90S Pre-Ribosomes Include the 35S Pre-rRNA, the U3 snoRNP, and 40S Subunit Processing Factors but Predominantly Lack 60S Synthesis Factors

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90S Pre-Ribosomes Include the 35S Pre-rRNA, the U3 snoRNP, and 40S Subunit Processing Factors but Predominantly Lack 60S Synthesis Factors

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Summary

We report the characterization of early pre-ribosomal particles. Twelve TAP-tagged components each showed nucleolar localization, sedimented at approximately 90S on sucrose gradients, and coprecipitated both the 35S pre-rRNA and the U3 snoRNA. Thirty-five non-ribosomal proteins were coprecipitated, including proteins associated with U3 (Nop56p, Nop58p, Sof1p, Rrp9, Dhr1p, Imp3p, Imp4p, and Mpp10p) and other factors required for 18S rRNA synthesis (Nop14p, Bms1p, and Krr1p). Mutations in components of the 90S pre-ribosomes impaired 40S subunit assembly and export. Strikingly, few components of recently characterized pre-60S ribosomes were identified in the 90S pre-ribosomes. We conclude that the 40S synthesis machinery predominately associates with the 35S pre-rRNA factors, whereas factors required for 60S subunit synthesis largely bind later, showing an unexpected dichotomy in binding.

Introduction

The formation of eukaryotic ribosomes occurs predominantly in the nucleolus, but late maturation steps also take place in the nucleoplasm and cytoplasm (for recent review see Kressler et al., 1999). This most complicated assembly starts with the synthesis of more than 80 ribosomal (r-) proteins in the cytoplasm, which are subsequently imported into the nucleolus. The two rRNA precursors, the 35S pre-rRNA transcribed by RNA polymerase I and the 5S pre-rRNA transcribed by Pol III, are generated from rDNA in the nucleolus (Woolford, 1991). Following transcription and assembly of early r-proteins onto the pre-rRNAs, many rRNA processing and base-modification steps occur before mature 25S/28S, 18S, 5.8S, and 5S rRNA are generated (Warner, 1989, 1999; Tollervey, 1996). Moreover, many non-ribosomal factors transiently associate with the pre-rRNAs and r-proteins, forming the pre-ribosomal particles.

While the processing of the pre-rRNA is now fairly well understood, understanding of ribosome assembly and subsequent export to the cytoplasm is limited and just beginning to be examined in greater detail (Warner, 2001). Pioneering experiments in the early 1970s identified a small number of pre-ribosomal particles; a 90S pre-ribosome that contains the 35S pre-rRNA was processed to 66S and 43S particles, the precursors to the 60S and 40S subunits, respectively (Udem and Warner, 1972; Trapman et al., 1975). It is now clear that the pathway of ribosome synthesis is substantially more complex than these studies suggested. Analyses of pre-60S ribosomes have identified a number of particles that differ in their content of pre-rRNA species on the pathway of 25 rRNA and 5.8S rRNA synthesis and contain large numbers of non-ribosomal proteins (Baβler et al., 2001; Harnpicharnchai et al., 2001; Saveanu et al., 2001; Fatica et al., 2002). Among the associated components were rRNA processing factors, RNA helicases, GTPases, methlytransferases, ribosomal-like proteins, AAA-type ATPases, and factors required for intranuclear movement of pre-ribosomes.

A fundamental question in ribosome synthesis is how the machineries required for 40S and 60S synthesis interact. It has long been known that there is considerable autonomy in the two pathways, since many factors required for the early pre-rRNA processing events at sites A_0, A_1, and A_2 and for 18S rRNA synthesis are not required for synthesis of the 25S and 5.8S rRNAs. These early cleavages are dispensable in part because 25S/5.8S synthesis can be initiated independently by cleavages in ITS1 (see Figures 4A and 4B). In contrast, it subsequently became clear that mutation of almost any factor required for 60S subunit synthesis impacts the early processing steps. These observations were surprising because it might have been expected that early processing defects would impact the later pathway of 60S synthesis, which is not generally the case, whereas it was less clear why late defects would inhibit earlier steps. This led to the "processome" model in which it was envisaged that both the 40S and 60S synthesis machinery bound to the 35S pre-rRNA, thus making sure that all components were present to ensure rapid and efficient processing.

Here we will show that the early events during ribosome synthesis may happen differently than initially assumed but will offer an explanation for the observation that defects in early processing steps specifically inhibit 40S synthesis. Starting from a protein predicted to function in 40S subunit synthesis, we have identified many components of the early pre-ribosomes. These are associated with much of the 40S subunit synthesis machin-

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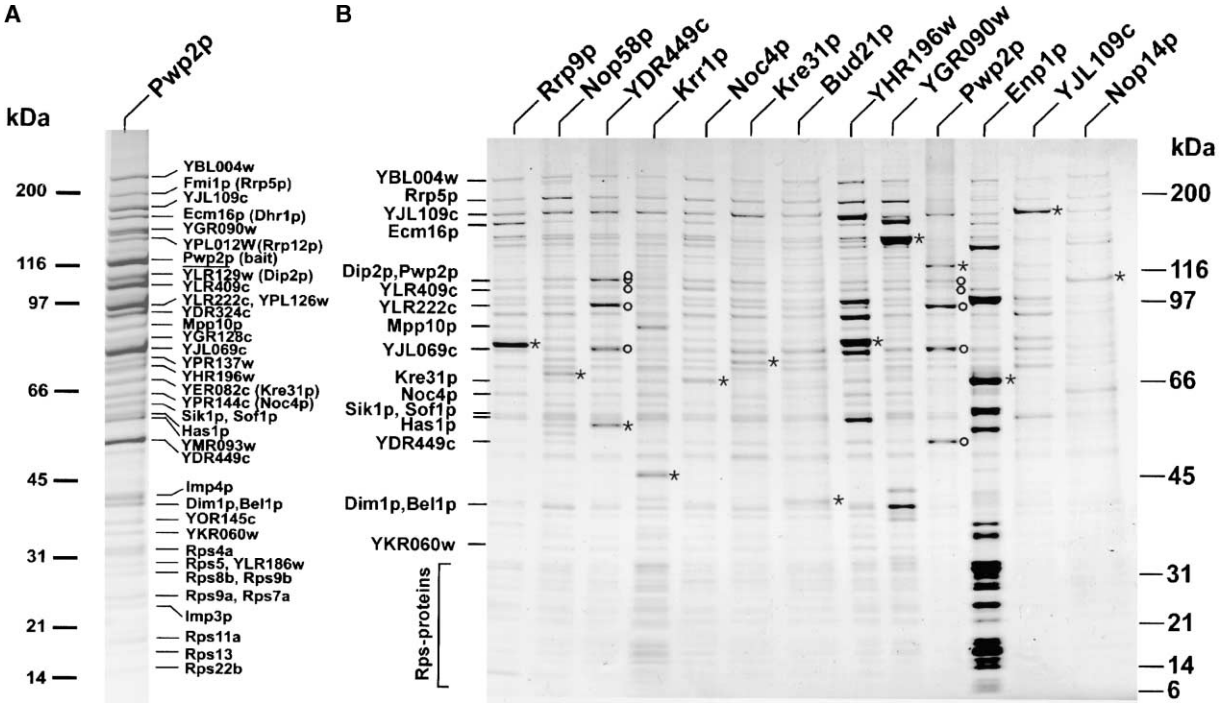

