Npa1p is an essential *trans*-acting factor required for an early step in the assembly of 60S ribosomal subunits in *Saccharomyces cerevisiae*

IVÁN V. ROSADO and JESÚS DE LA CRUZ
Departamento de Genética, Universidad de Sevilla, E-41012 Sevilla, Spain

ABSTRACT

Ribosome biogenesis requires >100 nonribosomal proteins, which are associated with different preribosomal particles. The substrates, the interacting partners, and the timing of action of most of these proteins are largely unknown. To elucidate the functional environment of the putative ATP-dependent RNA helicase Dbp6p from *Saccharomyces cerevisiae*, which is required for 60S ribosomal subunit assembly, we have previously performed a synthetic lethal screen and thereby revealed a genetic interaction network between Dbp6p, Rpl3p, Nop8p, and the novel Rsa3p. In this report, we extended the characterization of this functional network by performing a synthetic lethal screen with the *rsa3* null allele. This screen identified the so far uncharacterized Npa1p (YKL014C). Polysome profile analysis indicates that there is a deficit of 60S ribosomal subunits and an accumulation of half-mer polysomes in the slowly growing *npa1-1* mutant. Northern blotting and primer extension analysis shows that the *npa1-1* mutation negatively affects processing of all 27S pre-rRNAs and the normal accumulation of both mature 25S and 5.8S rRNAs. In addition, $27SA_2$ pre-rRNA is prematurely cleaved at site C_2. Moreover, GFP-tagged Npa1p localizes predominantly to the nucleolus and sediments with large complexes in sucrose gradients, which most likely correspond to pre-60S ribosomal particles. We conclude that Npa1p is required for ribosome biogenesis and operates in the same functional environment of Rsa3p and Dbp6p during early maturation of 60S ribosomal subunits.

Keywords: ribosome biogenesis; nucleolus; yeast genetics; DEAD-box RNA helicase; preribosomal particles

INTRODUCTION

Ribosome synthesis is a complex and highly energy-consuming process in eukaryotes (Warner et al. 2001). Therefore, the expression of the various ribosomal components is subjected to a tight coordinate control to ensure their production at equimolar amounts (Planta 1997; Leary and Huang 2001). In addition, ribosome content fluctuates to cope with the demand for protein synthesis in response to growth conditions and stresses (Planta et al. 1995; Warner 1999; Warner et al. 2001). Several proteins involved in ribosome biogenesis appear to have a function in regulating the cell cycle (Pestov et al. 2001; Du and Stillman 2002; Oeffinger and Tollervey 2003), and deregulation of ribosome biogenesis has been implicated in uncontrolled growth and tumorigenesis in mammalian cells (Ruggero and Pandolfi 2003).

Reprint requests to: Jesús de la Cruz, Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes, 6, E-41012 Sevilla, Spain; e-mail jdlcd@us.es.

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In eukaryotes, ribosome biogenesis takes place primarily in the nucleolus (Olson et al. 2000; Pederson and Politz 2000). The process starts with the transcription of the primary transcripts (35S pre-rRNA and pre-5S rRNA in yeast) by distinct RNA polymerases (Pol I and Pol III, respectively) and requires the import of most ribosomal proteins (r-proteins) from the cytoplasm (Nomura et al. 2004). The 35S pre-rRNA is processed at multiple sites to generate the mature 18S, 5.8S, and 25S rRNA. The pre-5S rRNA is only processed at its 3′ end to generate the mature 5S rRNA (Fig. 1; Venema and Tollervey 1999; Raué 2004). Concomitant to the pre-rRNA synthesis and processing reactions, the pre-rRNA intermediates are extensively modified (Decatur and Fournier 2002) and associate with the r-proteins and a large number of nonribosomal proteins to form preribosomal particles (Fatica and Tollervey 2002; Fromont-Racine et al. 2003; de la Cruz et al. 2004a). Most of our current knowledge on ribosome synthesis comes from studies with the yeast *Saccharomyces cerevisiae*; however, the basic outline of the process is highly conserved through the eukaryotic kingdom (Takahashi et al. 2003; Gerbi and Borovjagin 2004). The power of yeast genetics has led to the identification of

many of the different nonribosomal factors involved in ribosome synthesis (Kressler et al. 1999b; Kiss 2002). Recent advances in the biochemical purification of distinct preribosomal complexes have facilitated the identification of new nonribosomal factors and the location of many of them within the preribosomal particles (Milkereit et al. 2003). These analyses have also redefined the model of the r-subunit assembly pathway (Fatica and Tollervey 2002; Fromont-Racine et al. 2003; Tschochner and Hurt 2003). In this model, the 90S preribosomal particle contains the complete machinery responsible for the early cleavages of 35S pre-rRNA as well as several large and small r-proteins, but lacks most of the factors involved in 60S r-subunit formation (Dragon et al. 2002; Fatica and Tollervey 2002; Grandi et al. 2002; Fromont-Racine et al. 2003; de la Cruz et al. 2004a). Following cleavage at early sites, the 90S particle gives rise to early 43S and 66S preribosomal particles, which contain 20S and 27SA pre-rRNAs, respectively. It appears that most of the factors associated with the 35S pre-rRNA, with a few exceptions, are released after the 35S pre-rRNA is split into 20S and 27SA pre-rRNAs (Grandi et al. 2002; Nissan et al. 2002). The early 43S preribosomal particle is rapidly exported to the cytoplasm, where the conversion of 20S pre-rRNA into mature 18S rRNA and the last assembly reactions take place (Udem and Warner 1973). The study of the different purified pre-40S complexes indicates that only few *trans*-acting factors are needed to finish the maturation of 40S r-subunits from early 43S preribosomal particles (Schäfer et al. 2002; Fatica et al. 2003a; Vanrobays et al. 2003). However, the study of the different purified pre-60S complexes is consistent with the presence of distinct pre-60S intermediates that move from the nucleolus to the nucleoplasm and from there to the cytoplasm. These intermediates are termed, according to their position in the pathway, early, medium, late, and cytoplasmic pre-60S r-particles (Fatica and Tollervey 2002; Nissan et al. 2002). The earliest 66S preribosomal particle is likely the result of the association of >50 nonribosomal proteins and most large r-proteins with the 27SA pre-rRNA (Nissan et al. 2002). The complexity of the pre-60S r-particles decreases during their maturation from the nucleolus to the nucleoplasm (Nissan et al. 2002; Saveanu et al. 2003) and the export-competent pre-60S particle has completed the pre-rRNA processing reactions (Baßler et al. 2001; Nissan et al. 2002). As for the pre-40S particles, last assembly reactions occur in the cytoplasm (Senger et al. 2001; Kallstrom et al. 2003).

