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60S pre-ribosome formation viewed from assembly in the nucleolus until export to the cytoplasm

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60S ribosomes undergo initial assembly in the nucleolus before export to the cytoplasm and recent analyses have identified several nucleolar pre-60S particles. To unravel the steps in the pathway of ribosome formation, we have purified the pre-60S ribosomes associated with proteins predicted to act at different stages as the pre-ribosomes transit from the nucleolus through the nucleoplasm and are then exported to the cytoplasm for final maturation. About 50 non-ribosomal proteins are associated with the early nucleolar pre-60S ribosomes. During subsequent maturation and transport to the nucleoplasm, many of these factors are removed, while others remain attached and additional factors transiently associate. When the 60S precursor particles are close to exit from the nucleus they associate with at least two export factors, Nmd3 and Mtr2. As the 60S pre-ribosome reaches the cytoplasm, almost all of the factors are dissociated. These data provide an initial biochemical map of 60S ribosomal subunit formation on its path from the nucleolus to the cytoplasm.

Keywords: Nmd3/nuclear export/nucleolus/rRNA processing/60S subunit

Introduction

Ribosomal biogenesis is a complicated process starting with the transcription of rDNA repeats by RNA polymerase I (25S, 18S and 5.8S rRNA) and polymerase III (5S rRNA) (Kressler *et al*., 1999; Venema and Tollervey, 1999). Polymerase I transcription generates the 35S pre-rRNA, which then undergoes rapid processing to the mature rRNAs by endonucleases and exonucleases, with concomitant modification of the rRNA by pseudouridylation and methylation (Venema and Tollervey, 1999). Ribosomal RNA biogenesis has been extensively studied, and is fairly well understood. During these processing reactions, a large number of non-ribosomal proteins associate with the pre-rRNAs, and many of the ~80 ribosomal proteins are assembled onto the rRNA (Warner, 1989, 1999; Woolford, 1991). The process of ribosome assembly is, in contrast, just beginning to be understood (Warner, 2001). Early analyses reported that the ribosomal proteins and the 35S pre-rRNA primary transcript initially form a 90S particle (Trapman *et al*., 1975; Kruiswijk *et al*., 1978), which also includes many non-ribosomal proteins (Dragon *et al*., 2002; Grandi *et al*., 2002). Within the 90S pre-ribosomes the 35S pre-rRNA undergoes three rapid cleavage steps, which separate the precursors to the large and small subunits (Trapman *et al*., 1975; Venema and Tollervey, 1999). The precursor to the large subunit was initially described as the 66S particle, but is now known to consist of a family of pre-60S particles that carry many accessory proteins that show dynamic changes during maturation. Within the pre-60S particles the 27S pre-rRNAs are matured to the 25S and 5.8S rRNAs (Trapman *et al*., 1975), and the 5S rRNA associates with the complex. The early pre-60S particles are restricted to the nucleolus, but later particles are released into the nucleoplasm (Milkereit *et al*., 2001) and then exported to the cytoplasm. Nuclear export of the 60S pre-ribosome to the cytoplasm is facilitated by Nmd3, which is an NES-containing adapter protein that requires the export receptor Xpo1/Crm1/exportin-1 (Ho *et al*., 2000b; Gadal *et al*., 2001b), but may also require the function of other pre-60S associated proteins. The 40S subunit undergoes a comparatively simpler maturation (Venema and Tollervey, 1999) but its mechanism of export is still unknown (Moy and Silver, 1999).

Despite the speed with which the cell modifies and processes the pre-rRNA, several groups succeeded in isolating and characterizing stable pre-60S particles (Baßler *et al*., 2001; Harnpicharnchai *et al*., 2001; Saveanu *et al*., 2001; Fatica *et al*., 2002). These particles differed in protein and RNA composition and appeared to reflect the presence of a series of distinct nucleolar and/or nucleoplasmic pre-60S particles. To better define the pathway of 60S subunit synthesis we purified a series of particles to obtain 'snapshots' of the pre-60S ribosomes as they move from the nucleolus to the cytoplasm. Here, we report a detailed analysis of the composition of seven distinct 60S pre-ribosomes and describe their major location in the cell.

Results

Sedimentation of 60S pre-ribosomal particles

We previously isolated a pre-60S ribosomal particle by tandem-affinity purification of an associated component, Nug1, which is a putative GTPase (Baßler *et al*., 2001). This precursor contained ~20 non-ribosomal proteins, including both characterized factors and the products of many previously uncharacterized open reading frames. To unravel the spatio-temporal changes that occur during ribosome formation inside the nucleus, from early assembly in the nucleolus until final ribosomal export, we employed a 'reverse tagging' methodology. The

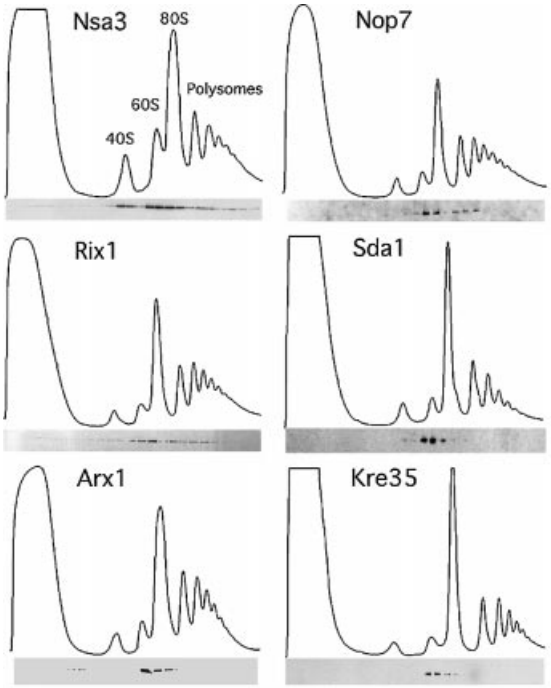

Fig. 1. Reverse-tagged protein baits of the Nug1-containing 60S pre-ribosome are associated with pre-ribosomal particles of different sizes. The sedimentation behavior of the indicated TAP-tagged proteins was analyzed on sucrose density gradients, and ribosomal fractions (40S, 60S, 80S and polysomes) were determined by OD_{254} measurement of the gradient fractions (upper graph). Western blot analysis of these gradient fractions using anti-ProtA antibodies reveals the position of the indicated TAP-tagged baits (lower panel). Note that some baits (e.g. Nsa3) exhibit a broad distribution on the sucrose gradient, whereas other baits (e.g. Kre35) exhibit a distinct peak at ~60S (see text).