Figure 1. Purification of TAP-Tagged Pwp2p Identifies Pre-Ribosomal Particles to 40S Subunits

(A) TAP-tagged Pwp2 was isolated from yeast by two-step affinity purification. Purified proteins were separated on a 4%–12% gradient gel and visualized by Coomassie staining. Copurifiying proteins were identified by mass spectrometry (MALDI-TOF) and are indicated. A molecular weight marker is also shown.

(B) Reverse TAP tagging of protein baits, which copurified with Pwp2p, and isolation of these baits from yeast by two-step affinity purification. Purified proteins were separated on a 4%–12% gradient gel and visualized by Coomassie staining. Prominent comigrating bands are indicated. The stars mark the bait proteins carrying the calmodulin binding peptide tag. Open circles indicate the proteins present in a stable Pwp2p subcomplex (see also Figure 6A and Supplemental Figures S1 and S2 at http://www.molecule.org/cgi/content/full/10/1/105/DC1), which are overstoichiometric in the Pwp2p and YDR449 preparations.

ery, but with almost no components that were previously shown to be associated with the 60S pre-ribosomes.

Results

Recent studies reported the isolation of different 60S precursor particles by employing a stringent tandem affinity purification (TAP) of tagged proteins that are implicated in ribosome synthesis and nuclear export (Baβler et al., 2001; Harnpicharnchai et al., 2001; Saveanu et al., 2001; Fatica et al., 2002). In a recent large-scale approach, several hundred TAP-tagged yeast proteins were purified, and their organization in multimeric complexes was determined (Gavin et al., 2002). To identify precursors to the 40S subunit, we screened for purified protein complexes that revealed a distinct enrichment of small subunit (Rps) over large subunit (Rpl) proteins (Figures 2 and 3).

The starting example was Pwp2p (periodic tryptophan protein 2; Shafaatian et al., 1996), which was reanalyzed and found to be associated with predominantly 40S subunit ribosomal proteins (Rps1p, Rps4, Rps5p, Rps7p, Rps9p, Rps11p, Rps13p, Rps16p, Rps22p) (Figures 1A, 2, and 3). Many non-ribosomal proteins copurified with Pwp2p, ranging in size from ~20 to ~200 kDa. Almost all of these are essential and conserved in evolution, supporting their function in ribosome synthesis. Importantly, several of the Pwp2p-associated proteins were previously characterized nucleolar proteins with known roles in 18S rRNA processing (e.g., Sof1p, Mpp10p, Nop56p, Nop14p, Rrp5, Imp3p, Imp4p, and Rrp9p).

To investigate the specificity of these associations, we generated TAP-tagged constructs for several of the Pwp2p-associated proteins and identified copurifying proteins (Figures 1B and 2). Comparison of these purifications revealed a pattern of many common bands in the Coomassie-stained SDS-polyacrylamide gel, which was particularly evident in the high molecular weight range of the gel (Figure 1B). The bands in the low molecular weight range represent predominantly Rps proteins (see also Figures 2 and 3). However, the different purified bait proteins also exhibit unique bands or comigrating bands that are present in overstoichiometric amounts (see also below). Some of the bands correspond to the bait proteins, which carry the calmodulin binding peptide tag and thus shift in molecular weight (Figure 1B, indicated by a star). Taken together these analyses defined a cluster of many (>30) common non-ribosomal proteins, which are predominantly associated with ribosomal S-proteins (~6 on average per entry point) and

	Function	Localization	Bait Bound	Pwp2p	Krr1p	Enp1p	YHR196w	Noc4p	Kre31 p	YJL109c	YGR090w	Rrp9p	YDR449c	Nop58p	Bud21p	Nop14p
1	40s biogenesis	nucleolus*	Krr1p													
2	40s biogenesis	nucleolus*	Nop14p													
3	unknown	unknown	Bfr2p													
4	unknown	unknown	Dip2p													
5	unknown	unknown	YKR060W													
6	rRNA processing	unknown	Rrp12p													
7	U3 snoRNP	nucleolus	Imp3p													
8	unknown	nucleolus*	Enp1p													
9	U3 snoRNP	nucleolus	Nop56p													
10	U3 snoRNP	nucleolus	Sof1p													
11	unknown	unknown	YMR093W													
12	40s biogenesis	nucleolus	Rrp5p													
13	rRNA processing	nucleolus	Bms1 p													
14	unknown	unknown	YBL004w													
15	unknown	nucleolus*	YDR449C													
16	unknown	nucleolus*	Pwp2p													
17	unknown	nucleolus*	YJL109c													
18	U3/U14 snoRNP	nucleolus	Nop1p													
19	unknown	unknown	YDR324C													
20	unknown	nucleolus*	Kre31p													
21	unknown	nucleolus*	YGR090w													
22	unknown	nucleolus	Nan1p													
23	unknown	unknown	YGR128c													
24	U3 snoRNP	nucleolus	Mpp10p													
25	NuAc	unknown	YGR145w													
26	rRNA processing	unknown	Emg1p													
27	unknown	unknown	Kre33p													
28	unknown	nucleolus*	Noc4p													
29	unknown	unknown	YLR222c													
30	unknown	unknown	YLR409c													
31	rRNA processing	nucleolus	Ecm16p													
32	unknown	nucleolus*	YHR196W													
33	U3 snoRNP	nucleolus*	Nop58p													
34	U3 snoRNP	nucleolus	Imp4p													
35	U3 snoRNP	nucleolus*	Rrp9p													
			S subunits	10	7	13	9	6	1	5	9	3	3	8	1	6
			L subunits		2	4		1	1	3	2			4	2	1

Figure 2. Identification of Precursor Particles to the 40S Subunit, which Contains More Than 30 Non-Ribosomal Proteins

TAP-tagged proteins are indicated in the top row (bait). Copurifying proteins identified by mass spectrometry are listed vertically (found) and indicated by an orange rectangle in the columns below each bait protein. TAP-tagged proteins are labeled by a blue rectangle. If known, the function and location of the proteins present in the purified complexes are indicated in two vertical columns adjacent to the column indicating the bound proteins. The number of L-proteins and S-proteins that copurified with the different TAP-tagged proteins is indicated within the orange rectangles. The star (*) indicates this study.