More than 150 protein *trans*-acting factors are so far known to be involved in ribosome biogenesis (Fromont-Racine et al. 2003; Milkereit et al. 2003). The roles of these factors have been inferred on the basis of their mutant phenotype with respect to pre-rRNA metabolism or their association with distinct preribosomal particles (for examples, see http://www.expasy.org/linder/proteins.html). Most of them lack conserved enzymatic domains and are termed generally r-subunit assembly factors. Few are predicted to encode enzymes such as endo- and exonucleases,

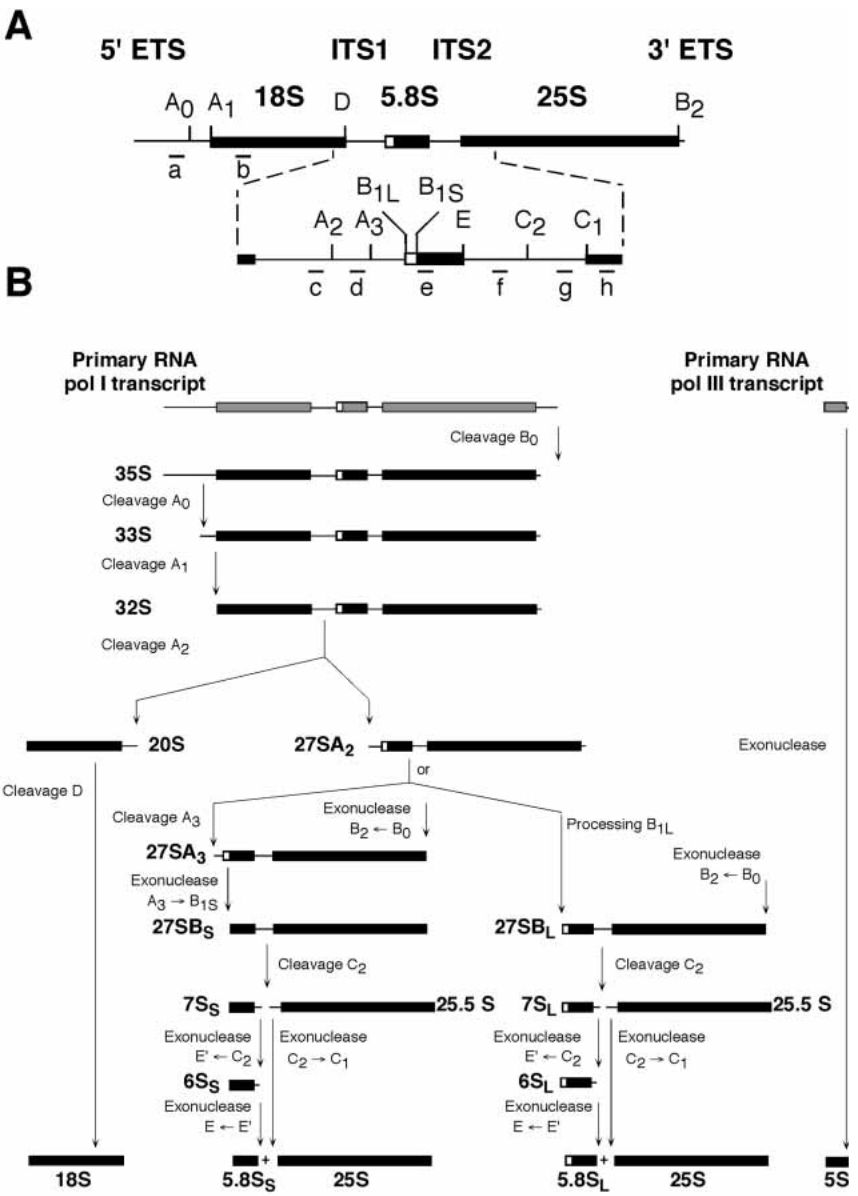

FIGURE 1. (Legend on next page)