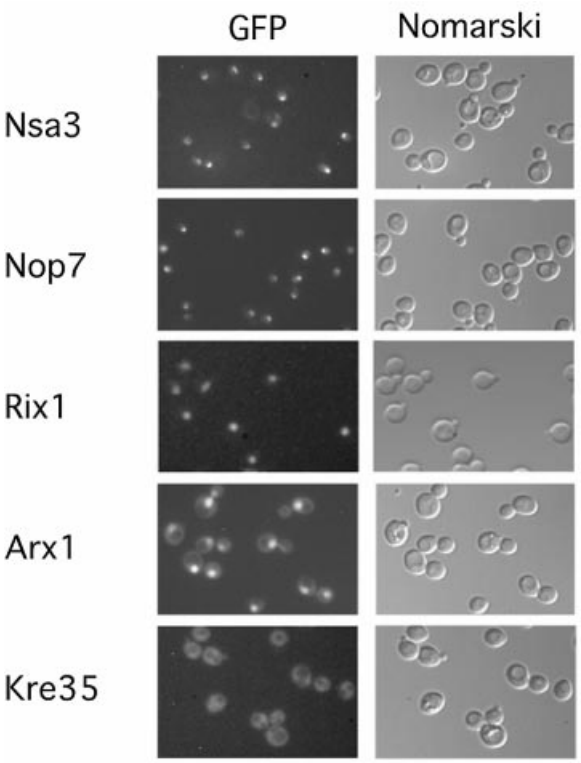

Fig. 2. Subcellular location of GFP-tagged protein baits in yeast cells. The *in vivo* location of the indicated tagged proteins, associated with different 60S pre-ribosomes, was analyzed by fluorescence microscopy. For microscopic inspection, cells were grown to mid-log phase, mounted on a microscope slide and photographed with identical exposure times.

rationale behind this approach was that some of the associated proteins in the Nug1 pre-ribosomal complex could also be present in other precursor particles and thus allow purification of pre-ribosomes from different stages of maturation. We have selected the non-ribosomal proteins Nsa3, Nop7, Sda1, Rix1 and Arx1, which are components of the Nug1-containing pre-ribosome (Baßler *et al*., 2001). Moreover, we also chose Kre35, which is a homolog of Nug1 and a putative GTPase (E.Hurt, unpublished data). These bait proteins were genomically tagged at the C-terminus with the TAP tag for tandem affinity purification (Puig *et al*., 2001) and sedimentation analysis. In addition, these factors were tagged with the green fluorescent protein (GFP) for subcellular localization studies.

We first determined the size of the pre-ribosomal particles that purify with the different tagged proteins. Previous analyses showed that Nug1 associated with pre-60S particles on sucrose gradients (Baßler *et al*., 2001). Similar sucrose gradient centrifugation showed that each of the bait proteins associated with large particles, but with markedly different sedimentation profiles. Nsa3 displays a complicated sedimentation pattern with one peak at ~40S, a second broader peak from 60S to 90S and some partitioning into fractions below 90S (Figure 1). In contrast, Sda1, Arx1 and Kre35 showed a distinct and confined peak at ~60S (Figure 1). Nop7 and Rix1 reveal an 'intermediate' sedimentation pattern, with a pronounced peak at 60S and a second broader peak below 80S (Figure 1). We conclude from these studies that the bait proteins analyzed are associated with different 60S pre-ribosomal particles, and in some cases are associated with more than one particle (see Discussion).

Intracellular location of 60S pre-ribosomes

To determine the steady state location of these pre-ribosomal particles in living cells, yeast strains expressing GFP-tagged Nsa3, Nop7, Rix1, Arx1 or Kre35 were examined by fluorescence microscopy (Figure 2). Our previous data showed Nug1–GFP to be located in both the nucleolus and nucleoplasm (Baßler *et al*., 2001). The studies shown here suggest that Nsa3 and Nop7 are present in earlier particles, since they are predominantly concentrated in the nucleolus (Figure 2; see also Harnpicharnchai *et al*., 2001; Adams *et al*., 2002). In contrast, Rix1–GFP localized throughout the nucleus (Figure 2; see also Baßler *et al*., 2001). Sda1–GFP could not be tested, since it was expressed very inefficiently (data not shown), but myc-tagged Sda1 co-localized with DAPI staining, showing a nucleoplasmic localization (Buscemi *et al*., 2000). Arx1–GFP accumulates in the nucleoplasm but is also present in cytoplasm (Figure 2), suggesting that it accompanies the pre-60S particles to the cytoplasm. Finally, Kre35–GFP exhibits an exclusive cytoplasmic distribution with nuclear exclusion (Figure 2). This analysis allows us to propose a pathway for pre-60S maturation, from the predominant nucleolar particles associated with Nsa3 and Nop7, to nucleolar/

nucleoplasmic particles (Nug1), nucleoplasmic particles (Rix1 and Sda1), nucleoplasmic/cytoplasmic particles (Arx1) and finally the cytoplasmic Kre35-associated particles.

Composition of 60S pre-ribosomes

To obtain information about the protein and RNA composition of the different 60S pre-ribosomes, we TAP-purified each of the six tagged proteins under standardized conditions of cell growth and cell lysis, and compared them with the previously purified Nug1 particle (Baßler *et al*., 2001). Co-precipitated proteins were compared on the same SDS–polyacrylamide gel stained with Coomassie Blue (Figure 3A). The loading of the various particles can be estimated from the recovery of ribosomal L-proteins, which correspond to prominent bands below 50 kDa (Figure 3A; e.g. Rpl3) and is approximately equal, with the exception of Arx1, which is slightly overloaded. The samples were loaded (from left to right) in the order corresponding to their position in the predicted maturation pathway. This showed a striking decrease during non-ribosomal protein complexity in the pre-60S ribosome's maturation.

