first, we attempted to assess the validity of the microarray data by checking the abundance of mRNA from various genes using Northern hybridisation. The discrepancies between the levels of mRNA predicted by microarray and those seen in the Northern hybridisation analysis showed that these microarray data required validation by conventional methods (Northern hybridisation, primer extension and others).

One of the genes shown to be accumulating by microarray and later verified by northern hybridisation was *NRD1*, which encodes a previously characterised RNA-binding protein (Steinmetz and Brow, 1996). This finding prompted us to investigate further the role apparently played by the exosome in regulating the expression of *NRD1*.The RNA-binding proteins Nrd1p and Nab3p and the putative helicase Sen1p are required in the pathway for 3'-end formation of snRNA and snoRNA precursor transcripts. Nrd1p was also found to auto-regulate the levels of the *NRD1* transcripts through binding to an Nrd1p-responsive element near the 5'-end of the *NRD1* mRNA (Steinmetz et al., 2001). To confirm that the regulation of the *NRD1* expression takes place at the level of nuclear turnover and involves the nuclear exosome, the accumulation of *NRD1* mRNA levels was assessed in mutants of the Ski complex which are required for the 3'-5' cytoplasmic RNA turnover pathway. The levels of the *NRD1* transcript were unaffected in these mutants indicating that observed regulation does not occur at the level of the cytoplasmic turnover.

Although the levels of the *NRD1* mRNA were seen to increase in the exosome mutants (*rrp6Δ*, *ski6-100* and *rrp47Δ*) and in the *sen1-1*mutant, the accumulation of the *NRD1* transcripts was not substantially increased in the double mutants *sen1-1/rrp6Δ* and *nrd1-102/rrp6Δ*. This result suggested that the effect of Nrd1p, Sen1p and Rrp6p on the regulation of *NRD1* levels are additive rather than synergistic and that all three proteins function in the same pathway. In addition, the accumulation of the *NRD1* transcripts in exosome mutants was accompanied by the appearance of 3'-truncated *NRD1* mRNA species. These truncated species are thought to be generated by the binding of Nrd1p to a sequence element present in the 5'-UTR of the *NRD1* transcript leading to premature transcription termination of RNA polymerase II. The stabilisation of these truncated species in the exosome mutants indicates that they are normally degraded by the exosome in a 3'-5' direction.

We propose that the 3'-truncated species sequester enough Nrd1p to cause the up-regulation of the *NRD1* mRNA. Interactions between Nab3p and Mtr4p or alternatively between Nrd1p and Rrp47p recruit the exosome to degrade the truncated *NRD1* transcripts.

VII.D Future directions

VII.D.1 An Rrp6p surveillance complex in the nucleus

The process of mRNA surveillance plays a key role in the biology of eukaryotic cells. mRNA surveillance functions to increase the fidelity of gene expression by degrading aberrant mRNAs that, if translated, would produce deleterious proteins. A second biological role for mRNA surveillance might be to restrict entry into the functional mRNA pool to only those transcripts that possess the proper regulatory elements.

Various studies have assigned the nuclear-specific exosome component Rrp6p a role in nuclear surveillance. The export and translation of RNAs with defects in processing is restricted by Rrp6p, which causes their retention in the nucleus and ultimate degradation (Bousquet-Antonelli et al., 2000;Hilleren et al., 2001;Torchet et al., 2002). The interaction of Rrp6p with various processing and export factors argues for the existence of an Rrp6p surveillance complex where Rrp6p acts independently of the core exosome.

Rrp6p was first identified via a mutation that suppressed the temperature sensitive lethality of a *pap1-1* mutation in the poly(A) polymerase and was shown to genetically interact with Pap1p (Briggs et al. 1998). Rrp6p was also shown to interact physically with the nuclear poly(A)$^+$ mRNA-binding protein Npl3p, indicating a possible role for Rrp6p in regulating and/or assessing the structure of mRNPs (Burkard and Bulter, 2000). Npl3p interacts with the transcripts during or soon after transcription and leaves the nucleus associated with poly(A)$^+$ mRNA (Lee et al., 1996;Lei et al., 2001). Npl3p also interacts with the large subunit of the cap-binding complex, which itself copurified from yeast cell extracts in a complex containing Rrp6p (Shen et al., 2000).

Gbp2p a novel poly(A)$^+$ mRNA binding protein which copurified with Rrp6p-TAP was shown to be homologous to Npl3p and to be also involved in the export of poly(A)$^+$

mRNA (Windgassen and Krebber, 2003). Gbp2p associates with the THO/TREX complex that consists of various transcription and export factors. Gbp2p and Npl3p are hnRNPs which are proposed to function in nearly every step of RNA maturation. The hnRNP proteins are thought to package the mature RNA into an mRNP particle ready for export. The presence of specific hnRNP proteins (like Npl3p and Gbp2p) within the mRNP particle may be recognised by an Rrp6p-surveillance complex that allows its export to the cytoplasm. Analysis of RNA stability in Gbp2p mutants would give more insight into the nature of the RNAs associated with Gbp2p. In addition, generating double mutants between Gbp2p and export factors would reveal whether such RNAs are restricted to the nucleus and targeted for degradation by a Gbp2p-Rrp6p-surveillance complex.

The identification of the NMD factor in the Rrp6p-associated complex further suggests a possible role for an Rrp6p complex in ensuring that RNAs harbouring premature termination codons are not exported to the cytoplasm. However, so far there is no indication of an NMD pathway occurring in the nucleus involving Rrp6p and the exosome in yeast (Lejeune et al., 2003). Since in mammalian cells, NMD was shown to be associated with the nucleus and to involve Rrp6p and the exosome further studies might yet reveal a role for nuclear NMD in yeast cells.

VII.D.1 Association of Rrp6p with pre-ribosomal particles

While many features of the pre-rRNA processing pathway are now relatively clear, the pathway of ribosome assembly is still being elucidated. Over the past years a large number of factors that are required for ribosome synthesis in yeast have been identified and it has become clear that a pathway must exist for their ordered assembly and disassembly from the pre-rRNAs and rRNAs.

Rrp6p was detected in pre-40S and pre-60S sized complexes that were shown to be sensitive to RNase treatment. As mentioned before Rrp6p is specifically required for the 3'-end processing of the 5.8S rRNA component of the 60S ribosomal particle. However

there is no evidence for its involvement in 40S biogenesis (Briggs et al., 1998). So far, many pre-60S particles and pre-40S particles have been identified (Nissan et al., 2002). It is likely that several further distinct pre-60S particles remain to be characterised since several known 60S synthesis factors (such as the exosome components) have not been detected in pre-ribosomal particles. In addition, the pre-40S particles are less well characterised and further studies might identify factors involved in their synthesis (Fatica and Tollervey, 2002). Alternatively, complex B might be an mRNP complex rather than a pre-40S ribosomal particle. Recent studies have identified complexes of similar size corresponding to mRNP complexes (Lang and Fridovich-Keil, 2000). Characterisation of the proteins and RNAs associated with complexes B and C might give more insight into the role played by Rrp6p in these complexes. Whether Rrp6p within these complexes is associated with specific sets of RNAs will be the subject of future studies.

VII.D.3. Nuclear turnover and the regulation of gene expression

Microarray analysis revealed the accumulation of *NRD1* mRNA in mutants lacking the exosome components, Rrp6p, Rrp41p and the cofactor Rrp47p. We propose a model in which the expression of *NRD1* mRNA, which is regulated by Nrd1p, involves the nuclear exosome. Down regulation of the *NRD1* mRNA depends on the putative RNA helicase Sen1p and the RNA-binding protein Nab3p. The link between the exosome and the Nrd1 complex (Nrd1p, Sen1p and NAb3p) could be mediated by in the interaction between Nab3p and Mtr4p, a putative helicase and cofactor of the nuclear exosome. Results presented in this work have characterised Rrp47p as a novel exosome cofactor. Two-hybrid analysis further revealed an interaction between Nrd1p and Rrp47p suggesting that it could constitute another mediator of the Nrd1p complex-exosome interaction. Rrp47p is thought to facilitate the interaction of Rrp6p with RNA substrates, possibly including the *NRD1* mRNA.

Rrp6p and the nuclear exosome were previously shown to be involved in the degradation of aberrant transcripts. However, recent studies have revealed that normal RNAs are retained in the nucleus of export mutants are degraded in a 3'-5' direction by a pathway involving Rrp6p and the large subunit of CBC, Cbc1p (Das et al., 2003). This is consistent with the co-purification of Cbc1p with Rrp6p and suggests that recognition of the cap might be a prerequisite for entry into the nuclear degradation pathway (Das et al., 2000).

Finding certain wild-type mRNAs that are particularly protected from degradation by the absence of the Nrd1p complex, the exosome or CBC would provide further evidence consistent with the view that the proposed Nrd1p pathway is a normal pathway acting to regulate the expression of a special class of mRNAs.