modifying enzymes, AAA ATPases, ATP-dependent RNA helicases, or GTPases (Kressler et al. 1999b; de la Cruz et al. 2004a). The putative RNA helicases represent the largest class of enzymes involved in ribosome biogenesis (Kressler et al. 1999b; de la Cruz et al. 2004a). These enzymes seem to unwind RNA duplex regions in an ATP-dependent manner and are supposed to assist the extensive structural rearrangements that are expected to occur during the r-subunit assembly reactions. So far, neither precise substrates nor clear interacting partners have been elucidated for these proteins. We have previously reported the functional characterization of one such putative RNA helicase, Dbp6p, which is an essential nucleolar protein that is required for 60S r-subunit assembly and has been proposed to act at an early step during this process (Kressler et al. 1998). In agreement with this, we have shown that Dbp6p functionally interacts with a set of *trans*-acting factors such as Dbp7p, Dbp9p, Nop8p, and Rsa3p and the r-protein Rpl3p, which have also been proposed to function early during 60S r-subunit assembly (de la Cruz et al. 2004b). Rsa3p is a nonessential protein (de la Cruz et al. 2004b); therefore, in order to gain more insight into the functional environment of Rsa3p and hence Dbp6p, we have performed a synthetic lethal (sl) screen with the *rsa3* null allele. Here, we describe the identification of the genes complementing the isolated sl mutants and study further the so far uncharacterized open reading frame (ORF) YKL014C. We provide evidence that the essential YKL014Cp is a nucleolar protein needed for optimal biogenesis of 60S r-subunits. Our results suggest that YKL014Cp is required for 27S pre-rRNA processing and for the normal accumulation of 25S and 5.8S mature rRNAs. YKL014Cp is associated with large complexes, which most likely represent pre-60S ribosomal particles. We conclude that YKL014Cp has an early role during 60S r-subunit assembly. While this work was in progress, YKL014Cp, thereafter named Npa1p (for Nucleolar Preribosomal Associated), was independently characterized and identified as a component of a very early pre-60S r-subunit particle, which contains the $27SA_2$ pre-rRNA and the *trans*-acting factors Dbp6p, Dbp7p, Dbp9p, and Nop8p but seems to lack Rsa3p (Dez et al. 2004).

RESULTS

Synthetic lethality with the *rsa3* null allele identifies Npa1p

Rsa3p is a nucleolar nonessential protein required for optimal biogenesis of 60S r-subunits (de la Cruz et al. 2004b). Several lines of experimental evidence suggests that Rsa3p and Dbp6p function together in vivo (de la Cruz et al. 2004b). To isolate novel factors belonging to the same functional environment as Dbp6p and Rsa3p, we carried out an sl screen with the *rsa3* null allele. To this end, strain IVY252 (pHT4467-RSA3) was mutagenized by UV irradiation and about 200,000 surviving colonies were screened for nonsectoring and 5-fluoroorotic acid (5-FOA) sensitivity phenotype. Thirteen positive candidates were obtained and subsequent transformation with YCplac111-RSA3 rescued growth on 5-FOA-containing medium, thus suggesting that all candidates indeed harbor bona fide genomic sl mutations. In addition, backcrossing the sl mutant strains to strain IVY262, a MATa derivative of IVY252, demonstrated that the sl phenotype of all mutants is caused by a single recessive mutation (see Materials and Methods).

In agreement with the previously revealed synthetic enhancement interactions (de la Cruz et al. 2004b), one mutant was complemented by *DBP6* and another one by *NOP8* (see Materials and Methods). Interestingly, seven mutants were complemented by *RPL3* and one by *DBP9* (see Materials and Methods). These likely represent allele-specific synthetic lethal relationships because the *rsa3* null mutant is not synthetically enhancing the growth phenotype of previously isolated *rpl3* or *dbp9* alleles (de la Cruz et al. 2004b). One of the sl mutant strains (sl1-4) is the subject of this study and the remaining two are neither complemented by any of the earlier-mentioned genes nor by *NPA1* and are still being characterized. To clone the gene complementing the sl mutation present in the sl1-4 strain, we took advantage of its recessive slow-growth (sg) phenotype. This strain was transformed with a yeast genomic library and the li-

FIGURE 1. Pre-rRNA processing in *S. cerevisiae*. (*A*) Structure and processing sites of the 35S pre-rRNA. This precursor contains the sequences for the mature 18S, 5.8S, and 25S rRNAs that are separated by two internal transcribed spacer sequences, ITS1 and ITS2, and flanked by two external transcribed spacer sequences, 5′ ETS and 3′ ETS. The mature rRNA species are shown as bars and the transcribed spacer sequences as lines. The processing sites and their locations as well as the various probes used are indicated. (*B*) Pre-rRNA processing pathway. The primary RNA Pol I transcript undergoes covalent modifications (not shown), and it is cleaved at its 3′ end to yield the 35S pre-rRNA, which is the longest detectable precursor. The 35S pre-rRNA is cleaved at site A_0 to generate the 33S pre-rRNA. This molecule is subsequently processed at sites A_1 and A_2, resulting in the separation of the pre-rRNAs destined for the small and large ribosomal subunits. The final maturation of the 20S precursor takes place in the cytoplasm, where cleavage at site D yields the mature 18S rRNA. The $27SA_2$ precursor is processed by two alternative pathways that both lead to the formation of mature 5.8S and 25S rRNAs. In the major pathway, the $27SA_2$ precursor is first cleaved at site A_3 and then the $27SA_3$ precursor is exonucleolytically digested 5′→3′ up to site B_{1S} to yield the $27SB_S$ precursor. A minor pathway processes the $27SA_2$ or the $27SA_3$ molecule at site B_{1L} by an as yet unknown mechanism, producing the $27SB_L$ pre-rRNA. While processing at sites B_{1S} and B_{1L} is being completed, the 3′ end of mature 25S rRNA is generated by 3′→5′ trimming to site B_2. The subsequent processing of both 27SB species appears to be identical. Cleavage at site C_2 generates the 25.5S and 7S pre-rRNAs. The 7S pre-rRNA is 3′→5′ trimmed to the 3′ end of the mature 5.8S rRNA. The 25.5S species is 5′→3′ digested to the mature 25S rRNA. The primary RNA Pol III transcript is trimmed to the 3′ end of the mature 5S rRNA. The data presented in this study suggest that Npa1p is required for efficient processing of the 27S pre-rRNAs. For reviews on pre-rRNA processing and the known processing enzymes, see Venema and Tollervey (1999) and Raué (2004).

brary plasmid was recovered from three colonies that grew to the wild-type extent at 37°C. Sequence and subcloning analysis of the insert demonstrated that the uncharacterized essential ORF, YKL014C, which was named *NPA1*, is sufficient to restore wild-type growth (see Materials and Methods). Furthermore, this ORF also restores red/white sectoring and growth on 5-FOA plates of the sl1-4 strain.