The earliest nucleolar particles that co-precipitated with tagged Nsa3 and Nop7 have similar protein profiles when the major bands are inspected following Coomassie staining (Figure 3A). Mass spectrometry (MALDI-TOF) identified additional non-ribosomal proteins associated with Nsa3 (~50 species were identified in total; Table I; Supplementary table SI available at *The EMBO Journal* Online), including several factors known to act in early pre-rRNA processing and ribosome assembly steps. Among these are the methyltransferase Spb1 and components of both the box C/D and H/ACA classes of snoRNPs (Nop1, Nop56, Nop58, Spb1, Rrp9 and Cbf5), which direct rRNA methylation and pseudouridinylation and are required for early pre-rRNA cleavage. Moreover, some of the associated factors have also been implicated in 40S biogenesis (Nop1, Nop58, Rrp5, Rrp8, Rrp9 and Nop14). However, many of the Nsa3-associated components were also found in the Nug1 particle, including the helicases Dbp2, Dbp10 and Has1, the methyltransferase Spb1, and the GTPases Nug1, Nug2 and Nog1 (Table I), all of which are implicated in 60S subunit synthesis. These results suggested that Nsa3 is predominately associated with pre-60S particles and has lower association with earlier 90S pre-ribosomes, consistent with the results of sucrose gradient analyses (Figure 1) and RNA co-precipitation (see below).

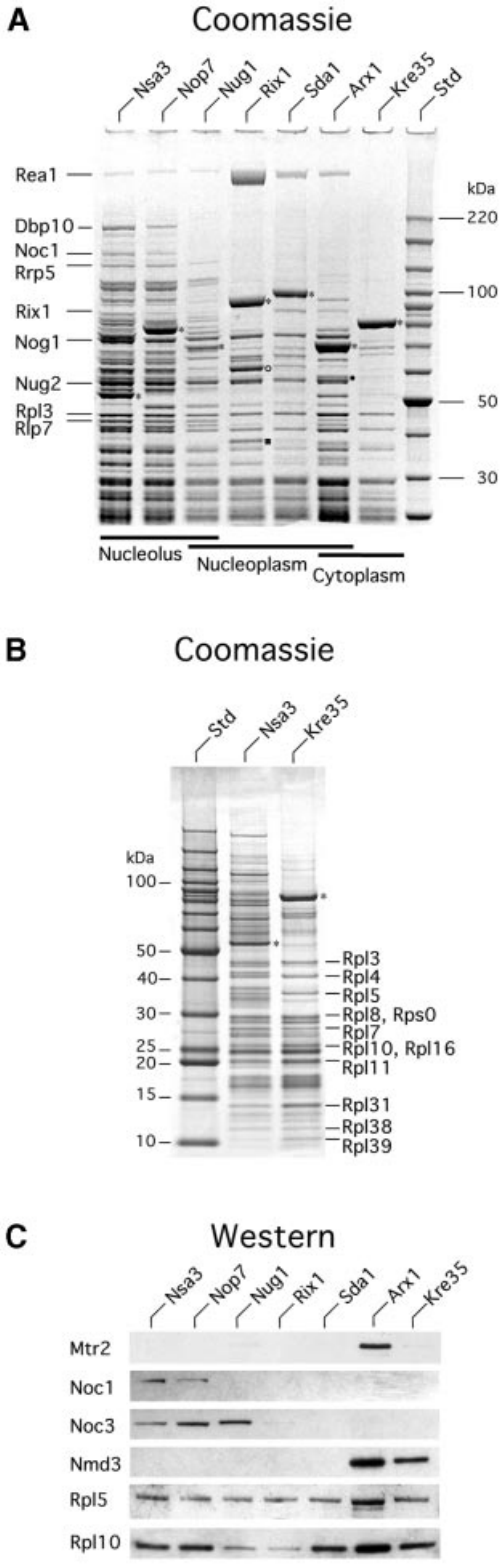

Fig. 3. SDS–PAGE analysis of the protein composition of the different pre-60S ribosomes. (**A**) The indicated TAP-tagged protein baits were isolated from yeast lysates by a two-step affinity purification (TAP method), and TAP-purified bait proteins were separated on an 4–12% SDS–polyacrylamide gradient gel and stained with colloidal Coomassie Blue. Co-purifiying proteins were identified by mass spectrometry (MALDI-TOF) and prominent co-migrating bands are indicated. The positions of tagged proteins are indicated by asterisks, Nmd3 in the Arx1 preparation by a filled circle, and Ynl182p and Yhr085p in the Rix1 preparation by an open circle and closed square, respectively. The molecular weight marker is indicated on the right. Note the relative higher loading of the Arx1 particle in comparison with the Rix1, Sda1 and Kre35 particle loading. (**B**) Lower molecular weight proteins in earliest (Nsa3) and latest (Kre35) particles as visualized with 10–20% SDS–PAGE. All bands, both in the high (Table I) and low molecular weight range (Table II), were identified by MS. Each bait protein is indicated with an asterisk. In addition, selected ribosomal proteins are indicated. (**C**) Western blot analysis of the purified bait proteins, analyzed by SDS–PAGE as shown in (A) and listed in Supplementary table SI. The western blot was incubated with the indicated antibodies to Mtr2, Noc1, Noc3, Nmd3, Rpl5 and Rpl10. Not shown are Sqt1, which was identified in all purifications, and Tif6, which was present in all except Kre35.