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Rrp47p Is an Exosome-Associated Protein Required for the 3′ Processing of Stable RNAs

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Related exosome complexes of 3′→5′ exonucleases are present in the nucleus and the cytoplasm. Purification of exosome complexes from whole-cell lysates identified a Mg^{2+}-labile factor present in substoichiometric amounts. This protein was identified as the nuclear protein Yhr081p, the homologue of human C1D, which we have designated Rrp47p (for rRNA processing). Immunoprecipitation of epitope-tagged Rrp47p confirmed its interaction with the exosome and revealed its association with Rrp6p, a 3′→5′ exonuclease specific to the nuclear exosome fraction. Northern analyses demonstrated that Rrp47p is required for the exosome-dependent processing of rRNA and small nucleolar RNA (snoRNA) precursors. Rrp47p also participates in the 3′ processing of U4 and U5 small nuclear RNAs (snRNAs). The defects in the processing of stable RNAs seen in *rrp47*-Δ strains closely resemble those of strains lacking Rrp6p. In contrast, Rrp47p is not required for the Rrp6p-dependent degradation of 3′-extended nuclear pre-mRNAs or the cytoplasmic 3′→5′ mRNA decay pathway. We propose that Rrp47p functions as a substrate-specific nuclear cofactor for exosome activity in the processing of stable RNAs.

The eukaryotic 18S, 5.8S, and 25S rRNAs (yeast nomenclature is given) are generated from a single large RNA polymerase I transcript by a series of endonucleolytic and exonucleolytic RNA processing reactions (reviewed in reference 39). The earliest detectable transcript in yeast, the 35S precursor rRNA (pre-rRNA), also undergoes extensive posttranscriptional modification involving predominantly pseudouridine formation and ribosyl-2′-O-methylation. These modifications are directed to specific nucleotides within the ~7-kb-long 35S pre-rRNA via complementary base-pairing mechanisms involving ~70 different small nucleolar RNAs (snoRNAs).

The snoRNAs can be divided into two major functional groups; the box C/D snoRNAs direct methylation of ribosyl-2′-hydroxyl groups, whereas the H/ACA snoRNAs direct the conversion of uridine to pseudouridine (for reviews, see references 4 and 21). Genes encoding snoRNAs have a varied organization, but in yeast and mammals all are transcribed by RNA polymerase II. Most yeast snoRNA genes are expressed as individual transcripts from their own promoters, whereas several are processed from common primary transcripts and a few are encoded within the introns of protein-coding genes. Gene clusters are the predominant organization of snoRNA genes in plants, whereas the majority of mammalian snoRNAs are intron encoded.

The synthesis of the mature 3′ ends of all characterized snoRNAs requires endonucleolytic cleavage of the transcript, followed by 3′→5′ exonucleolytic processing. Endonucleolytic cleavage is by Rnt1p, the yeast RNase III homologue, or by cleavage factor 1A (CF1A), which is also required for the 3′ end processing of mRNA transcripts (15). Maturation of intron-encoded snoRNAs involves linearization of the excised intron lariat, either by the debranching enzyme Dbr1p or endonucleolytic cleavage, followed by exonucleolytic processing (30, 31).

The 3′ processing of snoRNAs involves the exosome (1, 37), a complex of 9 to 10 distinct 3′→5′ exonucleases that functions in both RNA processing and degradation pathways. Nuclear and cytoplasmic forms of the exosome complex have been characterized that share 10 common components and differ by the presence of the RNase Rrp6p and the putative GTPase Ski7p, respectively (3). The 10 common components are all essential for cell viability. In contrast, *rrp6*-Δ mutants are viable but temperature-sensitive lethal (ts-lethal) and specifically impaired in exosome-mediated pathways in the nucleus (1, 8, 9, 19, 35, 37). In *rrp6*-Δ strains, most box C/D snoRNAs have short 3′ extensions, indicating a specific role for Rrp6p in the final trimming step. Longer, 3′-extended snoRNA precursors also accumulate in both *rrp6*-Δ mutants and in strains mutant for other exosome components and these species are polyadenylated by poly(A) polymerase.

The yeast spliceosomal snRNAs U1, U2, U4, and U5 are also transcribed by RNA polymerase II and are cleaved in their 3′ flanking regions by Rnt1p. In the case of U1, U4, and U5, it has been demonstrated that Rnt1p cleavage provides an entry site for 3′ exonucleolytic processing by the exosome (1, 37). However, snRNA synthesis is not blocked in strains mutant for either Rnt1p or components of the exosome complex, suggesting that alternative processing pathways exist.

The 3′-end processing of mRNAs typically involves the coordinated cleavage of the nascent transcript and polyadenyl-

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ation of the generated 3′ terminus. The cleavage and polyadenylation reaction is tightly coupled to transcription termination. In *rna14-1* mutants, which are defective in CF1A activity, cleavage is inefficient and readthrough transcripts are generated that extend several kilobases into downstream genes (7). These readthrough transcripts are rapidly processed by the nuclear exosome and then either polyadenylated or degraded by an Rrp6p-dependent mRNA surveillance mechanism (35).

The exosome was initially characterized during analyses of the pre-rRNA processing pathway. Mutation of exosome components inhibits the conversion of 7S pre-rRNA to 5.8S rRNA and the degradation of the 5′ external transcribed spacer (5′ETS) fragment, with the accumulation of heterogeneous populations of partially processed RNAs (1, 3, 12, 26). RNA analyses of *rrp6*-Δ mutants revealed a distinct defect in 7S pre-rRNA processing (9), with the accumulation of 5.8S rRNA species that are 3′ extended by ~30 nucleotides (nt). Like most mutations that inhibit the synthesis of 5.8S and 25S rRNA, exosome mutants also show indirect effects on early pre-rRNA cleavage events that are required for 18S rRNA synthesis (2, 43).

Genetic studies indicate that exosome function in vivo requires the activity of additional cofactors that are not found in exosome preparations or are present only at substoichiometric levels. All characterized nuclear functions of the exosome require the putative RNA helicase Mtr4p/Dob1p (12, 24), whereas exosome-mediated cytoplasmic mRNA turnover pathways are dependent upon Ski7p and the Ski complex, comprising the putative RNA helicase Ski2p and the proteins Ski3p and Ski8p (10, 20, 38).

Here we report that the *YHR081w* gene product is a novel exosome-associated factor that we designate Rrp47p. This protein was previously shown to be nuclear in a systematic localization study (22) (for an image, see http://ygac.med.yale.edu) and is homologous to the human nuclear protein C1D (14). Rrp47p and C1D are both implicated in DNA double-strand break repair (14, 41). We demonstrate that *rrp47*-Δ mutants are defective in the nuclear processing of pre-rRNA, snoRNAs, and snRNAs. In contrast, Rrp47p is not required for the exosome-mediated cytoplasmic 3′→5′ mRNA decay pathway or the nuclear degradation of 3′-extended readthrough transcripts generated in the *rna14-1* mutant. Rrp47p therefore exhibits characteristics of an exosome cofactor required specifically for the 3′ processing of stable RNAs.

MATERIALS AND METHODS

Strains. Yeast transformations were performed with plasmids or PCR-amplified DNA by using standard molecular biological techniques. Transformants were isolated by growth on selective media and integrants were screened by PCR directly on restreaked colonies. Strains were routinely grown in yeast extract-peptone-dextrose (YPD)-rich medium. The zz-Rrp44p strain (P203) was grown in rich medium containing galactose and sucrose as carbon sources. For the analysis of the *rna14-1 GAL::rrp41* mutant, the strain was pregrown in YPD medium for 24 h to deplete Rrp41p levels before transfer to 37°C.

Strains P203 (zz-Rrp44p), P414 (Rrp47p-zz), and YRH1 (Rrp6p-TAP) were generated by integration of PCR-amplified DNA by using plasmids pTL27 (23), pJE39 (a gift from J. Brown, University of Newcastle) and pBS1479 (32), respectively. The Rrp47p-zz allele was shown to be functional by growth rate assays and analyses of 5.8S rRNA species. The *rrp47*-Δ and *rna14-1 rrp47*-Δ mutants were generated by transferring the *rrp47*-Δ::Kan^r-null allele from genomic DNA of a strain (accession no. Y01909) obtained from EUROSCARF (University of Frankfurt, Frankfurt, Germany) into a haploid derivative of the wild-type strain BMA38 (5) and an *rna14-1* strain (35), respectively. The *rrp47*-Δ *rrp6*-Δ strain was generated by crossing the respective single mutant strains, sporulation, and selection for the auxotrophic markers.

Protein analyses. Crude cell extracts from zz-Rrp44p (P203), Rrp47p-zz (P414), Rrp44p-TAP (16), and Rrp6p-TAP (YRH1) strains were prepared by a glass bead extraction procedure in TMN-150 lysis buffer (50 mM Tris-HCl [pH 7.6], 5 mM $MgCl_2$, 150 mM NaCl, 0.1% NP-40). Immunoaffinity purification of exosome complexes on immunoglobulin G (IgG)-Sepharose, elution of retained proteins in TMN-150 containing an increasing gradient of $MgCl_2$, and matrix-assisted laser desorption ionization–nanoelectrospray MS analyses of gel-purified proteins were performed as previously described (3, 25, 34). Immunoprecipitates from strains expressing Rrp47p-zz were treated with RNase A (2 μg ml^{-1} [final concentration]) and incubated for 60 min on ice before a wash with 5 ml of TMN-150 buffer and subsequent digestion with tobacco etch virus (TEV) protease (Invitrogen). Polyclonal antisera were raised in rabbit against His_6-tagged fusion proteins expressed in *Escherichia coli* comprising full-length Rrp4p (26) and the N-terminal 222 residues of Rrp6p after purification from cell lysates by immobilized metal ion affinity chromatography on Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) and SDS-PAGE. Antisera were used at a dilution of 1:5,000 for Western analyses.