Bait Bound	Pwp2p	Krr1p	Enp1p	YHR196w	Noc4p	Kre31 p	YJL109c	YGR090w	Rrp9p	YDR449c	Nop58p	Bud21p	Nop14p
S subunits													
rpp0													
rppS0A													
rps1a													
rps1b													
rps3													
rps4a/b													
rps5													
rps6a													
rps7a													
rps8a/b													
rps9a													
rps9b													
rps11a													
rps11b													
rps13													
rps14a													
rps16a													
rps17a/b													
rps18a													
rps22a													
rps22b													
rps24a													
rps24b													
rps28b													
Total S	10	7	13	9	6	1	5	9	3	3	8	1	6
L subunits													
rpl4a													
rpl4b													
rpl5													
rpl7b													
rpl10													
rpl11a													
rpl17a													
rpl20a													
rpl20b													
rpl27													
rpl30													
Total L	0	2	4	0	1	1	3	2	0	0	4	2	1

Figure 3. Identification of the Ribosomal Proteins within the Precursor Particles to the 40S Subunit

Identification of the individual ribosomal (S- and L-) proteins found associated with the TAP-tagged proteins. Please note that the complete identification of ribosomal proteins by SDS-PAGE and mass spectrometrical analysis was not possible, since small ribosomal proteins were lost on the SDS 4%–12% gradient polyacrylamide gel. Moreover, the small size and sequence similarity between ribosomal proteins make it difficult to obtain specific peptides for a complete identification using MS or Edman analysis.

to a lower extent L-proteins (~1–2 on average per entry point; Figures 2 and 3). In addition to these core subunits, additional components (between 10 and 15) were coenriched with some of the core complex members (see Supplemental Table S1 at http://www.molecule.org/cgi/content/full/10/1/105/DC1). The specificity of their association with the particle is not established.

RNA components of these complexes were identified by primer extension and Northern hybridization on RNA coprecipitated with each of the TAP-tagged proteins. Strikingly, the 35S pre-rRNA transcript (for schematic drawing and rRNA processing, see Figures 4A and 4B) was coprecipitated in clear amounts with most of these proteins (Figure 4C). Moreover, the U3 snoRNA was found to be associated with many of the reverse-tagged and purified protein baits, but its yield was more variable (Figure 4C). In contrast, U14 snoRNA coprecipitated with the box C+D snRNP protein Nop58p and weakly with Yhr196w, but not in the other twelve purifications (Figure 4C). The variability of 35S pre-rRNA and U3 snoRNA coprecipitation may reflect differences in the stoichiometry or differential accessibility of the tags within the complexes, but may also be partly due to degradation of RNAs during the extensive purification procedures (see also Baβler et al., 2001).

Importantly, pre-rRNAs on the pathway of 60S synthesis, $27SA_2$ (shown by the primer extension stop at site A_2), 27SB, and 7S (shown by the primer extension stops and B_{1L} and B_{1S}) were coprecipitated with Rrp9p but showed only weak precipitation with the other tagged proteins (Figure 4C). The 6S pre-rRNA and the mature 5.8S rRNA (data not shown) were not detectably precipitated. Strikingly, precipitation of the 20S pre-rRNA was considerably weaker than 35S pre-rRNA for all tagged proteins, with the exception of Enp1p (Figure 4C). The 20S pre-rRNA undergoes late cytoplasmic dimethylation near the 3′ end of the 18S rRNA, which can be detected by primer extension (Brand et al., 1977; Lafontaine et al., 1995). The dimethylated pre-rRNA was efficiently coprecipitated only with Enp1p, although recovery above background was seen for some other of the tagged proteins (Figure 4C). We conclude that most of the TAP-tagged proteins are associated with pre-ribosomal particles that contain the 35S pre-rRNA and the U3 snoRNP. However, all of these except Enp1p are likely to be released from the pre-rRNA at or prior to formation of the 20S pre-rRNA and probably do not accompany the pre-40S particles to the cytoplasm.

We next sought to determine the size of the newly identified pre-ribosomal particles. Sucrose density gradient centrifugation was performed to analyze the sedimentation behavior of Pwp2p and the other bait proteins selected for TAP tagging. The OD_{260nm} profile of these gradients reveals the partitioning of 40S and 60S subunits, 80S ribosomes, and polysomes (Figure 5A, upper panels). The sedimentation of TAP-tagged proteins present in whole-cell lysates was determined by Western blot analysis (Figure 5A; lower panels). This analysis showed that none of the tagged proteins is monomeric in the cell and all are associated with particles larger than 40S subunits. Some of the bait proteins (Ygr090w, Yhr196w, Ydr449c, Noc4p) are predominantly present in very large particles that sediment broadly with a peak slightly below 80S (Figure 5A). The 35S pre-rRNA was also detected in this region of the sucrose gradient (Milkereit et al., 2001). Other bait proteins (Pwp2p, Krr1p, Kre31p, Nop14p, Bud21, Yjl109c) are not only detected in these dense fractions of the sucrose gradient but are also found at positions where 40S subunits and 43S precursors sediment (Figure 5A). These data show that all the tested tagged proteins are present in large particles, which are strongly predicted to be pre-ribosomes. Particles larger than 80S presumably represent early 90S pre-ribosomes. Enp1p exhibited distinct sedimentation behavior on sucrose gradients with a strong peak in the 40S and a diminished signal in the 90S region of the gradient (Figure 5A). This is consistent with the RNA data showing strong association of Enp1p with the 20S pre-rRNA. The steady state level of the 20S pre-rRNA is considerably higher than that of 35S, so a protein that is associated with both RNAs is expected to be enriched in the 40S fraction compared to 90S.

The intracellular location of TAP-tagged Pwp2 and the other twelve TAP-tagged proteins was determined by indirect immunofluorescence microscopy. This revealed that all the tested proteins are located in the nucleolus (Figure 5B), although a cytoplasmic signal was also observed for Enp1p in some cells (data not shown).