Table I. Non-ribosomal proteins associated with the different 60S pre-ribosomal particles

associated	Nsa3	Nug1	Rix1	Sda1	Arx1	Kre35	
Arx1							DEAD box RNA helicase
Dbp2		*					DEAD box RNA helicase
Dbp10							DEAD box RNA helicase
Erb1							WD40 repeats
Has1							DEAD box RNA helicase
Noc2							60S intranuclear transport
Nog1							Putative GTPase
Nop2		*					60S processing and synthesis
Nop7							Pescadillo homologue
Nsa3							Assembly of 26S protesome
Nug1							Putative GTPase
Nug2							Putative GTPase
Rea1							AAA ATPase
Sda1							rRNA synthesis
Spb1							Putative methyltransferase
Spb4							DEAD box RNA helicase
Ycr072c							WD40 repeats
Ypl146c							Unknown; purifies with NPC
Ytm1		*					WD40 repeats; 60S biogenesis
Rix1							60S biogenesis
Ynl182c							Unknown essential
Nap1							Nucleosome assembly
Kre35							Putative GTPase
Nmd3							Export adaptor
Ybr267w							C2H2 Zn finger
Cbf5							rRNA PsuS; part of H/ACA
Drs1							DEAD box RNA helicase; 60S bio.
Ebp2							60S processing and assembly
Mak11							WD40 repeats
Noc1							60S biogenesis
Nop1							35S processing and methylation
Nop4							rRNA processing and methylation
Nop12							25S pre-RNA synthesis
Nop14							40S biogenesis and rRNA process.
Nop56							Box C/D component
Nop58/5							Box C/D component
Prp43							Pre-mRNA splicing factor
Puf6							Unknown non-essential
Rrp5							Processing to 25S and 5.8S rRNA
Rrp8							A2 cleavage
Rrp9							Associated with U3 snoRNP
Ssf1							A2 cleavage associated
Ssf2							A2 cleavage associated
Yjl109c							Unknown essential
Xrn1							5'-3' exonuclease
Noc3							60S intranuclear transport
Pab1							Poly(A) binding protein
YHR085w							Unknown essential
Pse1							Karyopherin

TAP-tagged proteins are listed in the top row (bait, light gray). Co-purifying proteins larger than L3 (44 kDa) identified by mass spectrometry (row 'associated') are indicated vertically as gray rectangles in the columns below each bait protein. If known, the function of the associated proteins present in the purified 60S pre-ribosomes are indicated in the most right vertical column. The asterisk indicates proteins in the Nug1 preparation that have been newly identified in this study. Not indicated in the table are additional proteins associated with the bait proteins, which could be considered contaminants: Act1, Cdc10, Ded1, Gfa1, Mis1, Ssa1, Ssa2, Ssa4, Sbp1 and Tef1. Furthermore, selected proteins smaller than L3 (44 kDa) identified in the Arx1 particle using MS are Asc1, Nsa2, Pup2, YJL122w and Yvh1.

During transition from the nucleolar Nsa3- or Nop7-containing pre-ribosomes to the nucleolar/nucleoplasmic located Nug1-containing particles, many factors are dissociated causing a significant simplification of the protein composition (Figure 3A; Table I; Supplementary table SI). Among these are factors involved in processing in ITS1 (Rrp5, Rrp8, Rrp9, Ssf1 and Ssf2), the box C/D and H/ACA components, helicases and Noc1. Consistent with this, Noc1 was previously shown to be present in early nucleolar pre-ribosomes together with Noc2, but to be replaced by Noc3 upon release into the nucleoplasm (Milkereit *et al*., 2001). Western blot analysis (Figure 3C) confirms the exchange of Noc1 for Noc3. In the earliest particle (Nsa3), Noc1 is significantly enriched and Noc3 is present in lower amounts. As the particle matures (Nop7), Noc1 becomes less prevalent and Noc3 becomes more prominent. Subsequently, the Nug1 particle has largely lost Noc1 and is enriched for Noc3 (Figure 3C).

The next set of particles, which are associated with Rix1 and Sda1, showed a further reduction in complexity (Figure 3A). These nucleoplasmic particles (see Figure 2) are still associated with the putative GTPases Nug1, Nug2 and Nog1, but other factors, including the ribosomal-like protein Rlp7 (Dunbar *et al*., 2000; Gadal *et al*., 2002) and the putative helicases Dbp2, Dbp10 and Has1, are substantially reduced (Figure 3A; Table I). Moreover, western analysis revealed that Noc3 is largely absent from these latter particles (Figure 3C). Concomitant with the removal of certain factors, other components join these particles. In particular, the Rix1-containing pre-ribosome is associated with large amounts of Rea1, a putative AAA-type ATPase (for ribosome export/assembly; see Baßler *et al*., 2001), and Ynl182p, an essential unknown protein that exhibits homology to Rrp9 and Pwp2 (Figure 3A; Table I). A large amount of the uncharacterized protein Yhr085p is associated with Rix1 but has not been found in any other purified pre-ribosomal particle. The Sda1-containing particle exhibits decreased association of Rea1 and Ynl182p, and is somewhat simpler than the Rix1-associated particle (Figure 3A).

A next major stage in the biogenesis of 60S subunits is represented by the Arx1-containing pre-ribosome, which has a dual location in both the nucleoplasm and cytoplasm. Biochemical and western analysis of the TAP-purified Arx1 show that the NES-containing export factor Nmd3 joins the 60S pre-ribosome at this stage (Figure 3A and C). Nmd3 binds to the general nuclear export receptor Xpo1/Crm1/exportin-1, which is crucial for nuclear export of 60S ribosomal subunits (Ho *et al*., 2000b; Gadal *et al*., 2001b). In contrast, the ribosomal protein Rpl10, which interacts with Nmd3 both *in vivo* (Karl *et al*., 1999) and *in vitro* (Gadal *et al*., 2001b) and is also required for nuclear export of 60S subunits (Gadal *et al*., 2001b), is present in all particles, from early nucleolar (Nsa3) until late cytoplasmic 60S pre-ribosomes (Kre35) (Figure 3C). In addition to Nmd3, Ybr267p, which has a C2H2 zinc finger motif, and the putative GTPase Kre35, which is homologous to Nug1 and Nug2, were found to be associated with the Arx1-containing 60S pre-ribosome (Figure 3A; Table I). Western blot analysis also demonstrated that the nuclear export factor Mtr2 specifically associates with the Arx1 particle (Figure 3C). Mtr2 forms a complex with the conserved mRNA export factor Mex67 but the *mtr2-33* allele was previously observed to specifically inhibit pre-60S export (Baßler *et al*., 2001; see Discussion).