RNA analyses. RNA isolation, electrophoresis through acrylamide-urea or agarose-formaldehyde gels, Northern blot transfer, and hybridization with labeled oligonucleotides were performed as described previously (6). The following probes were used in the present study: 20S (oligonucleotide 2), GCTCTTTGCTCTTGCC; $27SA_2$ (oligonucleotide 3), TGTTACCTCTGGGCCC; 27S (oligonucleotide 6), AGATTAGCCGCAGTTGG; 25S (oligonucleotide 7), CTCCGCTTATTGATATGC; 18S (oligonucleotide 8), CATGGCTTAATCTTTGAGAC; 5.8S rRNA (oligonucleotide 17), GCGTTGTTCATCGATGC; 5.8S/ITS2 boundary (oligonucleotide 20), TGAGAAGGAAATGACGCT; 5′ETS (oligonucleotide 33), CGCTGCTCACCAATGG; U14 (oligonucleotide 202), TCACTCAGACATCCTAGG; U18-3′ (oligonucleotide 206), GCTCTGTGCTATCGTC; U14-3′ (oligonucleotide 210), GTATACGATCACTCAGAC; U18 (oligonucleotide 215), ATATATTATCTGTCTCCTC; snR13 (oligonucleotide 227), CACCGTTACTGATTTGGC; snR44 (oligonucleotide 237), CATGGGATTAAATATCCCGG; U4 (oligonucleotide 243), CCGTGCATAAGGAT; U5 (oligonucleotide 244), AATATGGCAAGCCC; U4-3′ (oligonucleotide 246), AAAGAATGAATATCGGTAATG; SCR1 (oligonucleotide 250), AAGGACCCAGAACTACCTTG; snR38 (oligonucleotide 255), GAGAGGTTACCTATTATTACCCATTCAGACAGGGATAACTG; snR8 (oligonucleotide 269), GCATGTTTAATATGTATCAT; ACT1 (oligonucleotide 400), TCTTGGTCTACCGACGATAGATGGGAAGACAGCA; CYH2 (oligonucleotide 405), GTGCTTTCTGTGCTTACCGATACGACCTTTACCG; and MFA2pG (oligonucleotide 487), ATATTGATTAGATCAGGAATTCC.

RESULTS

Purification of exosome-associated factors. To identify novel exosome cofactors, cell lysate from a strain expressing an epitope-tagged form of the exosome component Rrp44p (zz-Rrp44p), which contains two copies of the "z" domain of protein A fused to the N terminus, was passed over an IgG-Sepharose column. After being washed, associated proteins were eluted in a gradient of 0 to 2 M $MgCl_2$, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and identified by mass spectrometry (Fig. 1A).

In addition to Rrp6p (Fig. 1A, lanes 3 to 4), two polypeptides identified by mass spectrometry in the eluate fractions were recovered at substoichiometric levels, as judged by visual inspection of Coomassie-stained gels (Fig. 1A). A ~25-kDa protein that dissociated from zz-Rrp44p at ~0.2 to 0.4 M $MgCl_2$ (Fig. 1A, lanes 1 to 2) was identified as the product of the *YHR081w* gene. A similarly sized protein was observed in the equivalent eluate fractions from zz-Rrp4p retained material in our previous analyses (3) but not identified. We designated this protein Rrp47p on the basis of its role in rRNA processing (see below) and copurification with the exosome (which contains Rrp4p, Rrp40p-Rrp46p, and Rrp6p). An ~80-kDa protein that dissociated from zz-Rrp44p with the other

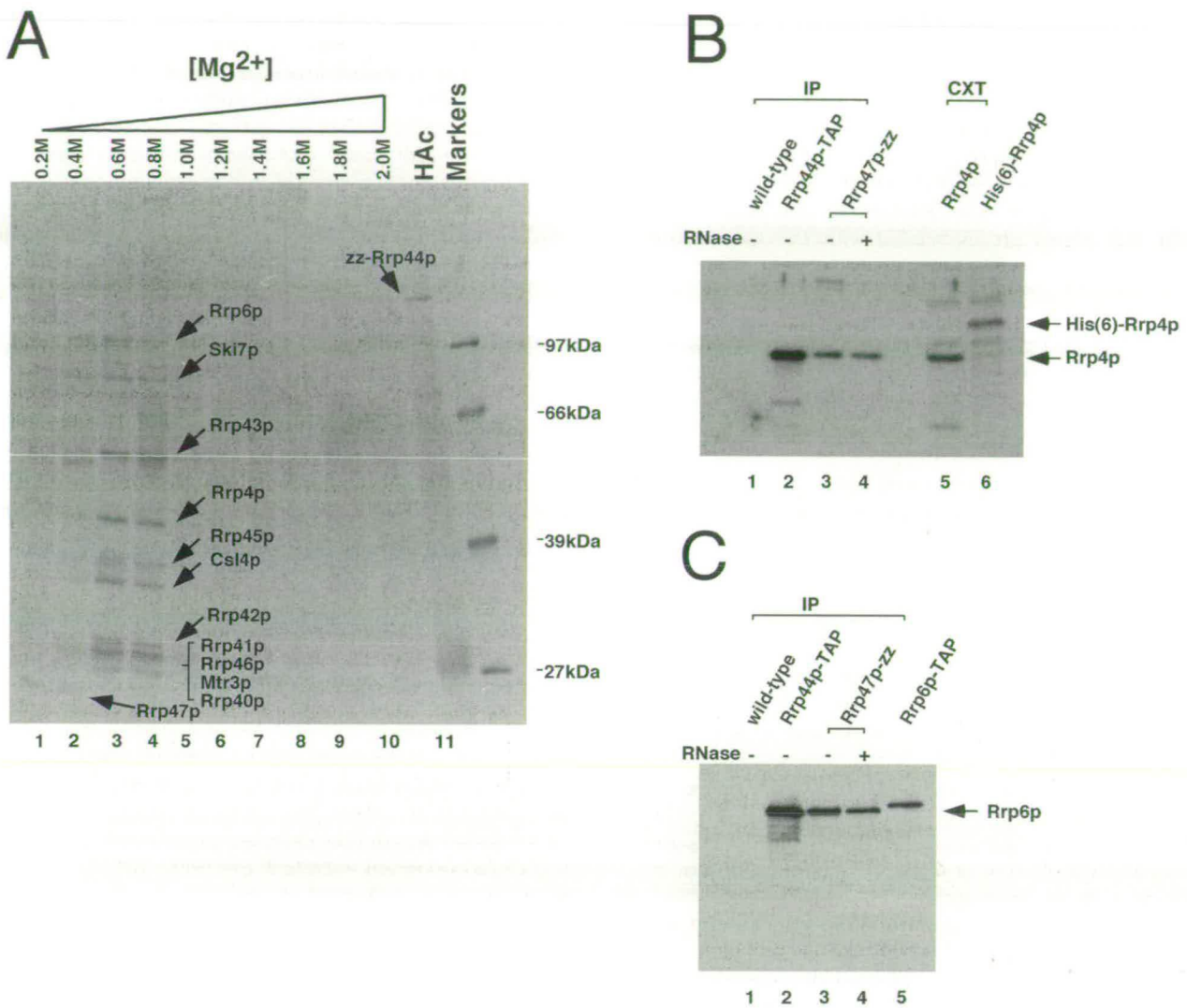

FIG. 1. Rrp47p is a novel exosome-associated factor. (A) Identification of Rrp44p-associated proteins. SDS-PAGE analysis of protein eluted from immunoprecipitated zz-Rrp44p was carried out. Lanes 1 to 10, linear 0 to 2 M $MgCl_2$ gradient in TMN-150 buffer; HAc lane, postgradient eluate in 0.5 M acetic acid. Proteins were visualized by staining with Coomassie G-250 and identified by mass spectrometry. Rrp47p was eluted in lanes 1 to 2 (~0.2 to 0.4 M $MgCl_2$). (B and C) Rrp47p is associated with Rrp4p and Rrp6p. Western blot analyses of immunoprecipitates from wild-type (lane 1), Rrp44p-TAP (lane 2), and Rrp47p-zz (lanes 3 to 4) strains, with antisera specific to Rrp4p (B) and Rrp6p (C) were done. Rrp47p-zz immunoprecipitates were eluted with or without prior digestion with RNase A. As controls for the specificity of the Rrp4p antiserum, cell extracts were loaded from strains expressing wild-type Rrp4p (B, lane 5) or epitope-tagged His (6)-Rrp4p (B, lane 6). Cleaved Rrp6p-TAP was included as a positive control for the Rrp6p Western analysis (C, lane 5). This protein is predicted to migrate slower than enodogenous Rrp6p in SDS-PAGE gels due to the presence of the calmodulin-binding domain.

exosome components (Fig. 1A, lanes 3 to 4) was identified as Ski7p (YOR076c), an exosome cofactor required for cytoplasmic 3′→5′ mRNA decay (38).