To determine how stably core components of the pre-40S ribosome particle associate with ribosomal and non-ribosomal proteins, the purified Pwp2p complex and several other TAP-tagged proteins were applied to a gel filtration column (Figure 6 and Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/10/1/105/DC1). Pwp2p eluted in two major peaks (Figure 6A). A peak in fraction 9–10, estimated at around 4–6 MDa, contains the numerous non-ribosomal factors and ribosomal proteins that were previously identified in the TAP-purified complex (see Figure 1B and Supplemental Figure S2A at http://www.molecule.org/cgi/content/full/10/1/105/DC1). Additionally, Pwp2 was found in fractions 19–22, together with Dip2p, YLR409c, YLR222c, YJL069c, and YDR449c (Figure 6A and Supplemental Figure S2A at http://www.molecule.org/cgi/content/full/10/1/105/DC1). Apparently, Pwp2p forms a stable 600 kDa subcomplex with these five proteins. Interestingly, these proteins are present in superstoichiometric amounts in the TAP-purified Pwp2p and YDR449c preparations but are apparently stoichiometric in the other tagged protein purifications (see Figure 1B). We conclude that the fraction of Pwp2p that is unassociated with pre-ribosomes is not free but is present in a 600 kDa complex. Whether it is this complex that binds to the assembling pre-ribosomes remains to be determined. Other TAP-tagged protein baits were purified and analyzed by gel filtration chromatography. Each of these resembled Pwp2-TAP with a peak in the 4–6 MDa range (corresponding to fractions 9–11) that yields a complex pattern of bands (shown for Krr1p in Figure 6B; for other baits see Supplemental Figures S1 and S2B at http://www.molecule.org/cgi/content/full/10/1/105/DC1). In addition, Krr1p-TAP in association with several other

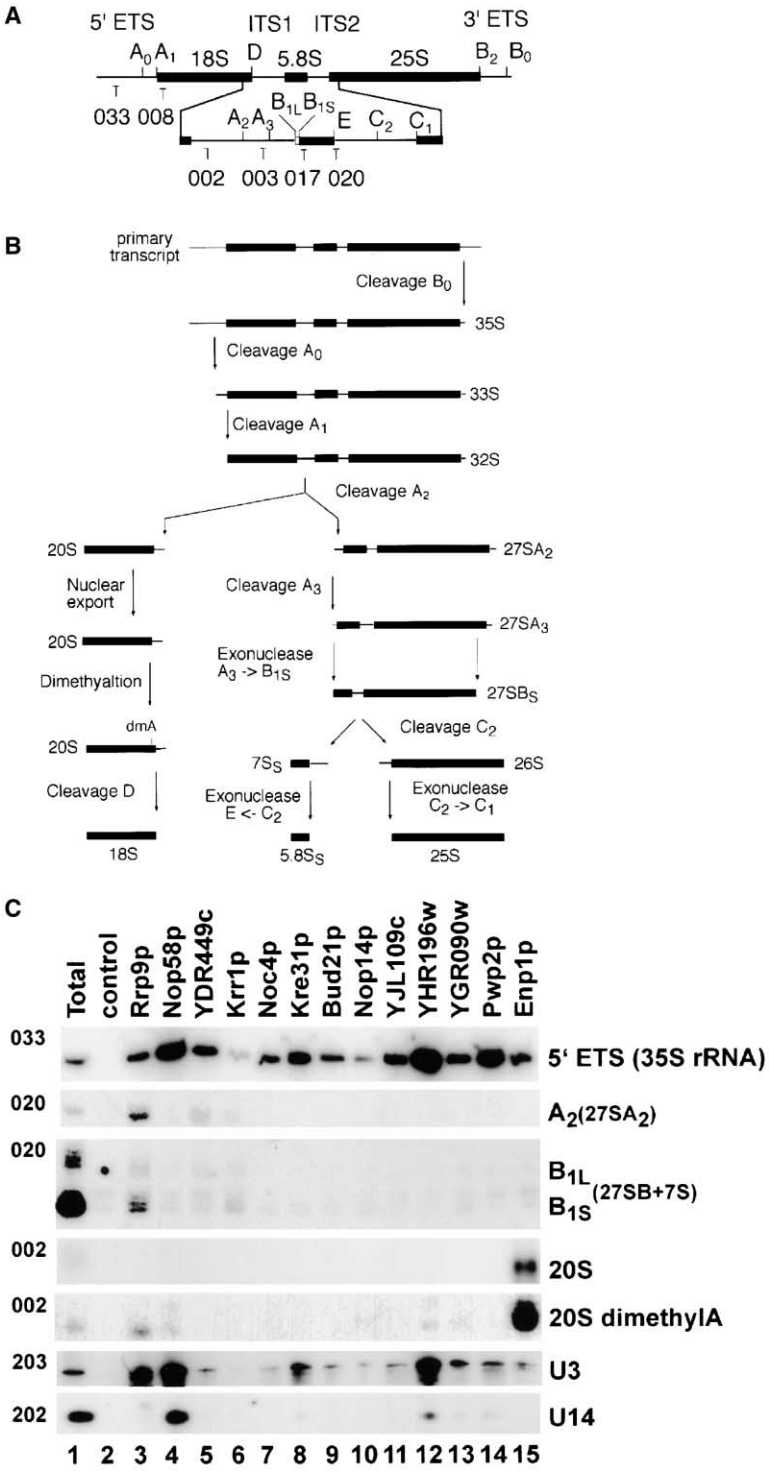

Figure 4. Northern Analysis of Pre-rRNA and rRNA Associated with the 40S Pre-Ribosomes

(A) The 35S pre-rRNA showing the processing sites and oligonucleotides used. The 35S pre-rRNA contains the sequences for the mature 18S, 5.8S, and 25S rRNAs, separated by the two internal transcribed spacers and flanked by the 5′-ETS and 3′-ETS.

(B) Schematic diagram of the major pre-rRNA processing pathway.

(C) Analysis of RNA species associated with TAP-tagged proteins. Lane 1, 5 μg of total RNA; lane 2, Adh4p-TAP (negative control); lanes 3–15, TAP-tagged proteins implicated in 40S subunit synthesis. RNA recovered after purification of the tagged proteins was analyzed by primer extension (5′ 35S, A_2, B_1, and dimethyladenine formation in the 20S pre-rRNA) or Northern hybridization (20S pre-rRNA, separated on an agarose/formaldehyde gel, and U3 and U14 snoRNA, separated on a polyacrylamide gel). Oligonucleotides used are indicated on the left (see Experimental Procedures).

bands was found in a second peak on the gel filtration column (Figure 6B, fractions 13–16), which is distinct from the ~600 kDa complex associated with Pwp2p-TAP (Figure 6A, fractions 19–22). The ~4–6 MDa complex that was seen for each of the TAP-tagged proteins analyzed is likely to correspond to the ~90S particles identified by sucrose gradient centrifugation (see Figure 5A).