The final particle characterized is associated with the putative GTPase Kre35. This late, cytoplasmic pre-60S ribosome was associated with many ribosomal proteins but few non-ribosomal proteins (Figure 3A; Tables I and II). These include Nmd3, which is speculated to also function in the cytoplasm (Ho *et al*., 2000a), and Arx1, consistent with the proposal that it accompanies the pre-60S particle to the cytoplasm (see above). In addition, an uncharacterized zinc finger protein Ybr267p was identified in the particle. Notably absent were the nucleolar and nucleoplasmic GTPases, Nug1, Nug2 and Nog1, as well as the AAA ATPase Rea1.

Ribosomal proteins were compared in detail for the early Nsa3-associated and late Kre35-associated particles. The pattern of bands in the low molecular weight range of the SDS–polyacrylamide gel was strikingly similar (Figure 3B). Mass spectrometry of these bands revealed

Table II. Identification of low molecular weight proteins in the Nsa3 and Kre35 preparations

associated	Nsa3	Kre35	
Brx1			BRIX family; 25S maturation
Mrt4			mRNA turnover
Nip7			60S biogenesis
Nop15			Unknown function, has RNA recog. motif
Nop16			60S biogenesis
Nsa2			Associated with 26S proteasome
Rlp7			C2 cleavage; similar to L7
Rlp24			60S biogenesis; Similar to L24
Rrp1			Protein involved in 25S maturation
Rpf1			BRIX family; Large subunit RNA maturation
Rpf2			BRIX family; Large subunit RNA maturation
Rpl2			RP
Rpl3			RP
Rpl4			RP
Rpl5			RP; Sole 5S rRNA-associated ribosomal protein
Rpl7			RP
Rpl8			RP
Rpl9			RP
Rpl10			RP; Mediates interaction with Nmd3
Rpl11			RP
Rpl12			RP
Rpl13			RP
Rpl14			RP
Rpl15			RP
Rpl16			RP; binds 5.8S RNA *in vitro*
Rpl17			RP
Rpl18			RP
Rpl19			RP
Rpl21			RP
Rpl23			RP
Rpl27			RP
Rpl30			RP; own mRNA splicing; rRNA processing
Rpl31			RP
Rpl32			RP
Rpl33			RP
Rpl35			RP
Rpl36			RP
Rpl38			RP
Rpl39			RP; required for translational accuracy
Rpl43			RP
Rpp0			Acidic ribosomal protein P0
Rps0			RP; component of a 45S penta-snRNP
Rps1			RP; component of a 45S penta-snRNP
Rps3			RP; component of a 45S penta-snRNP
Rps4			RP; component of a 45S penta-snRNP
Rps7			RP; component of a 45S penta-snRNP
Rps8			RP
Rps14			RP; component of a 45S penta-snRNP
Rps17			RP
Rps21			RP
Rps24			RP
Rps25			RP
Rps26			RP
Yvh1			Protein-tyrosine phosphatase

TAP-tagged proteins are listed in the top row (bait, light gray). Co-purifying proteins smaller than L3 (44 kDa) identified by mass spectrometry (row 'associated') are indicated vertically as gray rectangles in the columns below each bait protein. If known, the function of the associated proteins present in the purified 60S pre-ribosomes are indicated in the most right vertical column.

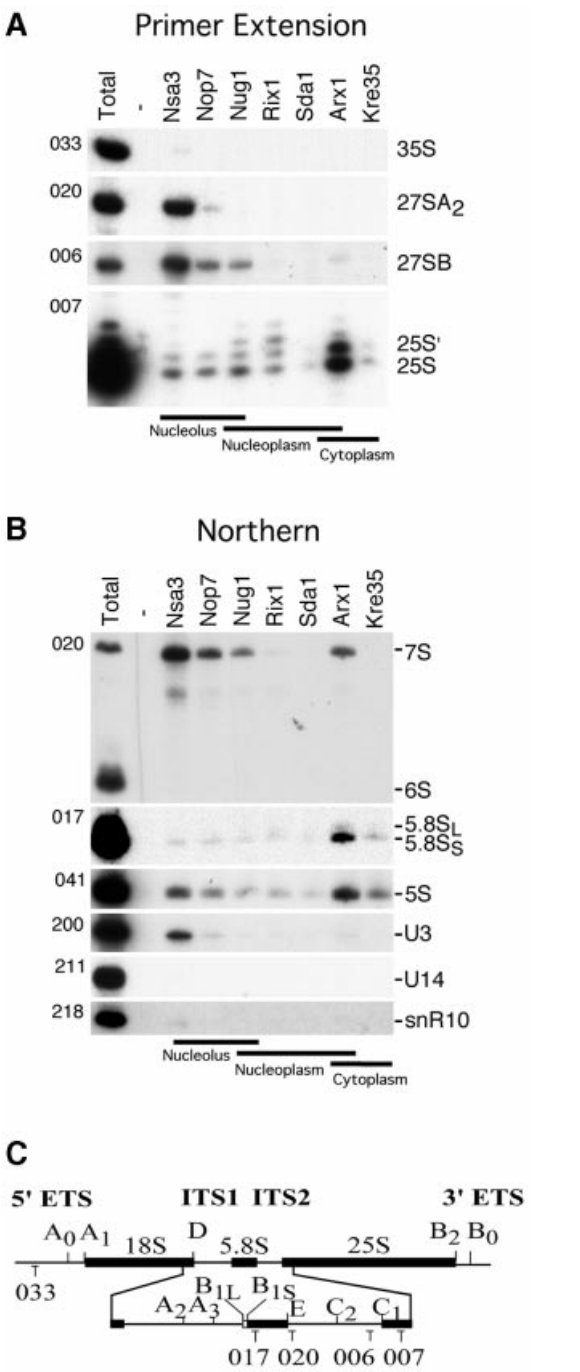

Fig. 4. RNA analysis of the different pre-60S particles. (**A**) Primer extension and (**B**) northern hybridization analyses for 35S, 27S and 25S rRNA were performed on RNA extracted from whole cells (Total) and affinity-purified tagged (Nsa3, Nop7, Nug1, Rix1, Sda1, Arx1 and Kre35) and non-tagged (Negative) wild-type strain. RNA was loaded proportionally to a Coomassie stained protein SDS–PAGE from the same purifications. The oligonucleotide used for each panel is indicated on the left side of each gel (see Materials and methods). The annealing location of oligonucleotides used for primer extension and northern hybridization analysis are indicated (**C**).

that both particles contain predominantly the same L-proteins, suggesting that most of the L-proteins are already assembled onto early pre-60S particles (Table II; Supplementary table SI). Moreover, we also found a low number of S-proteins in the Nsa3 and Kre35 preparations. Nsa3 is expected to co-precipitate with some small subunit proteins due to its association with 90S pre-ribosomes. The recovery of small subunit proteins with Kre35 could reflect some association with 40S subunits in the cytoplasm. However TAP purifications are frequently contaminated by ribosomal proteins (Gavin *et al*., 2002) and therefore this could be non-specific.