To confirm the interaction between Rrp47p and the exosome, a strain was constructed that expressed a C-terminal fusion protein linked by a TEV protease cleavage site (Rrp47p-zz). Lysate from the Rrp47p-zz strain was passed over an IgG-Sepharose column, and bound proteins were eluted by digestion with TEV protease. The eluate was assayed for the presence of Rrp4p by Western blot analyses, by using an anti-Rrp4p antiserum (Fig. 1B, lanes 3 to 4) (see Materials and Methods). As a positive control, lysate from a strain expressing TAP-tagged Rrp44p (Rrp44p-TAP) (16) was also analyzed (Fig. 1B, lane 2). Rrp4p was recovered from both the Rrp47p-zz and Rrp44p-TAP columns but was not detected in the eluate from the wild-type control strain (Fig. 1B, lane 1), confirming the interaction between Rrp47p and Rrp4p.

Previous analyses failed to detect any RNA species that copurified with the exosome complex (P. Mitchell and D. Tollervey, unpublished data). However, it remained formally possible that Rrp47p is associated with a ribonucleoprotein substrate of the exosome. Immunoprecipitated Rrp47p-zz was therefore treated with RNase A at 2 μg ml^{-1} for 60 min prior to digestion with TEV protease. Rrp4p was detected at comparable levels with or without RNase treatment (Fig. 1B, lanes 3 and 4). The immunoprecipitation analyses shown were per-

formed in buffer containing 0.5 M NaCl, and the coimmunoprecipitation of Rrp47p-zz with Rrp4p therefore reflects a stable interaction.

The relative intensities of Rrp47p and the other exosome components observed in Coomassie blue-stained gels (Fig. 1A) suggests that Rrp47p is present in ~20% of the exosome complexes. Similarly, ~20% of the immunoprecipitated exosome complex is associated with Rrp6p (3). To assess whether Rrp47p and Rrp6p are associated with the same exosome fraction, the Rrp47p-zz immunoprecipitates were assayed for the presence of Rrp6p with an anti-Rrp6p antiserum (Fig. 1C, see Materials and Methods). Western blot analyses strongly decorated a single band of the predicted size in the Rrp47p-zz immunoprecipitate (Fig. 1C). This band was absent from total protein extracted from an *rrp6*-Δ strain (data not shown).

The *RRP47* gene is known to be nonessential for cell viability (17). To analyze the requirement for Rrp47p in exosome function, we generated an *rrp47*-Δ null allele (see Materials and Methods). The *rrp47*-Δ mutant exhibited a slow-growth phenotype at 25, 30, and 37°C, with a doubling time of 6.3 h at 37°C compared to 2.4 h for the isogenic wild-type strain (data not shown). Western blot analyses demonstrated that Rrp6p levels were not significantly altered in the *rrp47*-Δ mutant and that the coimmunoprecipitation of Rrp6p with an epitope-tagged Rrp4p fusion protein (zz-Rrp4p) was unaffected in the absence of Rrp47p (data not shown). We conclude that Rrp47p is not required for the expression of Rrp6p or its association with the exosome.

Rrp47p is required for normal pre-rRNA processing. Previous analyses revealed that exosome mutants are delayed in the early pre-rRNA cleavages at sites A_0, A_1, and A_2 (2, 43) (Fig. 2A). To address the role of Rrp47p in rRNA synthesis, RNA was isolated from the isogenic wild type, from *rrp47*-Δ and *rrp6*-Δ mutants, and from the ts-lethal *rrp4-1* mutant during growth at 30°C and after a 3 h shift to 37°C.

Large pre-rRNA processing intermediates were analyzed by Northern blot hybridization of RNA resolved in agarose-formaldehyde gels (Fig. 2). The *rrp47*-Δ mutant exhibited a mild accumulation of the 35S pre-rRNA, accompanied by an accumulation of the 23S and 21S RNAs (Fig. 2B) that are generated by cleavage at site A_3 in the absence of prior processing at sites A_0 to A_2 (18). These results indicate that early processing events at sites A_0, A_1, and A_2 are slowed in the *rrp47*-Δ mutant. The $27SA_2$ pre-rRNA also accumulated in the *rrp47*-Δ mutant (Fig. 2B). Primer extension (Fig. 2C) confirmed the accumulation of $27SA_2$ and showed mild depletion of $27SA_3$, as well as depletion of both $27SB_L$ and $27SB_S$ in the *rrp47*-Δ strain at 37°C. These results indicate that subsequent processing of the $27SA_2$ pre-rRNA was also impaired, as seen in previous analyses of other mutants of the exosome complex (2). The aberrant 17S′ species (Fig. 2B) extends from heterogeneous sites within the 18S rRNA to the 3′ end of 5.8S rRNA. This species has also been observed in exosome mutants (2) and is produced by a 5′→3′ degradation pathway when both processing in ITS1 and 3′ degradation are inhibited (40).

Ethidium bromide-stained acrylamide-urea gels of RNA from the *rrp47*-Δ mutant revealed a decrease in the level of 5.8S rRNA and the appearance of an aberrant RNA of ~200 nt (data not shown). The size and abundance of this RNA is similar to the 3′-extended "5.8S+30" rRNA that accumulates in strains lacking Rrp6p (3, 9). Northern blot hybridization with probes complementary to the mature 5.8S rRNA and to the 5.8S-ITS2 boundary hybridized to the same 5.8S+30 species in the *rrp47*-Δ and *rrp6*-Δ mutants (Fig. 3A). This 5.8S rRNA processing defect is distinct from that observed in strains mutant for "core" components of the exosome, such as Rrp4p, which is characterized by the accumulation of longer, 3′-extended 5.8S species (Fig. 3A) (26). Exosome mutants are also defective in the degradation of the 5′ETS fragment that is generated upon initial cleavage of the 35S pre-rRNA at site A_0 (1, 12). Hybridization with a probe complementary to a sequence 5′ of A_0 demonstrated that the 5′ETS fragment accumulated to a comparable level in the *rrp47*-Δ and *rrp6*-Δ mutants (Fig. 3B). Substantially stronger accumulation was seen in the *rrp4-1* mutant (Fig. 3B) and in strains depleted of other exosome components (1, 12).

Exosome mutants show a moderate inhibition of the endonucleolytic cleavages at sites A_0, A_1, and A_2. However, this phenotype is also seen for many other strains defective in 60S subunit synthesis (reviewed in reference 39) and is likely to be indirect. The defects seen in these early pre-rRNA processing steps in the absence of Rrp47p are similar to those of core exosome mutations. In contrast, the role of the exosome in the exonucleolytic processing of the 5.8S rRNA and in 5′ETS degradation are very likely to be direct and here the role of Rrp47p appears to resemble closely that of Rrp6p rather than the core exosome.

Rrp47p is required for snoRNA synthesis. Mutations in the exosome complex cause the accumulation of 3′-extended and polyadenylated snoRNA precursors. In *rrp6*-Δ mutants, but not in strains mutant for core exosome components, most box C/D snoRNAs also retain discrete 3′ extensions of ~3 nt (1, 37). To assay the role of Rrp47p in snoRNA synthesis, acrylamide gel-Northern blots were hybridized with probes specific for snR8 (box H/ACA) and snR13 (box C/D), which are transcribed from their own promoters, the dicistronic U14 snoRNA (box C/D) and the intron-encoded snoRNAs U18, snR38 (box C/D), and snR44 (box H/ACA) (Fig. 4).

As previously reported (1, 31), precursors to the intron-encoded snoRNAs were observed in the wild-type strain (labeled U18-3′, snR38-3′, and snR44-3′ in Fig. 4D to F) that have mature 5′ ends but are 3′ unprocessed. Similar 3′-extended precursors were also observed for snR8 and U14 (Fig. 4A and C). In the *rrp47*-Δ mutant, increased levels of these 3′-extended pre-snoRNAs and shorter 3′-extended species were observed for all snoRNAs tested. This indicates a requirement for Rrp47p in the 3′ processing of all classes of snoRNAs. A very similar pattern of 3′-extended snoRNA species was observed in the *rrp6*-Δ mutant, whereas a distinct pattern was observed in the *rrp4-1* mutant (Fig. 4). The 3′-extended snoRNA species observed in the *rrp6*-Δ mutant are polyadenylated (1, 37), yielding diffuse hybridization signals in the region of some bands (Fig. 4). The similar hybridization patterns observed in the *rrp47*-Δ and *rrp6*-Δ mutants strongly indicate that the extended species observed in the *rrp47*-Δ mutant are also polyadenylated.