To isolate the sl mutation from the sl1-4 strain, thereafter named *npa1-1*, we crossed this strain to an isogenic wild-type strain, W303-1A, sporulated the resulting diploid, and dissected the tetrads. In all complete tetrads, two sg spore clones were obtained. For further analyses, the meiotic segregant IVY325 (see Table 1 for its relevant genotype) was selected. The *npa1-1* mutation, which causes by itself the sg phenotype (doubling time of 4 h in liquid YPD medium at 30°C, 5 h at 37°C), is complemented to the wild-type extent by a centromeric plasmid harboring the *NPA1* gene at both 30°C and 37°C (Fig. 2A; data not shown). The *npa1-1* mutant is, as expected, sl with the *rsa3* null allele (Fig. 2B). To test for linkage between the *npa1-1* mutation and the ORF YKL014C, we crossed IVY325 with IVY507 (YCplac33-NPA1), a haploid strain that harbors an *npa1*::kanMX4 disruption at the chromosomal locus and contains as a sole *NPA1* allele the centromeric plasmid YCplac33-NPA1. After sporulation and tetrad analysis, all complete tetrads showed a 2^+:2^- segregation for cell viability on plates containing 5-FOA, with all the viable spore clones having an sg phenotype (data not shown). This result indicated that the *npa1-1* mutation is linked to the *NPA1* gene.

Npa1p encodes a protein of 1764 amino acids with a predicted molecular mass of 203.3 kDa. The protein seems to be neutral (pI = 7.31) and probably exists in low intracellular abundance on the basis of a codon adaptation index of 0.158. A database search revealed no obvious protein motifs for Npa1p, but its first 693 amino acids seem to have a predicted structure related to proteins of the HEAT-repeat family such as the PR65A subunit of protein phosphatase 2A (Yeast Resource Center Informatics Platform, http://www.yeastrc.org; Hazbun et al. 2003) and a low homology with Tpd3p, the yeast PR65A protein. We could also identify potential homologs of yeast Npa1p in human, *Drosophila*, *Arabidopsis*, and other fungi (Sequence Similarity Query Tool, *Saccharomyces Genome Database*; Christie et al. 2004). The strong sequence conservation suggests that Npa1p carries out an important, evolutionarily conserved function in all eukaryotes. This function might be related to 60S r-subunit synthesis, as suggested by the genetic interaction between Npa1p and Rsa3p. Moreover, searches in the preribosomal network (http://www.pre-ribosome.de; Milkereit et al. 2003) and the YEAST protein complex database (http://yeast.cellzome.com) found Npa1p associated with 90S preribosomal and early pre-60S particles. In the next sections, we investigated the possible involvement of Npa1p in 60S r-subunit biogenesis.

TABLE 1. Yeast strains used in this study

Name[a]	Relevant genotype	Source
W303-1A	*MAT***a**	
W303-1B	*MAT*α	
YDK11-5A	*MAT*α *ade3*::kanMX4	Kressler et al. 1999a
YMD3-1A	*MAT*α *rsa3*::HIS3MX6	de la Cruz et al. 2004b
YMD3-2D	*MAT***a** *rsa3*::HIS3MX6	de la Cruz et al. 2004b
IVY252	*MAT*α *rsa3*::kanMX4 *ade3*::kanMX4 [pHT4467-RSA3]	This study
IVY262	*MAT***a** *rsa3*::kanMX4 *ade3*::kanMX4 [pHT4467-RSA3]	This study
IVY325	*MAT*α *npa1-1*	This study
IVY507[b]	*MAT*α *LYS2 MET15 npa1*::*kanMX4* [YCplac111-NPA1]	This study

[a]All strains used in this study, except the IVY507 strain, are derivatives of the diploid strain W303. IVY507 is a derivative of the diploid strain Y24863 (see Materials and Methods for other genotypic markers).
[b]Depending on the experimental conditions, the original YCplac111-NPA1 was replaced by other *NPA1*-containing plasmids.

Npa1p is a nucleolar protein

As a first step in the functional characterization of Npa1p, we studied the subcellular localization of Npa1p using an *NPA1*-eGFP fusion allele (see Materials and Methods). This allele, which is expressed from the *NPA1* promoter, is fully functional because it complemented the *npa1* null allele to the wild-type extent at 30°C and 37°C (data not shown). Western blot analysis with anti-GFP antibodies detected a single protein that migrated at the expected molecular mass of about 220–230 kDa (data not shown; see Fig. 8). In the strain IVY507 (YCplac33-NPA1-eGFP pUN100-DsRed-NOP1), the green fluorescence is detected in the nucleolus (Fig. 3B). The nucleolar localization is confirmed by colocalization of the fusion DsRed-Nop1p protein and exclusion of the nucleoplasm, which was visualized by staining DNA with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Fig. 3E–F). Our results are in full agreement with those described in a recent systematic protein localization study (Huh et al. 2003). The predominant localization of Npa1-eGFPp in the nucleolus suggests a specific role of Npa1p in ribosome biogenesis.