The RNA composition of the pre-ribosomal particles was also compared by primer extension (Figure 4A) and northern blot hybridization (Figure 4B). The Nsa3 particle predominantly contained the $27SA_2$ and 27SB pre-rRNAs, as shown by primer extension (Figure 4A), and by northern hybridization, the 7S rRNA (Figure 4B). The Nsa3 particle is unique in the presence of the U3 snoRNA, but in

contrast no U14 or snR10 snoRNA could be observed (Figure 4B). Furthermore, none of the particles contained the 18S rRNA (data not shown). Taken together, these data mark Nsa3 as the earliest ribosomal pre-60S particle.

Similar to Nsa3, both Nop7 and Nug1 contained mainly 27SB, 7S pre-rRNA and 5S rRNA, but less of the $27SA_2$ precursor RNA and a higher ratio of matured 25S rRNA. These results are similar to previous results (Baßler *et al*., 2001; Harnpicharnchai *et al*., 2001) with only small variations in relative abundance, probably due to alternative purification procedures or strain background differences (see Harnpicharnchai *et al*., 2001).

For the Rix1, Arx1 and Kre35 particles, the most abundant ribosomal RNA species were mature 25S, 5.8S and 5S with smaller quantities of 27S and 7S precursors (Figure 4A and B). These data strongly suggest that rRNA maturation of the 60S ribosomes is largely completed at the nucleoplasmic stage of the Rix1 particle. This is supported by the finding that the *rix1-1* mutant very efficiently accumulates the Rpl25–GFP reporter inside the nucleus, but does not show any rRNA processing defect (Baßler *et al*., 2001). In several independent preparations Sda1 did not detectably co-precipitate any pre-rRNAs, despite good protein precipitation, leading to the conclusion that it is predominately associated with the mature rRNAs.

These RNA analyses further confirm the ordering of these particles suggested by sedimentation, localization and protein composition analysis. Overall, this ordering showed that the particles represent five stages: (i) highly complex nucleolar pre-60S particles (Nsa3, Nop7); (ii) complex nucleolar/nuclear pre-60S particles (Nug1); (iii) intermediate complex nucleoplasmic particles (Rix1, Sda1); (iv) intermediate complex nuclear–cytoplasmic 60S pre-ribosome (Arx1); and (v) simple cytoplasmic 60S pre-ribosome (Kre35). Finally, it is important to note that the 5S rRNA (Figure 4B) and its binding partner the L5 protein (Figure 3C) were detected in each of the particles, indicating that the 5S RNP joins an early pre-60S particle. Together these results strongly indicate that maturation of the 25S rRNA is largely or fully completed prior to release of the particle from the nucleolus, whereas the final maturation of 7S to 5.8S rRNA occurs in the nucleoplasm.

Discussion

We have adopted a proteomic approach to follow the 60S pre-ribosomal particles from its assembly in the nucleolus until appearance in the cytoplasm. To this end, we isolated and compared the pre-60S ribosomes that were associated with seven proteins. Notably, a given bait protein can occur in different pre-ribosomal particles (for example Arx1), yet when purified is preferentially associated with a class (early, medium or late) of 60S pre-ribosomes. An explanation for this could be that the concentration of a given bait protein differs in the various precursor particles at steady state. Accordingly, Arx1 may be at its highest concentration in late nuclear/cytoplasmic particles, and occur in lower amounts in earlier particles. We have succeeded in ordering the purified complexes from early to late, consistent with the protein sedimentation and localization as well as the rRNA content of these particles. Thus, these analyses provide sequential 'snapshots' of the biochemical composition of these particles along the maturation and export pathway from nucleolar processing through nuclear export to cytoplasmic maturation. The particles characterized here contain many non-ribosomal proteins that were not isolated with previous 60S pre-ribosome purifications. The TAP purifications of Nug1, Sda1, Kre35 and Nop7 have been reported previously and the results are generally similar to our findings (Baßler *et al*., 2001; Harnpicharnchai *et al*., 2001; Gavin *et al*., 2002). Here we purified all seven bait proteins under identical conditions and further analyzed them for localization, sedimentation and RNA content.

These data shed new light on the earliest and late intermediates and give insights into how pre-ribosomes gain export competence. The earlier pre-60S particles, which are associated with Nsa3, have many known 60S biogenesis factors. However, these particles also contain a few components that also play a role in 40S subunit biogenesis. Among these are core factors of both box C/D and box H/ACA snoRNAs (Maden, 1998), which are required for modification of the pre-rRNA. Most snoRNA-directed modification takes place on the 35S rRNA, but the presence of the snoRNP proteins in the pre-60S particle could also indicate that this is not fully completed prior to the early cleavages. A similar observation has been made for *Xenopus* pre-rRNA methylation (Yu *et al*., 1997). Also present is Rrp5, which is required for cleavage of the $27SA_2$ pre-rRNA at site A_3, as well as for cleavage at earlier sites on the 40S synthesis pathway (Venema and Tollervey, 1996). Its presence in an early 60S pre-ribosome is not unexpected, but it was not identified in previous analyses. Less expected was the presence of the U3 snoRNP protein Rrp9p, which is required for cleavage at sites A_0, A_1 and A_2 within the 35S pre-rRNA (Venema and Tollervey, 1996; Venema *et al*., 2000). Whether Rrp9 remains on the pre-rRNA after the other U3 snoRNP components have dissociated or has a separate function in 60S synthesis remains to be determined. Notably, Rrp9 was recently found to be associated with early 90S pre-ribosomes including the 35S pre-rRNA, the U3 snoRNP and 40S subunit processing factors, but predominantly lacking 60S synthesis factors (Grandi *et al*., 2002). However, purified Rrp9 also contains $27SA_2$ pre-rRNA, whereas the other purified 40S biogenesis factors do not contain this intermediate (Grandi *et al*., 2002). Thus, Rrp9 could be one among fewer factors that bridge between 60S and 40S biogenesis and are already present in 90S pre-ribosomes. Thus, it is possible that Rrp9, Nsa3 and a few other 60S biogenesis factors become associated with 35S pre-rRNA to trigger cleavage at A_2 site as a prerequisite for generating 27S pre-rRNA and subsequent recruitment of the bulk 60S biogenesis machinery. Ribosomal RNA analysis (Figure 4) and sucrose gradient sedimentation (Figure 1) suggest that Nsa3 might bind to very early pre-60S particles and possibly already to 90S particles.