Box C/D snoRNAs with short 3′ extensions of ~3 nt were observed in the *rrp6*-Δ mutant (indicated as "+3" species for snR13, U14, U18, and snR38 in Fig. 4) and these RNAs were also detected in the *rrp47*-Δ mutant. The levels of the U14+3

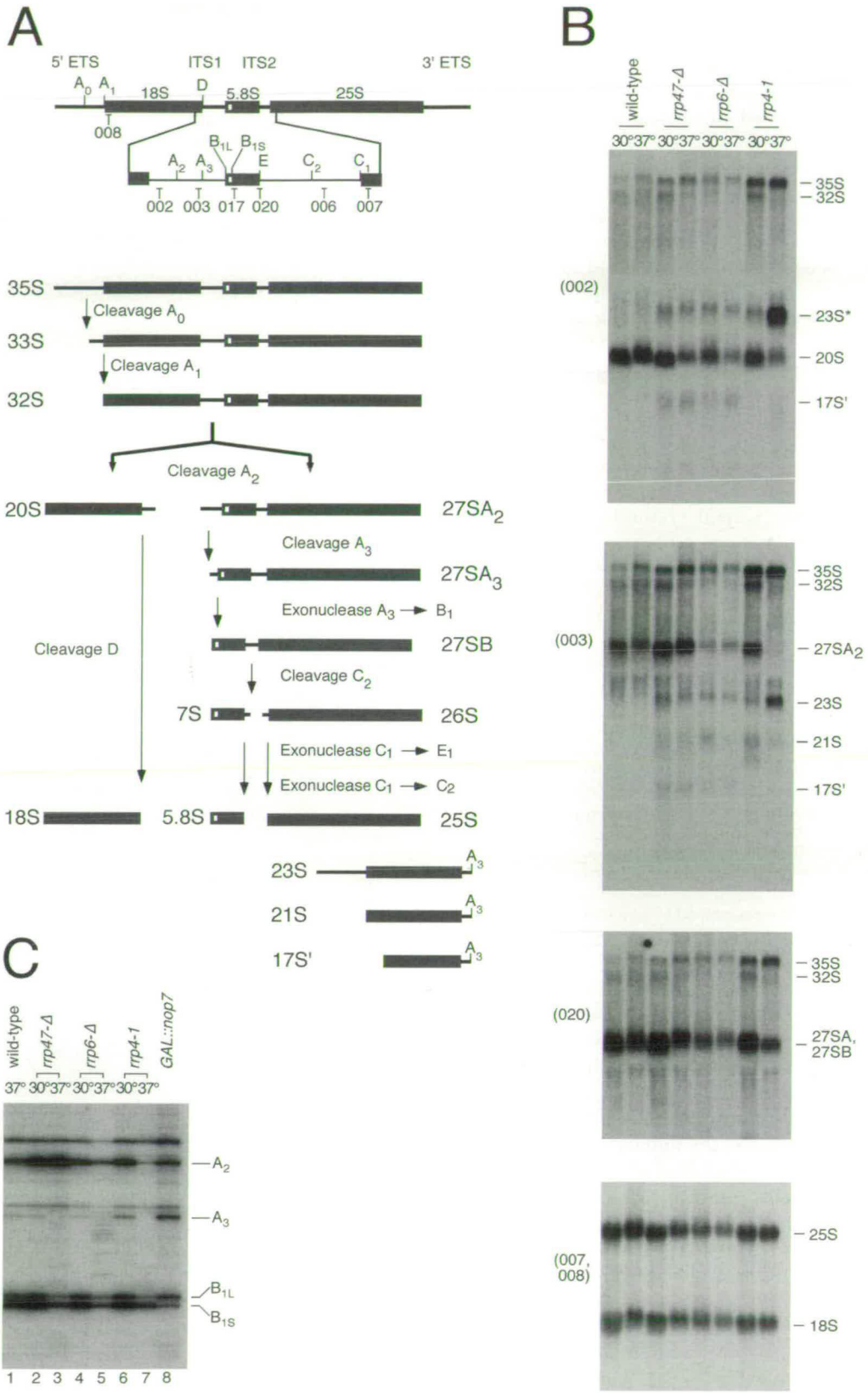

FIG. 2. Rrp47p is required for early pre-rRNA processing events. (A) Organization of the yeast pre-rRNA and processing pathway. The 18S, 5.8S, and 25S rRNAs are separated by internal transcribed spacers ITS1 and ITS2 and flanked by the external transcribed spacers 5′ETS and 3′ETS. Coding regions are indicated by thick bars; spacer regions are indicated by thin lines. Sites within the pre-rRNA complementary to probes used in the present study are indicated. Early cleavages at sites A_0, A_1, and A_2 generate the 20S and $27SA_2$ pre-rRNAs. The 20S pre-rRNA is processed to 18S rRNA by cleavage at site D. $27SA_2$ is processed in ITS1 and ITS2 to generate the 5.8S and 25S rRNAs. Two forms of 27SB, 7S, and 5.8S that differ by 7 nt at their 5′ ends (boxed) are generated by alternative processing pathways. The 23S and 21S pre-rRNAs arise through premature cleavage at site A_3. The 17S′ species results from 5′ degradation of pre-rRNA blocked for processing in ITS1. (B) Northern blot analyses

and snR38+3 species were reduced in the *rrp47-Δ* mutant compared to the levels observed in the *rrp6-Δ* mutant, whereas the U18+3 species appeared to be slightly shorter in the *rrp47-Δ* mutant after transfer to 37°C. Low levels of the snR13+3 species were observed in both the *rrp47-Δ* and *rrp6-Δ* mutants during growth at 30°C (Fig. 4B). In addition, a truncated fragment of snR13 ($snR13_T$) accumulated in the *rrp47-Δ*, *rrp6-Δ*, and *rrp4-1* mutants. This probably corresponds to the 5′-truncated snR13 species previously observed in *sen1-1* and *ssu72-2* mutants (13, 33). The reason for the appearance of this species is unclear but it strongly correlates with the generation or stabilization of snR13 readthrough transcripts, which are seen in several exosome mutant strains (A. Fatica and D. Tollervey, unpublished data).

We conclude that Rrp47p and Rrp6p play related roles in the initial processing of 3′-extended snoRNA precursors. However, the lack of Rrp47p has milder effects on the removal of the last few nucleotides than the absence of Rrp6p.

Rrp47p is required for normal synthesis of U4 and U5 snRNAs. The absence of Rrp6p or mutations in core components of the exosome complex also causes defects in the 3′ processing of snRNAs (1, 37). We therefore analyzed the levels of the U4 and U5 snRNAs and their processing intermediates in the *rrp47-Δ* mutant (Fig. 5).

Two 3′-extended pre-U4 species are detected in wild-type cells (1), denoted U4-3′I and U4-3′II (Fig. 5A). The U4-3′I intermediate has a short 3′ extension of ~6 nt, whereas the larger U4-3′II precursor is a pair of similarly sized RNAs ~140 nt longer than the mature U4 snRNA. Higher levels of the U4-3′II intermediate were observed in the *rrp47-Δ* mutant than in the wild-type strain (Fig. 5A). Shorter 3′-extended species were also observed in the *rrp47-Δ* mutant but not in the wild-type strain. A similar phenotype was observed for the *rrp6-Δ* and *rrp4-1* mutants (1, 37). No clear effect was observed on the levels of the U4-3′I or mature U4 snRNA in the *rrp47-Δ* mutant compared to the isogenic wild-type control strain.

In wild-type cells, mature U5 snRNA is present as a major, long form ($U5_L$) and a less-abundant short form ($U5_S$) that differ by ~35 nt at their 3′ ends. Precursor species with short extensions are observed in wild-type strains for both $U5_L$ and $U5_S$ (denoted $U5_L$-3′ and $U5_S$-3′, respectively), as well as a longer precursor denoted U5-3′I (Fig. 5B). The $U5_L$-3′, $U5_S$-3′, and U5-3′I precursors accumulated in the *rrp47-Δ*, *rrp6-Δ*, and *rrp4-1* mutants compared to the wild-type strain (Fig. 5B). Mutants in the exosome complex lead to increased levels of $U5_S$, while having little effect on the levels of $U5_L$ (1). Comparable levels of the minor $U5_S$ and major $U5_L$ snRNA were observed in the *rrp47-Δ* mutant, whereas the $U5_S$ form was the more abundant form in the *rrp6-Δ* and *rrp4-1* mutants (Fig. 5). Quantitative analyses revealed that the $U5_S/U5_L$ ratio increased in the *rrp47-Δ*, *rrp6-Δ* and *rrp4-1* mutants by 1.6-, 3.1-, and 2.9-fold, respectively, compared to the wild-type control.