Npa1p is required for 60S r-subunit accumulation

To assess the function of Npa1p in ribosome biogenesis, we used the *npa1-1* mutant strain. We first checked whether this mutation causes a deficiency in r-subunits by polysome profile analysis. Compared with the isogenic wild-type strain, there

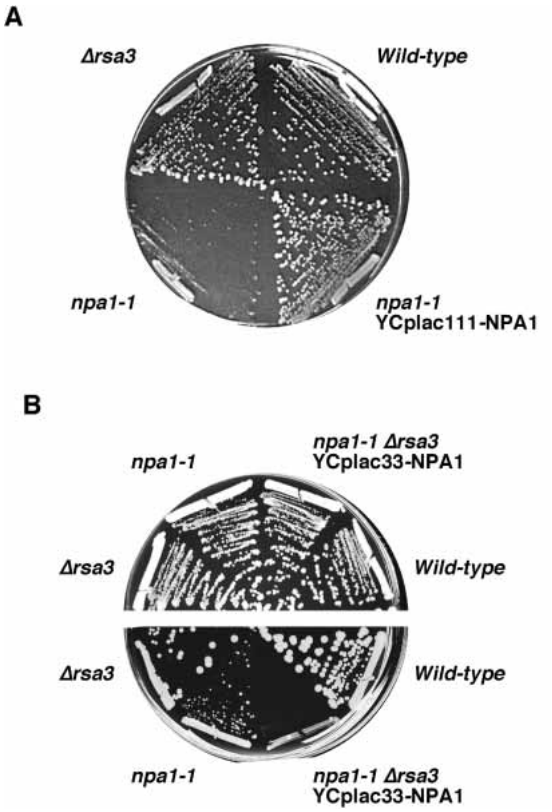

FIGURE 2. The *npa1-1* mutation confers a slow-growth phenotype and it is synthetically lethal with the *rsa3* null allele. (*A*) The strain IVY325 (*npa1-1*) was transformed with either YCplac111 or the complementing plasmid YCplac111-NPA1. As controls, strains W303-1B (wild type) and YMD3-1A (Δ*rsa3*) were transformed with YCplac111. Transformants were grown on SD-Leu plates for 4 d at 30°C. (*B*) The strains IVY325 (*npa1-1*), carrying the plasmid YCplac33-NPA1, and YMD3-2D (Δ*rsa3*) were crossed, the resulting diploid was sporulated, and the tetrads were dissected. Complete tetrads were streaked on YPD plates and restreaked on 5-FOA-containing plates to counter select YCplac33-NPA1. A representative tetratype tetrad is shown on a YPD plate (*top* half) or on a 5-FOA containing plate (*bottom* half). Plates were incubated for 4 d at 30°C.

was a reduction of free 60S r-subunits relative to 40S r-subunits and also a decrease in 80S and polysomes in the *npa1-1* strain at both 30°C and 37°C (Fig. 4A,B; data not shown). As a result of the shortage of 60S r-subunits, there was also an accumulation of half-mer polysomes (Fig. 4B). The relative reduction of the 60S r-subunits was further analyzed by completely dissociating mono- and polysomes into 40S and 60S r-subunits by using low Mg^{2+} sucrose gradients. Indeed, in the *npa1-1* strain, we observed a 30% reduction in the overall quantity of 60S r-subunits relative to the wild-type strain (A_{254} ratio ~1.8 in the wild-type strain and A_{254} ~1.2 in the *npa1-1* strain at 30°C, data not shown). These results indicate that the partial loss of function of Npa1p by the *npa1-1* mutation causes a deficit in 60S r-subunits. In agreement with this deficit, the *npa1-1* mutant strain is hypersensitive to antibiotics such as cycloheximide and neomycin that interfere with the translation process (Fig. 4C; data not shown). Together with the nucleolar localization of Npa1p, these results strongly suggest that Npa1p is a *trans*-acting factor involved in 60S r-subunit biogenesis.

Npa1p is required for normal pre-rRNA processing

Pre-rRNA processing is intimately linked to the r-subunit assembly process (see Introduction). Therefore, to study in more detail the function of Npa1p in the biogenesis of 60S r-subunits, we assessed the steady-state levels of mature rRNA and precursors by Northern blot hybridization and primer extension analyses in the *npa1-1* strain and compared them to those in the isogenic null *rsa3* and wild-type strain. Strains IVY325 (*npa1-1*), YMD3-1A (Δ*rsa3*), and W303-1B (wild type) were first grown at 30°C in liquid YPD medium and then shifted to 37°C for up to 9 h. Cells were maintained in mid-log phase (OD_{600} of ~0.8) and harvested at different time points and total RNA was extracted and

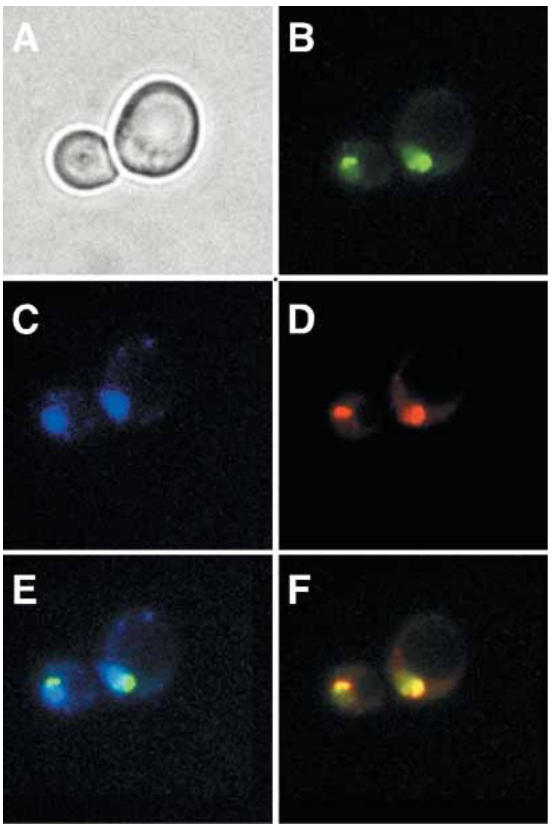

FIGURE 3. Npa1p localized predominantly to the nucleolus. Fluorescence microscopy was performed with exponentially growing cells expressing the Npa1-eGFPp and DsRed-Nop1p from their cognate promoters. (*A*) Phase contrast acquisition (Nomarski) of the same field as in *B–F*. (*B*) Npa1-eGFPp was detected by the GFP fluorescence. (*C*) Chromatin DNA was stained with DAPI. (*D*) DsRed-Nop1p was detected by the DsRed fluorescence. (*E*) Colocalization of Npa1-eGFPp and chromatin DNA. (*F*) Colocalization of Npa1-eGFPp and DsRed-Nop1p.