Previously purified 60S pre-ribosomes that were associated with Nop7, Nug1, Nog2/Nug2 and Ssf1 (Baßler *et al*., 2001; Harnpicharnchai *et al*., 2001; Saveanu *et al*., 2001; Fatica *et al*., 2002) represent nucleolar intermediates that are located prior to the pre-60S particles associated with Sda1, Arx1 and Kre35 in the 60S synthesis pathway (the protein profile of TAP-tagged purified Nug2 is similar

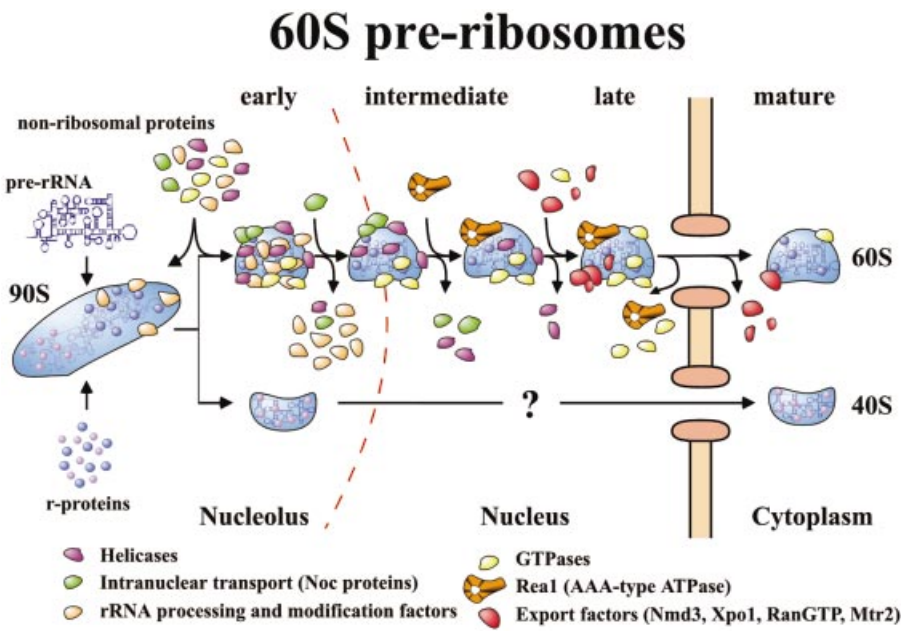

Fig. 5. Model of the pathway of 60S pre-ribosome maturation and export. The maturation of the pre-ribosome is depicted. The various classes of factors that are associated with the particle are identified by color. The factors coming on and off the particles represent actual identified proteins by mass spectrometry. For simplicity, the alterations in RNA composition are not depicted.

to that of Nug1; T.A.Nissan and J.Baßler, unpublished data).

The pre-ribosomal particles isolated with Arx1 and Kre35 baits represent, respectively, the 60S pre-ribosome just prior and after export into the cytoplasm. Strikingly, these particles are relatively simple and have only a few non-ribosomal proteins bound. The nuclear export factor Nmd3 joins the 60S pre-ribosome at the level of Arx1 (Ydr101c, termed Arx1 for associated with ribosomal export complex), which could be the trigger for the acquisition of export competence. The adapter Nmd3 can interact with both the large subunit protein Rpl10 on the ribosome and the nuclear export receptor Xpo1 (Ho *et al*., 2000b; Gadal *et al*., 2001b). Xpo1 is thought to bind to nucleoplasmic NES-containing cargoes, such as Nmd3, in a RanGTP-dependent manner. However, Rpl10 was found to be present in all the purified 60S pre-ribosomes. It is thus conceivable that the Nmd3 binding site on Rpl10 is concealed in earlier 60S pre-ribosomes, but revealed in a later stages of maturation, e.g. by the action of ATPases or GTPases. In earlier studies, Rpl10p was reported to associate with 60S ribosomal subunits in the cytoplasm where it functions to mediate joining of 60S and 40S subunits (Eisinger *et al*., 1997). Our previous work suggested that Rpl10p may have a late nuclear function, as indicated by defects in ribosome export in *rpl10* temperature-sensitive (ts) mutants (Gadal *et al*., 2001b).

Another nuclear export factor, Mtr2, was also found associated with the Arx1 particle. Most ts mutants of *MTR2* show an mRNA export defect (Santos-Rosa *et al*., 1998). However, the *mtr2-33* allele did not impair mRNA export, but showed nuclear accumulation of 60S pre-ribosomes at the restrictive temperature and a synthetic lethal relationship with the Nug1 GTPase, Ecm1 and Nmd3 (Baßler *et al*., 2001). Mtr2 was not identified in earlier or later 60S pre-ribosomal particles, suggesting a more direct role of Mtr2 in 60S subunit export. These data provide a biochemical link between the bona fide mRNA export factor Mtr2 and ribosomal export. We also found the importin Kap121/Pse1 associated with the Arx1-containing 60S pre-ribosomal particles (see Table I). The significance of this interaction is not clear. Kap121/Pse1 is known to be involved in the import of ribosomal proteins (Rout *et al*., 1997). However, a role of Kap121 in nuclear export of pre-ribosomes cannot be excluded. Notably, the karyopherin Kap142p/Msn5p mediates nuclear import and export of different cargo proteins (Yoshida and Blobel, 2001).