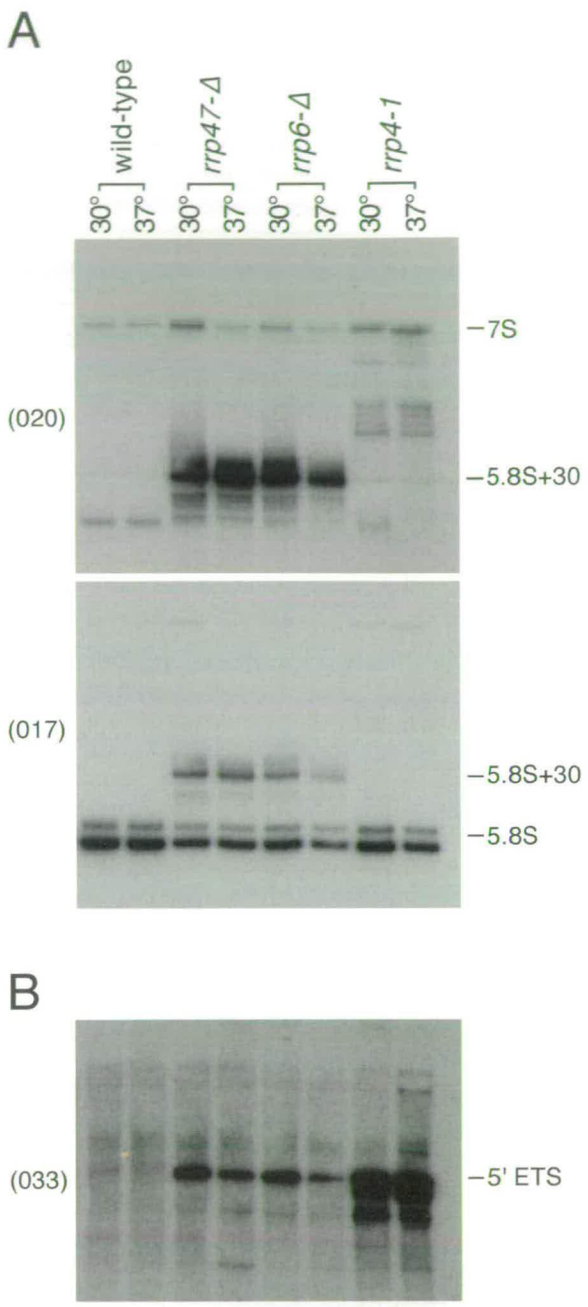

FIG. 3. Rrp47p is required for 7S pre-rRNA processing and 5′ETS degradation. (A) Northern blot analyses of 5.8S rRNA species in a wild-type strain and in exosome mutants. RNA was recovered from wild-type and mutant strains, as in Fig. 2. (Upper panel) Hybridization with a probe specific for 5.8S species extended into ITS2; (lower panel) hybridization with probe specific for the mature 5.8S rRNA. (B) Hybridization with a probe specific for the 5′ETS species. Probes used are indicated in parentheses to the left of each panel. The long and short forms of 5.8S rRNA are clearly resolved.

of pre-rRNAs from wild-type, *rrp47-Δ*, *rrp6-Δ*, and *rrp4-1* strains during growth at 30°C and after transfer to 37°C for 3 h. The probes used are given in brackets to the left of each panel; the species detected are indicated to the right (see panel A). The 23S* species detected with probe 002 is more abundant than 23S detected with probe 003 due to the limited 3′ degradation of 23S pre-rRNA in the *rrp4-1* mutant grown at 37°C. (C) Primer extension analyses of 27S pre-rRNAs with probe 006. Extension products corresponding to 27S species processed to sites A_2, A_3, B_{1L}, and B_{1S} are indicated. RNA from a *GAL::nop7* mutant grown in YPD medium for 24 h is included to provide a marker for the 5′ end of $27SA_3$ (29).

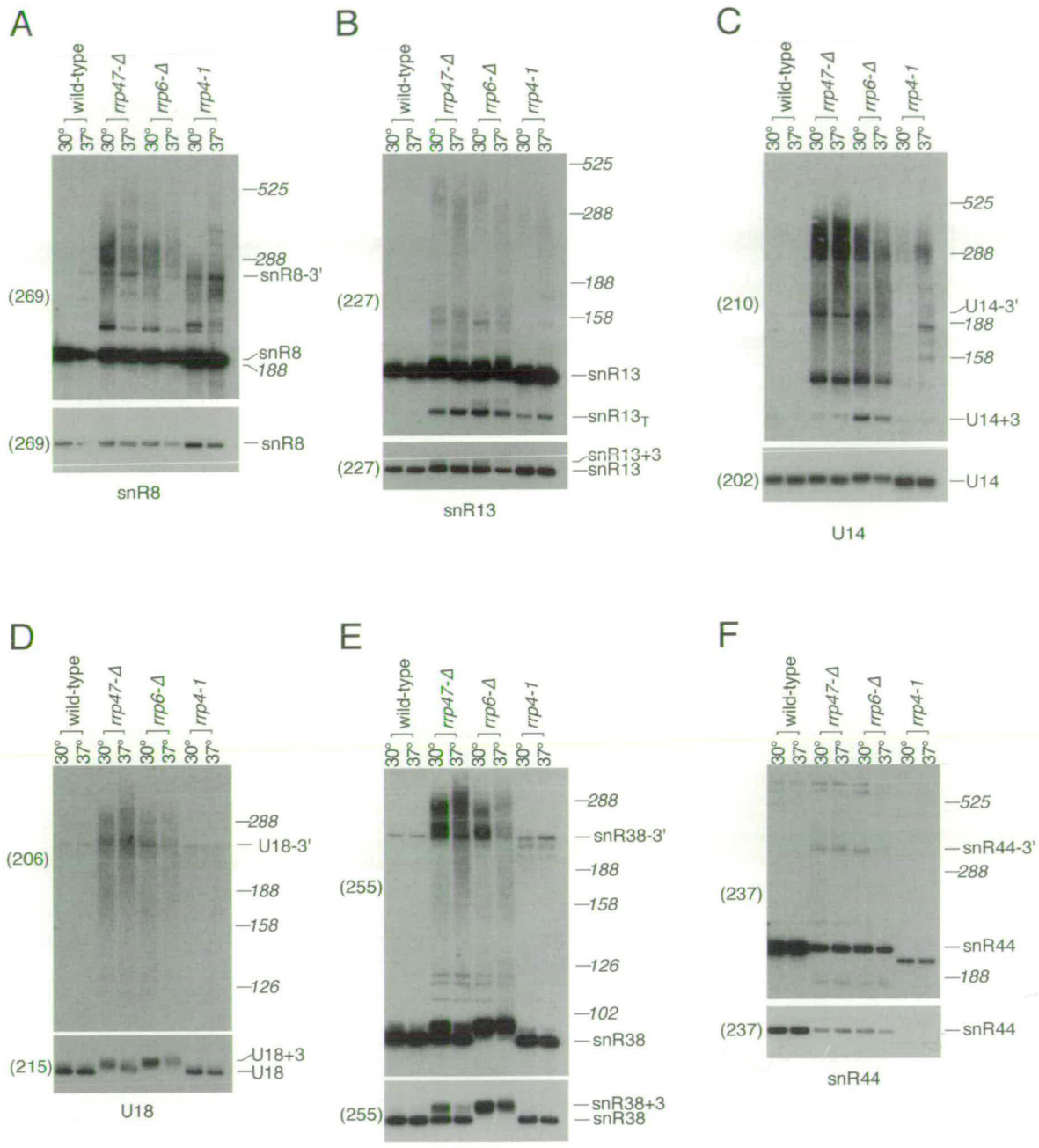

FIG. 4. Rrp47p is required for 3′ processing of snoRNAs. Northern blot analyses of snoRNA species were resolved in 6% (A and F) or 8% (B to E) acrylamide-urea gels. RNA was recovered from wild-type and mutant strains as in Fig. 2. (A) Hybridization with an snR8-specific probe; (B) hybridization with an snR13-specific probe (a truncated form [$snR13_T$] detected in the exosome mutants is indicated); (C) hybridization with a probe specific for 3′-extended U14 species; (D) hybridization with a probe specific for 3′-extended U18 species; (E) hybridization with an snR38-specific probe; (F) hybridization with an snR44-specific probe. Discrete, 3′-extended snoRNA precursors detected in the wild-type strain (snR8-3′, U14-3′, U18-3′, snR38-3′ and snR44-3′), and the snoRNAs with short, 3-nt extensions that are specifically detected in the *rrp47*-Δ and *rrp6*-Δ mutants (snR13+3, U14+3, U18+3, and snR38+3) are indicated. The lower panels show appropriate exposures of the hybridized blots to reveal clearly the mature snoRNAs. The probes used to detect snoRNA species are indicated in brackets to the left of each panel. The electrophoretic mobilities of *SCR1* (525 nt), 7S pre-rRNA (288 nt), 5.8S+30 (188 nt), 5.8S rRNA (158 nt), U14 (126 nt), and U18 (102 nt), determined by hybridization of the same filters, are indicated as size markers. The faster mobility of snR44 in the *rrp4-1* mutant is due to differences in the strain background.

Strains lacking Rrp47p therefore exhibit mild defects in U4 and U5 snRNA processing similar to those seen in strains lacking Rrp6p or mutant for core components of the exosome. We conclude that Rrp47p is required for the exosome-mediated 3′ processing of U4 and U5 snRNAs.