We wanted to gain functional evidence that core components of the ~90S particles are essential for 40S subunit biogenesis and export to the cytoplasm. Previous work showed that 18S rRNA is not produced in *krr1* mutant strains, whereas 25S rRNA is made in normal amounts (Sasaki et al., 2000). We therefore tested characterized *krr1* ts mutants (*krr1-17* and *krr1-18*; see Sasaki et al., 2000) for defects in nuclear export of 40S

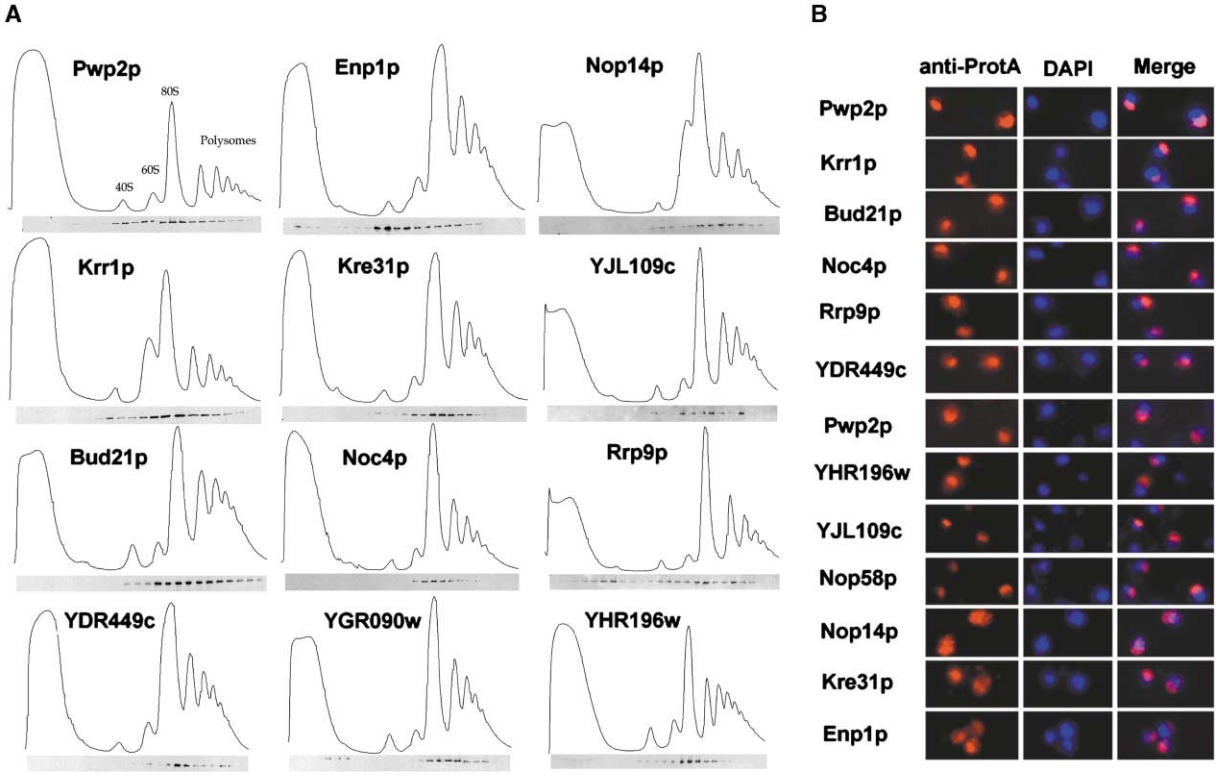

Figure 5. Pwp2p and the Other Reverse-Tagged Protein Baits Are Associated with Pre-Ribosomal Particles in the Nucleolus

(A) Sedimentation behavior of Pwp2p and the other indicated reverse-tagged baits on sucrose gradients. Ribosomal profiles were determined by OD_{260nm} measurement of the gradient fractions (upper part). Western blot analysis of these gradient fractions reveals the position of the indicated TAP-tagged baits (lower panel).

(B) Indirect immunofluorescence assay of TAP-tagged Pwp2p and the other reverse-tagged protein baits in yeast cells. The location of the tagged proteins (anti-ProtA; red) and the corresponding DAPI-staining (DNA; blue) in the fluorescence microscope is shown. Both pictures were merged, which reveals that the proteins are located in the nucleolus. For Enp1p, cytoplasmic staining was also observed in some cells (data not shown).

subunits. Strikingly, *krr1* mutants exhibited strong nucleolar accumulation of a 40S subunit reporter (Rps2p-GFP) upon shift to the restrictive temperature (Figure 7A). In contrast, nuclear export of 60S subunits was not impaired in *krr1* ts mutants (Figure 7A). The presence of 40S subunits was severely decreased, and the 60S subunit peak significantly increased in the *krr1* ts strain when incubated at the nonpermissive temperature (Figure 7B; see also Sasaki et al., 2000).

The Rps2p-GFP reporter was used in a fluorescence-based visual assay to isolate novel *rix* mutants, which are defective in 40S subunit export (see Experimental Procedures). This screen identified a mutation in Kre33p, a previously uncharacterized component of the 40S pre-ribosomes (see Figure 2). In the *kre33-1* ts strain, export of 40S but not 60S subunits was impaired at the restrictive temperature, which can be seen by a distinct nucleolar accumulation of the small subunit reporter (Figure 7A). Moreover, a strong decrease in 40S subunits on polysomal gradients was observed in the *kre33-1* ts strain upon shift to 37°C (Figure 7B). This shows that Krr1p and Kre33p are each essential factors for 40S subunit assembly and subsequent export to the cytoplasm.

Discussion

In this study we have reported the identification of an early pre-ribosomal particle. The major form of the complex sediments below 80S ribosomes, contains the 35S pre-rRNA primary transcript, and presumably represents the 90S pre-ribosomes previously identified by metabolic labeling (Udem and Warner, 1972). The complex also contains a large number of non-ribosomal proteins, including the U3 snoRNP particle and other factors specifically required for 40S subunit synthesis (Figure 8). Notably absent are the components of the characterized pre-60S complexes (Baβler et al., 2001; Harnpicharnchai et al., 2001; Saveanu et al., 2001; Fatica et al., 2002) indicating that the processing factors involved in 40S synthesis assemble onto the 35S pre-rRNA prior to assembly of the 60S synthesis machinery.