analyzed. As shown in Figure 5, a mild increase of the 35S pre-rRNA was detected in all strains after transfer to 37°C. This can be due to the effects of the heat shock, although 9 h at 37°C is likely enough time for adaptation. However, in the *npa1-1* strain, this increase was accompanied by a reduction of 20S pre-rRNA and an accumulation of an aberrant 23S pre-rRNA and, to a lesser extent, of an aberrant 21S pre-rRNA. This suggests that pre-rRNA processing at early sites is delayed in the *npa1-1* strain at 37°C. The appearance of 21S pre-rRNA suggests that cleavage at site A_2 is more affected than cleavage at sites A_0 and A_1. In good agreement with this, levels of mature 18S rRNA were slightly decreased in the *npa1-1* strain at 37°C. Most important, there was a significant decrease in the 27SA and 27SB pre-rRNAs in the *npa1-1* mutant at 37°C. The decrease of the 27SB pre-rRNA is even observed when *npa1-1* is grown at 30°C. Consistently, there was a reduction of the steady-state levels of 25S rRNA (Fig. 5). In general, the levels of pre-rRNAs and mature rRNAs in the *rsa3* null strain were similar to those of the wild-type strain, although we could detect some decrease in the 27SB pre-rRNAs at 37°C (Fig. 5).

Analysis of low-molecular-weight RNA species showed that although levels of mature 5S rRNA remained unaffected, both the mature $5.8S_S$ and $5.8S_L$ rRNAs slightly underaccumulated in the *npa1-1* strain at 37°C (Fig. 6). This reduction is due to decreased levels of $7S_S$ and $7S_L$ pre-rRNAs and it is fully consistent with the decreased levels of the 27SB pre-rRNAs (Fig. 6). In addition, a fragment that extends from A_2 to C_2 was accumulated (Fig. 6). This suggests that inactivation of Npa1p allows a premature cleavage of $27SA_2$ at site C_2 that reduces the conversion of $27SA_2$ into 27SB and 7S pre-rRNAs.

In order to resolve the $27SA_2$ and $27SA_3$ pre-rRNAs and distinguish between the $27SB_L$ and $27SB_S$ pre-rRNA, we performed primer extension analyses. As shown in Figure 7, the signal from the primer extension stop at sites B_{1L} and B_{1S} and, to a lesser extent, at sites A_3 and A_2 was found to be significantly decreased in the *npa1-1* strain compared with the *rsa3* null and the wild-type strain. In addition, primer extension analysis through site C_2 showed a clear reduction in the level of the 25.5S pre-rRNA (Fig. 7). Finally, these analyses confirmed that levels of 27SB pre-rRNAs were also slightly decreased in the *rsa3* null strain (Fig. 7).

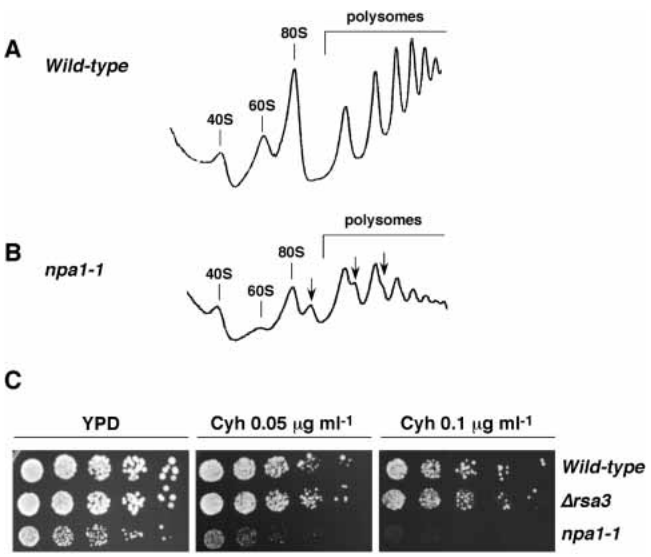

FIGURE 4. The *npa1-1* mutant has a deficit in free 60S r-subunits, accumulates half-mer polysomes, and is hypersensitive to cycloheximide. (*A*) W303-1B (wild type) and (*B*) IVY325 (*npa1-1*) were grown in YPD at 30°C. Cells were harvested at an OD_{600} of 0.8, and cell extracts were resolved in 7%–50% sucrose gradients. The A_{254} was continuously measured. Sedimentation is from *left* to *right*. The peaks of free 40S and 60S ribosomal subunits, 80S free couples/monosomes, and polysomes are indicated. Half-mers are indicated by arrows. (*C*) Drug sensitivity assay. Different dilutions of log-phase cultures from W303-1B (wild type), YMD3-1A (Δ*rsa3*), and IVY325 (*npa1-1*) were tested for their sensitivities to two different concentration of cycloheximide (Cyh).