A striking feature of both early and late 60S pre-ribosomes is their association with several putative GTPases (Baßler *et al*., 2001; Harnpicharnchai *et al*., 2001; Saveanu *et al*., 2001; Fatica *et al*., 2002). The Nug1, Nug2 and Nog1 GTPases have nucleolar/nuclear locations (Baßler *et al*., 2001; Park *et al*., 2001; Saveanu *et al*., 2001) and co-purify with nuclear pre-ribosomal particles (from Nsa3 until Arx1), whereas the Kre35 GTPase is predominately localized in the cytoplasm and associates with the three later 60S pre-ribosomes (Sda1, Arx1 and Kre35). Thus, pre-60S associated GTPases could play regulatory roles, e.g. in the maturation and export of 60S subunits, or in signaling between ribosome synthesis and other cellular pathways.

Another conspicuous factor of the 60S pre-ribosome is the very large (559 kDa) protein Rea1. Rea1 is a member of the AAA family of ATPases (Baßler *et al*., 2001), which have a chaperone activity that has been implicated in dissociating protein–protein interactions (Vale, 2000). It is thus tempting to speculate that Rea1 functions in the late nucleoplasmic 60S pre-ribosome to dissociate non-ribosomal proteins at or prior to export. This class of ATPases may have another function as molecular motors (Vale, 2000), which could be relevant in the case of Rea1,

as it has recently been shown to be related to dynein (Garbarino and Gibbons, 2002). Therefore, an alternative role of Rea1 may be in assisting transportation of the pre-ribosomal particle to the nuclear pore or through the nuclear pore complex.

Taken together, our data allow us to draw a new model for maturation and export of the 60S ribosomal subunit (Figure 5). The initial ribosomal precursor particle is a 90S assembly, which after cleavage at A_2 separates into 40S and 60S pre-subunits. The derived pre-60S ribosomes undergo a series of RNA processing reactions, which are likely to be exclusively nucleolar. These particles carry many associated factors that fulfill many different functions (see Fatica and Tollervey, 2002). Following rRNA maturation, the 60S pre-ribosomes move from the nucleolus to the nucleoplasm, accompanied by major changes in the protein composition of the particles. In the nucleoplasm, maturation and removal of factors from the particle continues. However, new components join the 60S pre-ribosome in a sequential manner, of which the huge AAA-type ATPase may catalyze the restructuring or the export of the 60S pre-ribosomes. Prior or concomitantly to export, most of the GTPases are removed from the 60S pre-ribosome and an Nmd3 binding site is revealed, which triggers recruitment of the general export receptor exportin-1. The final cytoplasmic pre-60S ribosomes have lost almost all of the non-ribosomal proteins and await the final structural rearrangements that will convert them into mature subunits competent for translation.

Materials and methods

Yeast strains and plasmids

Genomic integration of GFP (*HIS3MX6*-Marker) and TAP (*TRP1*-Marker) C-terminal tags resulting in fusion proteins of Nsa3 (*YHR052w*), Nop7 (*YGR103w*), Rix1 (*YHR197w*), Sda1 (*YGR245c*), Arx1 (*YDR101c*) and Kre35 (*YGL099w*) were performed as described previously (Longtine *et al*., 1998; Puig *et al*., 2001) into the yeast strain DS1-2b (*MAT***a**, *ura3, trp1, his3, leu2*) derived from cross of FY23 with FY86. The following yeast strains and plasmids used in this study were as described previously: Nug1-TAP (Baßler *et al*., 2001), FY23 and FY86 (Gadal *et al*., 2001b).

Sucrose density gradient centrifugation

Isolation of ribosomes under low salt conditions by sucrose gradient centrifugation was performed as described previously (Baßler *et al*., 2001). Briefly, cycloheximide was added to yeast grown to OD_{600} 0.5, after a 15 min incubation, cells were washed and lysed. After ultracentrifugation, a gradient collector was used to record the UV profile and collect fractions for analysis.

RNA analysis

Northern hybridization and primer extension of RNA extracted from the reverse-tagged and purified protein baits, which were isolated using the complete TAP purification protocol with modifications to minimize RNA degradation. Briefly, the purification were performed using 1 mM ribonucleoside vanadyl complex (Sigma) as an RNase inhibitor, with time reduction in centrifugation and TEV cleavage steps to further reduce ribonuclease degradation. After purifications, samples were flash-frozen in liquid nitrogen. The northern hybridization and primer extention were performed as described previously (Beltrame and Tollervey, 1992; Tollervey *et al*., 1993). Oligonucleotides used were: 006, AGATTAGCCGCAGTTGG; 007, CTCCGCTTATTGATATGC; 017, GCGTTGTTCATCGATGC; 020, TGAGAAGGAAATGACGCT; 033, CGCTGCTCACCAATGG; 041, CTACTCGGTCAGGCTC; 070, CTCCGCTTATTGATATGC; 200, UUAUGGGACUUGUU (2′-*O*-methyl RNA); 211, TGCGAATGTTAAGGAACC; 218, CUIUUAAAUUUICIUU (2′-*O*-methyl RNA).

Mass spectrometry

Mass spectrometry using tryptic digest from Coomassie-stained SDS–PAGE was performed as described previously (Baßler *et al*., 2001). Proteins were identified using Mascot (Matrix Science) and the MSDB protein database. The MS/MS results were supported by peptide fingerprints (involving less than five peptides).

Miscellaneous

Affinity purification of TAP-tagged proteins was performed as described previously using 2–6 l of yeast culture (Puig *et al*., 2001). Protein was visualized using Novex 4–12% gradient or NuPAGE 10–20% gradient SDS–PAGE (Invitrogen) stained with colloidal Coomassie (Sigma). Western blot analysis were performed according to Siniossoglou *et al*. (1996). Fluorescence microscopy was carried out as described previously (Gadal *et al*., 2001a).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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