Some variation was observed in the particles purified with different bait proteins. This may reflect genuine differences, perhaps indicative of an ordered assembly pathway. However, as previously discussed (Gavin et al., 2002) the efficiency of TAP coprecipitation is influenced by the properties of the individual bait proteins (the TAP-tag on the bait could weaken the interaction

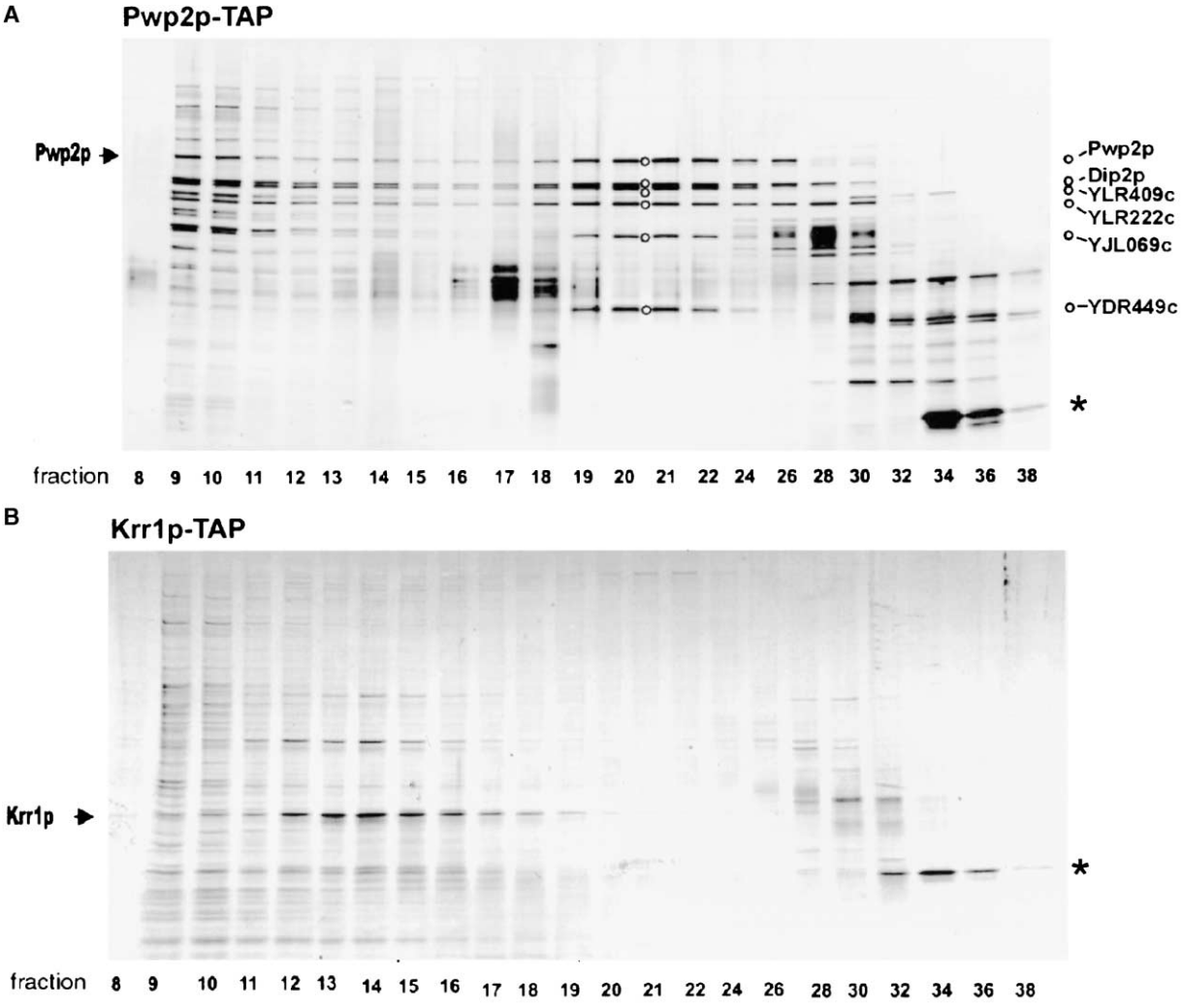

Figure 6. Pwp2p and Other Reverse-Tagged Protein Baits Are Found in Large Pre-Ribosomal Particles

(A and B) The TEV eluate of a Pwp2p-TAP purification (A) and a Krr1p-TAP purification (B) was applied on a FPLC Superose 6 column. Fractions were analyzed by SDS-PAGE and Coomassie staining. Open circles in (A) indicate the six proteins present in a 600 kDa Pwp2p subcomplex (fraction 20), which are overstoichiometric in the Pwp2p and YDR449c preparations (see also Figure 1B). The asterisk indicates the TEV protease. The proteins present in fraction 34 (Pwp2p-TAP) were also identified by mass spectrometry and are mainly contaminants frequently found in TAP purifications: Eft2p, human keratin, Tkl1p, Hsp70p, Pdc1p, Cdc19p, Eno2p, Eno1p, Pgi1p, Pgk1p, Sam1p, Tif2p, Fba1p, Adh1p, Tdh2p, Tdh3p, Ilv5p, Gpm1p, and Tpi1p. The Superose 6 column was calibrated with molecular weight marker proteins of 600, 158, 44, and 17 kDa. In a similar way, other reverse-tagged purified protein baits were analyzed by gel filtration chromatography (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/10/1/105/DC1).

with associated proteins) and the limitations of mass spectrometry (in particular, when several proteins comigrate in the same band). In particular, this makes the identification of small proteins less reliable (e.g., Imp3p; see Figure 2), since their identification relies on a smaller number of peptides.

The early endonucleolytic cleavages at sites A_0, A_1, and A_2 in the 35S pre-rRNA are required for 18S rRNA synthesis and 40S biogenesis. These require base-pairing of the U3 snoRNA to specific sites in the 5′ end of the 35S pre-rRNA and 18S rRNA (Beltrame and Tollervey, 1995; Sharma and Tollervey, 1999). The assembly of U3 and its associated proteins (Nop1p/fibrillarin, Nop56p, Nop58p, Sof1p, Mpp10p, Imp3p, Imp4p, Rrp9p, and Dhr1p) with the pre-rRNA may trigger the successive pre-rRNA cleavage and 40S subunit assembly steps. The U3-associated processing machinery has been proposed to correspond to the terminal knobs visualized in EM spreads at the 5′ end of nascent pre-rRNA transcripts (Miller and Beatty, 1969; Mougey et al., 1993). The protein components purified here have a calculated molecular weight ~2.7 MDa (adding together the M_r of the core subunits) and could represent or be a part of the terminal knobs.