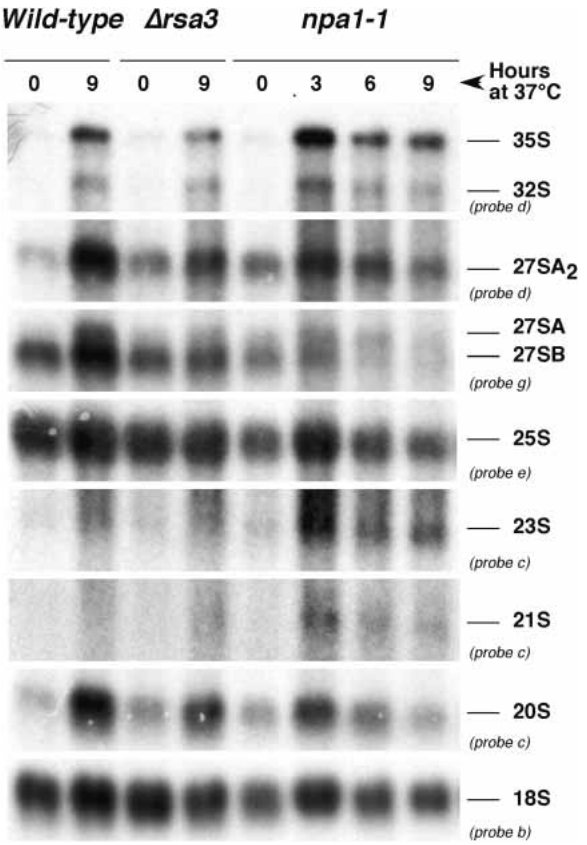

FIGURE 5. Effects of the *npa1-1* mutation on steady-state levels of high-molecular-weight pre-rRNA and mature rRNA species. RNA was extracted from W303-1B (wild type), YMD3-1A (Δ*rsa3*), and IVY325 (*npa1-1*) following growth in YPD at 30°C or at various time points after a shift to 37°C. RNA corresponding to an equivalent number of cells was resolved on a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane for Northern hybridization. The same filter was hybridized consecutively with different probes. Probe names are indicated in parentheses on the *right* (see Fig. 1A for their location in the 35S pre-rRNA).

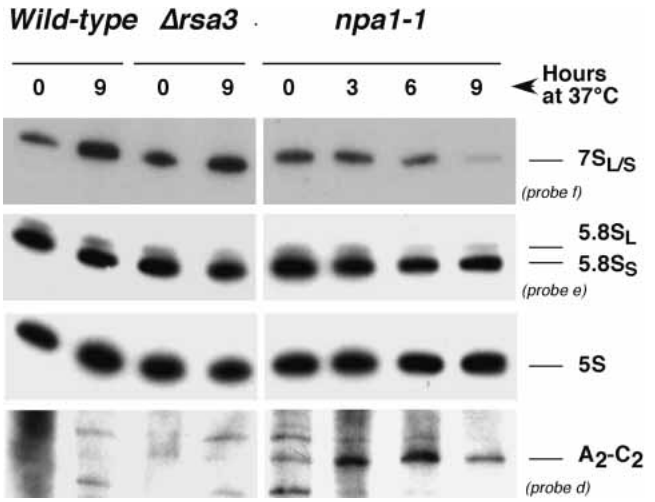

FIGURE 6. Effects of the *npa1-1* mutation on steady-state levels of low-molecular-weight pre-rRNA and mature rRNA species. The same RNA samples described in the legend of Figure 5 were separated on a 7% polyacrylamide-urea gel, transferred to a nylon membrane, and hybridized consecutively with different probes. Probe names, except the one used to detect mature 5S rRNA, are indicated in parentheses on the *right* (see Fig. 1A for their location in the 35S pre-rRNA).

Together, these data strongly suggest that Npa1p is mainly required for proper maturation of both 25S and 5.8S rRNAs. The inactivation of Npa1p by the *npa1-1* mutation has a major impact on processing of all 27S pre-rRNAs, although there is also an effect on the cleavage efficiency at site A_2 and to a lesser extent at sites A_0 and A_1. Finally, the appearance of the aberrant A_2-C_2 fragment molecule indicates that Npa1p is also required to maintain the normal order of processing of the $27SA_2$ pre-rRNA. Our results with the *npa1-1* strain are similar to those reported for a *GAL::NPA1* strain on in vivo depletion of Npa1p (Dez et al. 2004).

Npa1p is associated to high-molecular-weight particles

Recent reports have identified Npa1p in 90S particles as well as in several pre-60S particles (see earlier). In order to determine the steady-state levels of Npa1p associated with large preribosomal particles, we analyzed the sedimentation behavior of the Npa1-eGFPp fusion protein in sucrose density gradients. For this purpose, total extracts were prepared from the IVY507 (YCplac33-NPA1-eGFP) strain and subjected to low Mg^{2+} sucrose gradient ultracentrifugation. Then, fractions were collected and analyzed by Western blot to detect Npa1-eGFPp. As shown in Figure 8, a peak of Npa1-eGFPp was found associated with high-molecular-weight particles. The maximum of this peak sediments slightly lower than mature 60S r-subunits (Fig. 8, fraction 14 versus fraction 12). It has been previously described that different pre-60S r-particles migrate to this region of the sucrose gradient (de la Cruz et al. 1998a, 2004b). No protein was detected at the positions expected for the free proteins (Fig. 8, fraction 1–3). Taking also into account the nucleolar localization of Npa1-eGFP (see Fig. 3), we conclude that the majority of Npa1p is stably associated with pre-60S r-particles.

DISCUSSION

Sl screens have proven to be very helpful tools in identifying components of multiprotein complexes, such as the nuclear pore complex or preribosomal particles (Doye and Hurt 1995; Venema and Tollervey 1999). We have previously described the function of Dbp6p, a putative RNA helicase that is required for an early step during the assembly of 60S r-subunits (Kressler et al. 1998). We have elucidated the functional environment of Dbp6p by establishing a genetic interaction network with the *trans*-acting factors Dbp7p, Dbp9p, Nop8p, Rsa1p, and Rsa3p and the 60S r-protein Rpl3p (Kressler et al. 1999a; de la Cruz et al. 2004b). All of these proteins have been implicated in similar early nucleolar steps during the assembly of 60S r-subunits, with the exception of Rsa1p, which localizes to the nucleoplasm. Rpl3p belongs to a group of r-proteins that associate early with preribosomal particles (Kruiswijk et al. 1978). In order to learn more about the functional environment of Rsa3p and hence Dbp6p, we have performed a sl screen with the *rsa3* null allele. We found mutations in Rpl3p, Dbp6p, Dbp9p, and Nop8p. We also identified a mutation in the so far uncharacterized ORF YKL014C/*NPA1*. Interestingly, this