Analysis of the *rrp47*-Δ *rrp6*-Δ double mutant. To analyze further the functional relationship between Rrp47p and Rrp6p, we generated *rrp47*-Δ *rrp6*-Δ double mutants by genetic crossing. Sister strains from full tetrads showing a tetratype segregation for the *rrp47*-Δ and *rrp6*-Δ alleles were grown at

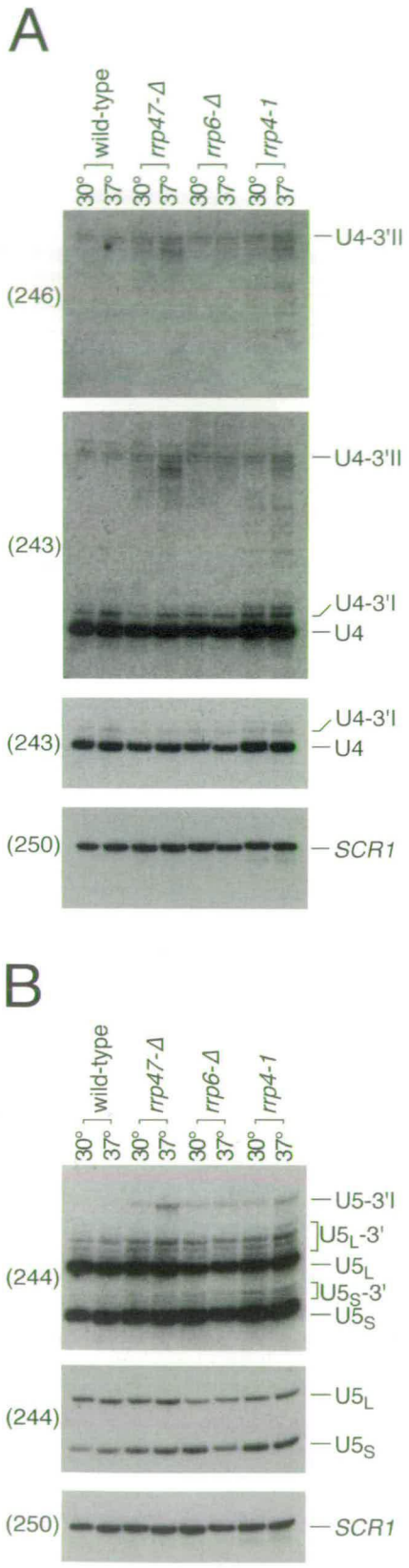

FIG. 5. Analysis of U4 and U5 snRNA species in the *rrp47-Δ* mutant. RNA was recovered from wild-type and mutant strains as in Fig. 2 and resolved in a 6% acrylamide-urea gel. (A) Analysis of U4 snRNA. (Upper panel) Hybridization with a probe specific for 3′-extended U4 snRNA species; (center panels) hybridization with a probe complementary to the mature U4 snRNA; (lower panel) control hybridization with a probe complementary to *SCR1*. (B) Analysis of U5 snRNA. Hybridization was performed with a probe complementary to the mature U5 snRNA. (Upper panel) long exposure (2 days) to reveal the 3′-extended U5 snRNA precursors; (middle panel) short exposure (3 h) to reveal the relative levels of the $U5_L$ and $U5_S$ snRNAs; (lower panel) hybridization with a probe complementary to *SCR1*. The probes used are indicated in brackets on the left of each panel.

30°C in YPD medium. The double-mutant strains were viable, with a growth rate comparable to that of the *rrp6-Δ* single mutant strain, indicating that the proteins do not have redundant functions.

This conclusion was supported by RNA analyses, which showed that the level of the 5.8S+30 intermediate in the *rrp47-Δ rrp6-Δ* double mutant was very similar to either single mutant (Fig. 6A). The 3′ processing of the U14 and snR38 snoRNAs in the *rrp47-Δ rrp6-Δ* double mutant resembled the *rrp6-Δ* single mutant rather than the *rrp47-Δ* single mutant, with a substantial accumulation of the short, 3′-extended species (Fig. 6B and C).

Recombinant Rrp6p has been demonstrated to have exonuclease activity (11), whereas sequence analyses do not suggest such an activity for Rrp47p. We therefore propose that Rrp47p is required for the activity of Rrp6p in 7S pre-rRNA processing and in the initial processing of snoRNA precursors. In contrast, Rrp47p promotes, but is not strictly required for, the Rrp6p-dependent final trimming of box C/D snoRNAs.

Rrp47p is not required for nuclear mRNA surveillance or cytoplasmic mRNA turnover. Rna14p is required for transcription termination of RNA polymerase II and the cotranscriptional cleavage and polyadenylation of mRNA transcripts. The absence of Rrp6p and the depletion of the core exosome component Rrp41p have distinct phenotypes in the mRNA surveillance pathways that degrade the readthrough transcripts generated in the ts-lethal *rna14-1* mutant (35). To address the role of Rrp47p in nuclear mRNA surveillance, we recovered RNA from *rna14-1*, *rna14-1 rrp47-Δ*, *rna14-1 rrp6-Δ*, and *rna14-1 GAL::rrp41* strains at time points after transfer to 37°C and then analyzed the *ACT1* and *CYH2* transcripts by hybridization of agarose gel-Northern blots (Fig. 7A).

Upon transfer to 37°C, the *ACT1* and *CYH2* mRNAs were rapidly depleted in the *rna14-1* mutant. Approximately normal length transcripts were stabilized in the *rna14-1 rrp6-Δ* mutant, whereas long extended transcripts accumulated in the *rna14-1 GAL::rrp41* mutant, as previously reported (35). In contrast, no mRNA stabilization was observed in the *rna14-1 rrp47-Δ* mutant and the *ACT1* and *CYH2* mRNAs were depleted upon transfer to 37°C with kinetics similar to the *rna14-1* single mutant. Consistent with the lack of suppression of *rna14-1* by the absence of Rrp47p, the *rna14-1 rrp6-Δ* mutant was viable at 37°C, whereas the *rna14-1 rrp47-Δ* mutant was not (Fig. 7B).

These data demonstrate that Rrp47p is not required for the nuclear, exosome-mediated initial degradation of the long readthrough transcripts generated in the *rna14-1* mutant or for the subsequent Rrp6p-dependent degradation of the truncated mRNAs.

Reporter constructs containing poly(G) tracts within the 3′

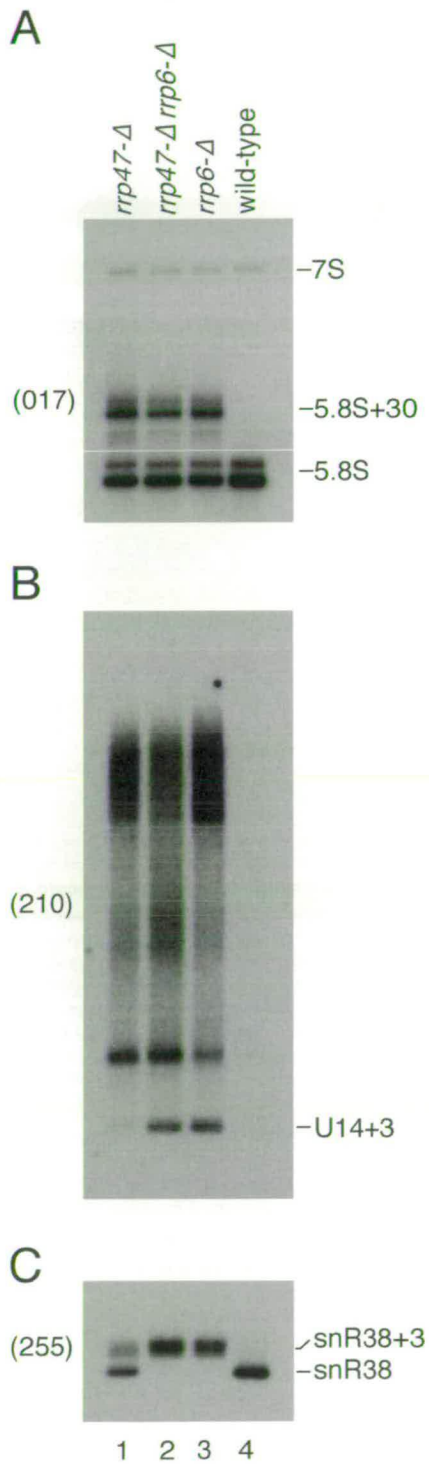

FIG. 6. Northern blot analyses of the *rrp47-Δ rrp6-Δ* double mutant. RNA from *rrp47-Δ* (lane 1), *rrp47-Δ rrp6-Δ* (lane 2), *rrp6-Δ* (lane 3), and wild-type (lane 4) sister strains grown at 30°C were resolved through an 8% acrylamide gel, transferred, and hybridized with probes specific for mature 5.8S rRNA (A), U14 3′-extended species (B), and mature snR38 (C). The probes used are indicated in brackets to the left of each panel.

untranslated region have been extensively used to trap and analyze intermediates in the exonucleolytic decay of cytoplasmic mRNA (reviewed in reference 36). In mutants of the exosome that are defective in cytoplasmic 3′→5′ mRNA decay, the turnover of the poly(G)→3′ end fragment of the *MFA2pG* reporter transcript is impeded compared to wild-type strains and shorter degradation intermediates accumulate (20). Constructs encoding the *MFA2pG* transcript were transformed into isogenic wild-type and *rrp47-Δ* strains, as well as a *ski7-Δ* mutant that is specifically defective for cytoplasmic mRNA decay. RNA recovered from the transformants was resolved in 8% polyacrylamide-urea gels and analyzed by Northern hybridization by using a probe complementary to the 3′ end of the poly(G) cassette. Degradation intermediates from the poly(G)→3′ fragment were observed in the *ski7-Δ* mutant but not in the *rrp47-Δ* mutant or the wild-type strain (Fig. 7C). This result demonstrates that Rrp47p is not required for the exosome-mediated cytoplasmic 3′→5′ decay pathway.