Besides the U3-specific proteins, other nucleolar proteins with a known role in 18S rRNA processing and 40S

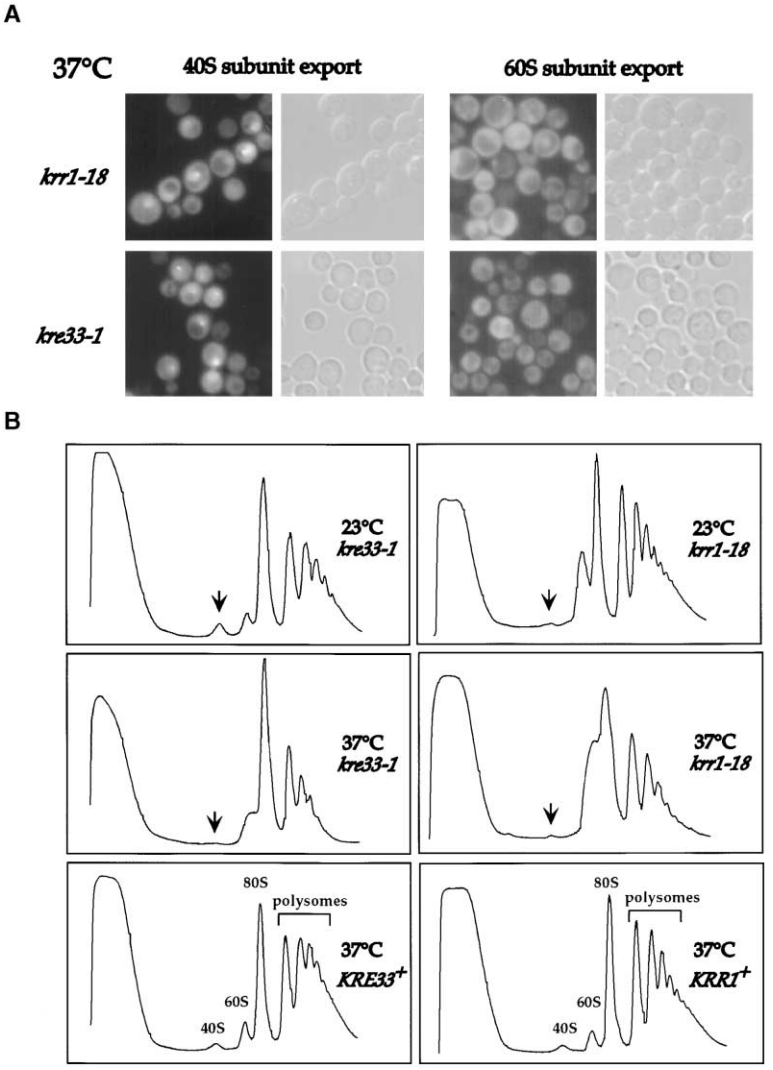

Figure 7. 40S Subunit Biogenesis and Nuclear Export Is Inhibited in *krr1* and *kre33* ts Mutants

(A) Temperature-sensitive *krr1-18* and *kre33-1* strains are impaired in 40S subunit export. Fluorescence-based in vivo assays were used to analyze ribosomal export of 40S and 60S subunits. Cells were shifted for 4 hr to 37°C before nuclear accumulation of Rps2-eGFP (40S subunit reporter) and Rpl25p-eGFP (60S subunit reporter) was determined by fluorescence microscopy.

(B) Analysis of ribosomal and polysomal fractions derived from the *krr1-18* and *kre33-1* ts strains, grown at 23°C or shifted for 4 hr (*kre33-1* or isogenic *KRE33*$^+$) or 2 hr (*krr1-18* or isogenic *KRR1*$^+$) to 37°C. The UV profiles (OD_{260nm}) of the sucrose gradient are depicted. The arrows indicate the decrease in the amount of 40S subunits occurring at 37°C in the mutant strains.

subunit assembly are present in the purified 90S pre-ribosomes. Among these are Nop14p (Liu and Thiele, 2001), Krr1p (Sasaki et al., 2000), Rrp5p (Venema and Tollervey, 1996), and the putative GTPase Bsm1p (Gelperin et al., 2001; Wegierski et al., 2001). Other GTPases (Nug1p, Nug2p, Nog1p) were found in pre-60S precursor particles and are implicated in 60S subunit assembly and nuclear export (Baβler et al., 2001; Harnpicharnchai et al., 2001; Saveanu et al., 2001). The GTPases may induce or monitor structural rearrangements of the rRNA and/or protein interactions. The 90S pre-ribosomes contain at least 19 additional factors of unknown function, 18 of which are encoded by essential genes and seven of which have orthologs in humans. Several of these exhibit WD-repeats, which may mediate protein-protein interactions. A stable hexameric subcomplex was isolated that includes four WD-repeat proteins, Pwp2p, Dip2p, YLR409c, and YLR222c. It is possible that this subcomplex helps to nucleate the assembly of the pre-ribosomal particle. Pre-40S subunit assembly may be coupled to export to the cytoplasm, as proposed for pre-60S particles (Gadal et al., 2001a; Milkereit et al., 2001). Mutation of Krr1p or Kre33p specifically impaired 40S subunit export but not 60S subunit export, and Noc4p (Ypr144c) exhibits a Noc-domain (Milkereit et al., 2001). This domain is also found in Noc1p and Noc3p, two nucleolar/nuclear proteins implicated in intranuclear transport and export of pre-60S particles (Milkereit et al., 2001). Future studies will elucidate how the numerous subunits in the 90S pre-ribosomes orchestrate pre-18S rRNA processing and 40S subunit assembly and export.