DISCUSSION

The exosome complex functions in a wide range of RNA processing and degradation pathways. It remains largely unclear how different classes of exosome substrates are initially identified and subsequently targeted to the very distinct fates of either accurate 3′ end processing or rapid and complete degradation. This differentiation is likely to be largely dependent upon additional cofactors that are predicted to be present at substoichiometric levels in exosome preparations and may be only weakly associated with the complex. We used a one-step immunoaffinity chromatography procedure coupled with elution of retained proteins in an increasing $MgCl_2$ concentration gradient to identify substoichiometric proteins associated with the exosome component Rrp44p. This allowed the identification of a novel exosome cofactor, Rrp47p. The association of Rrp47p with the exosome was insensitive to RNase treatment and therefore probably reflects a direct interaction. The substoichiometric levels of Rrp47p in exosome preparations and its electrophoretic mobility, which is similar to that of the five smallest core exosome components, presumably precluded its identification in previous analyses. Rrp47p was previously shown to be localized to the nucleus (22), suggesting that it associates with only the nuclear exosome. Consistent with this finding, Rrp47p was not required for cytoplasmic mRNA turnover. The nuclear-specific exosome component Rrp6p was efficiently coprecipitated with epitope-tagged Rrp47p, indicating that they are components of the same complex. A genome-wide analysis of protein complexes in yeast identified Rrp47p in the immunoprecipitates of the exosome components Rrp44p and Rrp46p (16). It should, however, be noted that the product of the *YIR035c* gene was also identified in association with exosome components in the same study, whereas a deletion mutant showed no defect in pre-rRNA or snoRNA processing or in cytoplasmic 3′→5′ mRNA decay (P. Mitchell, unpublished observations).

The RNA processing defects in the *rrp47-Δ* mutant resembled those previously observed in strains lacking Rrp6p. For several substrates, these are distinct from the defects seen in strains mutant for core exosome components or Mtr4p. Comparison of the phenotypes of *rrp47-Δ*, *rrp6-Δ*, and *rrp47-Δ*

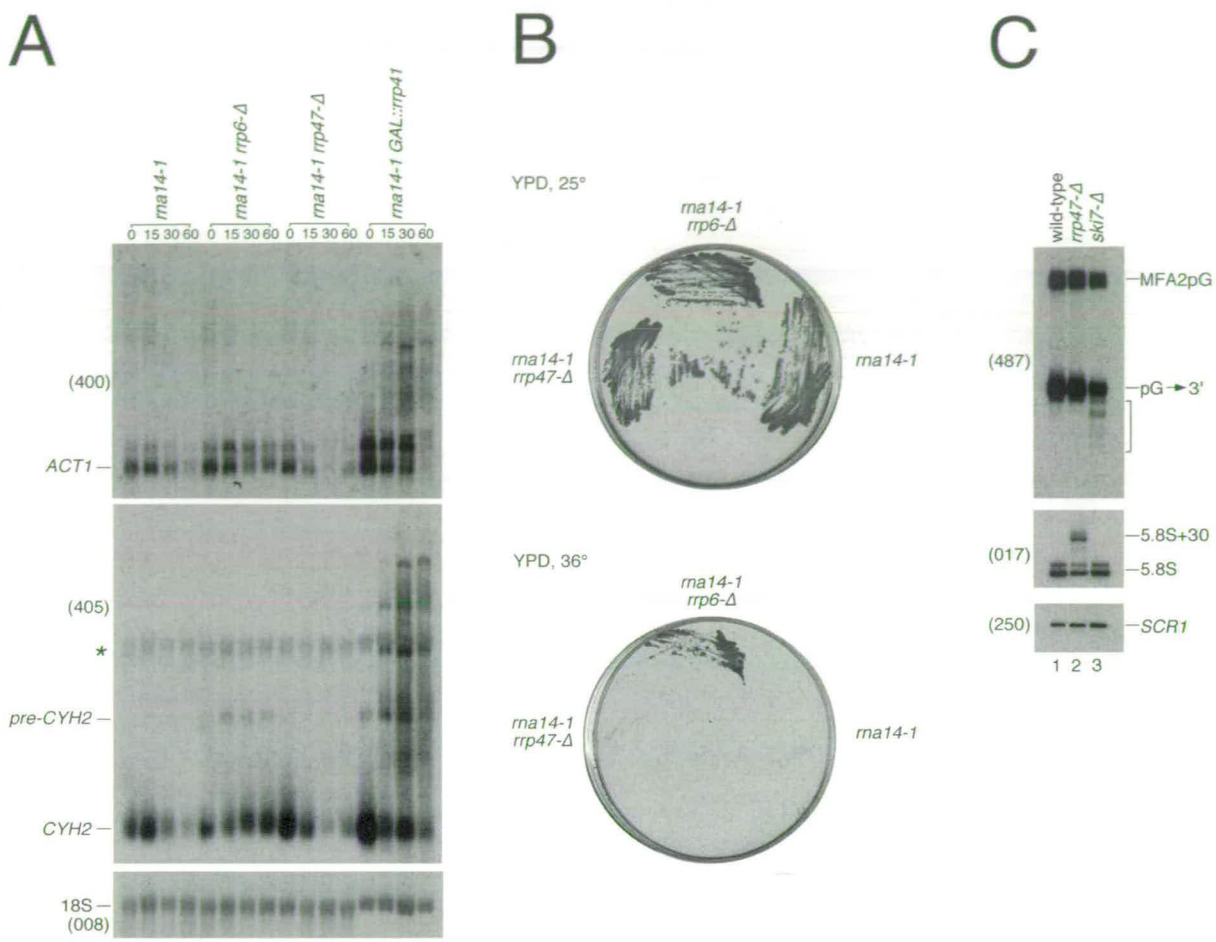

FIG. 7. Rrp47p is not required for nuclear mRNA surveillance or cytoplasmic mRNA decay. (A) Northern blot analysis of RNA isolated from *rna14-1*, *rna14-1 rrp6-Δ*, *rna4-1 rrp47-Δ*, and *rna14-1 GAL::rrp41* mutants during growth at 23°C (zero time points) and after transfer to 37°C for 15, 30, and 60 min. Blots were hybridized with probes specific to *ACT1* and *CYH2* mRNAs and to 18S rRNA as a loading control. A nonspecific RNA detected with the *CYH2* probe is indicated with an asterisk. The probes used are indicated in brackets to the left of each panel. (B) Plate growth assay of the *rna14-1*, *rna14-1 rrp47-Δ*, and *rna14-1 rrp6-Δ* mutants on YPD medium at 25 and 36°C. Plates were incubated for 5 days. (C) Northern blot analysis of RNA from wild-type (lane 1), *rrp47-Δ* (lane 2), and *ski7-Δ* (lane 3) strains expressing the *MFA2pG* reporter transcript. Hybridization was performed with probe 487, complementary to the 3′ end of the poly(G) cassette within the *MFA2* 3′ untranslated region. The probe detects the full-length *MFA2pG* transcript and the poly(G)→3′ fragment (pG→3′). Shortened degradation intermediates of the poly(G)→3′ fragment detectable in the *ski7-Δ* mutant are indicated by a bracket on the right-hand side of the panel. The lower panels show control hybridizations of the same Northern with probes complementary to the 5.8S rRNA (oligonucleotide 017) and the *SCR1* RNA (oligonucleotide 250).

rrp6-Δ double mutants suggested that Rrp47p functions to promote Rrp6p activity. The absence of Rrp47p did not significantly affect either the expression level of Rrp6p or its coimmunoprecipitation with Rrp4p (R. Houalla and D. Tollervey, unpublished observations), suggesting that Rrp47p is not required for Rrp6p expression or its assembly into the exosome. Although Rrp47p shares no similarity with characterized 3′→5′ exoribonucleases, the homologous human protein C1D binds strongly to nucleic acids (28). Rrp47p may therefore function in substrate recruitment and targeting to Rrp6p.

Significantly, the effects of the absence of Rrp47p and Rrp6p, although related, were not identical, and not all nuclear functions of the exosome required Rrp47p. In particular, no effect was observed in the absence of Rrp47p on the exosome-mediated degradation of readthrough transcripts observed in the *rna14-1* mutant, which is defective in pre-mRNA cleavage and transcription termination (7, 42). This indicates that Rrp47p may facilitate interactions between the Rrp6p-exosome complex and specific classes of exosome substrates.

Rrp47p was previously reported to function in DNA double-strand break repair and the homologous human protein C1D binds with high affinity to free 3′ ends of DNA (14, 28). Both nonhomologous end joining and homologous recombination events involve nucleotide removal from the free ends by exonucleases. The RNase D family of 3′→5′ exonucleases, which includes Rrp6p, is closely related to the proofreading domain of RNA polymerases (27). Rrp47p may therefore regulate exonucleolytic activities required for both stable RNA processing and DNA repair.

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