The most striking finding of these analyses was the lack of characterized pre-60S processing factors associated with the complexes. This appears to show that many factors required for 40S synthesis bind to the 35S pre-rRNA. In contrast, only few components of the recently characterized pre-60S ribosomes were identified in the 90S pre-ribosomes (Figure 8). The early pre-60S complexes that were associated with Ssf1p lacked all known 40S processing factors (Fatica et al., 2002). The Ssf1p-associated complexes contained the $27SA_2$ pre-rRNA but little 35S, consistent with association of

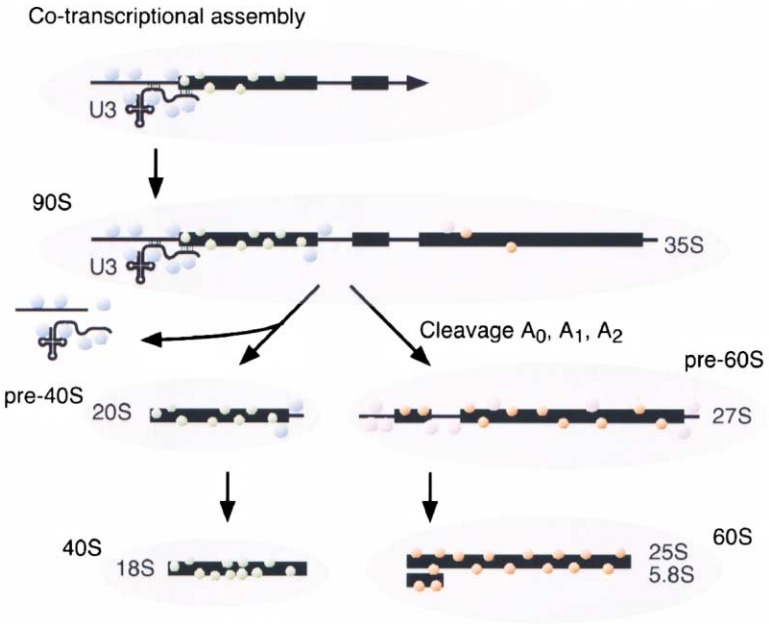

Figure 8. Model of the 90S Pre-Ribosome with Associated Factors and Its Separation into 40S and 60S Pre-Ribosomal Particles

See text for details.

the majority of 60S synthesis factors only after processing of 35S to 27SA_2. The absence of the 60S synthesis factors from the pre-rRNA during processing at sites A_0–A_2 provides an explanation for the long-standing observation that many mutations that inhibit these cleavages prevent only 18S rRNA synthesis (see, for example, Tollervey, 1987). Moreover, few ribosomal proteins of the 60S subunit were recovered with the TAP-tagged components of the 90S pre-ribosomes, indicating that these also largely associate only with pre-60S ribosomes, in which they were readily detected. This clear separation of assembly of the 40S and 60S subunits was an unexpected feature of ribosome synthesis.

The simplest explanation for this phenomenon is that the assembly pathway reflects the 5′–3′ synthesis of the pre-rRNA. Transcription of the 7 kb long 35S pre-rRNA requires approximately 5 min. This suggests that during early transcription binding sites in the 5′-ETS first become available to initiate 40S subunit processing factor assembly. Later on with a delay of several minutes, when the 3′ sequences of the 35S pre-rRNA become synthesized, the ribosomal L-proteins and the processing/assembly factors of the 60S subunit can be recruited. It may well be the case that a large fraction of the purified complexes actually represent factors that were assembled onto nascent transcripts. Consistent with this idea, examination of the nascent yeast pre-rRNAs in "Miller" spreads (Saffer and Miller, 1986) showed the presence of the terminal balls on the nascent transcripts. Due to the nature of the analysis by mass spectrometry, we identify only proteins that are approximately stoichiometric, so any pre-60S synthesis factors assembling later onto the 35S pre-rRNA will have been overlooked.

A second unexpected finding was that most factors associated with the 35S pre-rRNA showed little coprecipitation of the 20S pre-rRNA, indicating that they dissociate from the pre-rRNA at or immediately following A_2 cleavage (Figure 8). Of the proteins tested, only Enp1p remained significantly associated with the 20S rRNA and is likely to accompany the pre-40S ribosomes to the cytoplasm, where final modification and processing of the 20S pre-rRNA occurs.

While this manuscript was under revision, the identification of a related but apparently not identical complex was reported (Dragon et al., 2002). This complex was designated the small subunit (SSU) processome, although it is unclear whether it actually contains any of the pre-rRNA processing enzymes. All of the known enzymes that participate in large subunit synthesis are absent from the purified pre-60S particles (reviewed in Fatica and Tollervey, 2002). The SSU processome complex is reported to contain fewer proteins than those identified here and to cosediment with 80S ribosomes, suggesting that it might represent a precursor in 90S pre-ribosome assembly.

Experimental Procedures

Microbiological techniques and yeast work were done essentially as described (Baβler et al., 2001; Gavin et al., 2002). DNA recombinant work was performed as described (Maniatis et al., 1982). Isolation of ribosomes under low salt conditions by sucrose gradient centrifugation was done as described (Baβler et al., 2001). These ribosomes were extracted from yeast cultures grown in YPD medium to an OD_{600nm} of 0.8 before cycloheximide was added to a final concentration of 100 μg/ml. TAP-tagged yeast proteins were purified and analysis of associated proteins by mass spectrometry was performed essentially as described (Gavin et al., 2002). Gel filtration analysis was performed in lysis buffer (50 mM TRIS-HCl [pH 7.5], 100 mM NaCl, 0.15% Igepal [NP40], 1.5 mM $MgCl_2$, 0.5 mM DTT, protease inhibitors, and 1 mM PMSF) using the FPLC System (Amersham/Pharmacia). The TEV eluate from the IgG column of each TAP-tagged protein was loaded on a Superose 6 HR 30/10 column (Amersham/Pharmacia). During gel filtration, 0.4 ml fractions were collected and analyzed by SDS-PAGE. The fluorescence-based vi-

sual assay to analyze nuclear export of large ribosomal subunits using the Rlp25p-eGFP reporter in living cells has been published (Gadal et al., 2001b). A similar fluorescence-based visual assay to analyze nuclear export of small ribosomal subunits using the Rps2p-eGFP reporter and isolation of *rix* mutants with a specific defect in 40S subunit export from a bank of random ts mutants will be published elsewhere (D.S. and E.H., unpublished data). The indirect immunofluorescence assay to detect ProtA-tagged baits in yeast cells was performed as described (Gautier et al., 1997). Northern analysis was done essentially as described (Baβler et al., 2001). Oligonucleotides used: 002, GCTCTTTGCTCTTGCC; 003, TGTTAC CTCTGGGCCC; 008, CATGGCTTAATCTTTGAGAC; 017, GCGTTGT TCATCGATGC; 020, TGAGAAGGAAATGACGCT; 033 CGCTGCTCAC CAATGG; 200, UUAUGGGACUUGUU (2'-O-methyl RNA); 020 TGA GAAGGAAATGACGCT; 202 TCACTCAGACATCCTAGG; 203 CUA UAGAAAUGAUCCU (2'-O-methyl RNA). Function and localization information about yeast proteins was retrieved from the YPD database (Hodges et al., 1999; Costanzo, 2001).

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