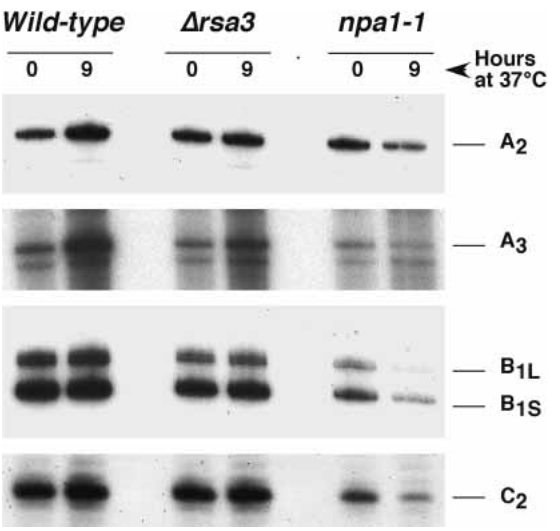

FIGURE 7. Effects of the *npa1-1* mutation on steady-state levels of 27S and 25.5S pre-rRNA species. The same RNA samples described in the legend of Figure 5 were used for primer extension analysis. Probe g (see Fig. 1A for its location in the 35S pre-rRNA) was labeled and used for the reactions. Note that this probe allows detection of $27SA_2$ (as the stop at site A_2), $27SA_3$ (as the stop at site A_3), both 27SB (as stops at sites B_{1L} and B_{1S}), and 25.5S (as the stop at site C_2).

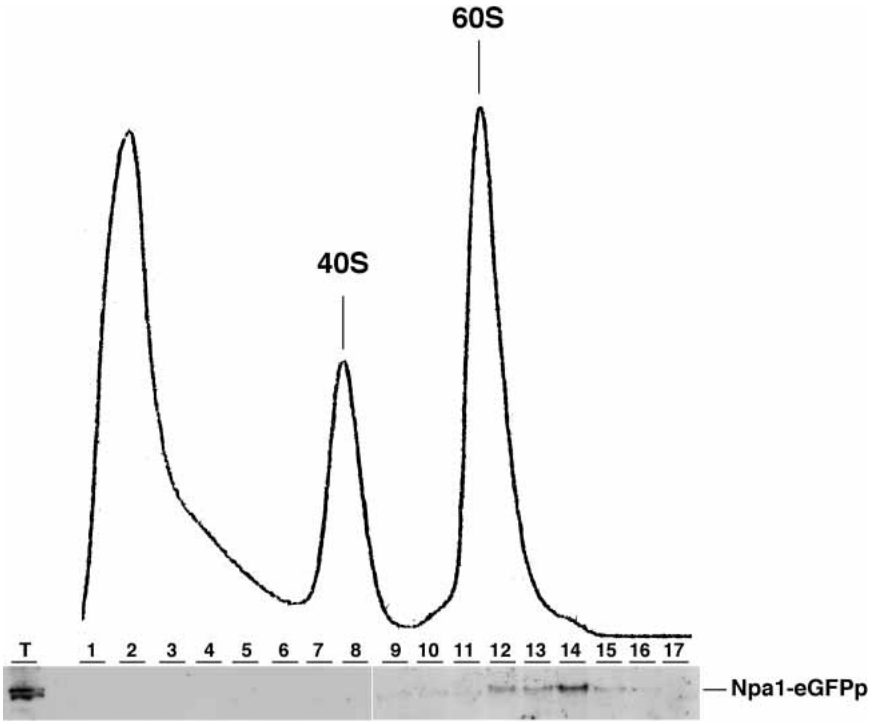

FIGURE 8. Analysis of the sedimentation of Npa1-eGFPp in sucrose gradients. Total cell extracts were done from IVY507 (YCplac33-*NPA1*-eGFP) cells following growth at 30°C and resolved in 7%–50% sucrose gradients containing a low concentration of Mg^{2+} to dissociate ribosomes into subunits. The A_{254} was continuously measured. The profile obtained is shown; sedimentation is from *left* to *right*. The peaks for 40S and 60S ribosomal subunits are indicated. Fractions were collected from the gradients, proteins were extracted from each fraction, and equal volumes were resolved on 7% SDS-polyacrylamide gels and subjected to Western blotting. T stands for total extract and numbers correspond to fraction number. A blot decorated with anti-GFP antibody is shown and the position of Npa1-eGFP is indicated.

mutation, *npa1-1*, is synthetically lethal not only with the *rsa3* null allele but also with different *dbp6*, *dbp9*, *nop8*, and *rpl3* alleles and the *dbp7* null mutant (I.V. Rosado, unpubl.).

In this work, we show that Npa1p is a *trans*-acting factor that, like Dbp6p, Dbp7p, Dbp9p, Nop8p, and Rsa3p, is required for an early step in the pathway of 60S r-subunit synthesis. Npa1p is, like most factors involved in ribosome biogenesis (Fromont-Racine et al. 2003), localized predominantly in the nucleolus in exponentially growing cells. The *npa1-1* mutation resulted in the deficit of 60S r-subunit and the appearance of half-mer polysomes. Most likely, as a consequence of this deficit, the *npa1-1* strain is hypersensitive to antibiotics that interfere with the translation process. Similar results have been described for many mutants defective in 60S r-proteins or factors involved in 60S r-subunit biogenesis (for examples, see Hess et al. 1994; Benard et al. 1998; de la Cruz et al. 1998b).

When pre-rRNA processing was examined in *npa1-1* cells, we found that the most pronounced phenotype was the drastic reduction in the steady-state levels of all 27S pre-rRNA at 37°C, especially both 27SB pre-rRNAs. Consistently, lower levels of both 7S pre-rRNAs were detected. Primer extension reveals that 25.5S levels were also reduced. As a consequence of all these pre-rRNA processing phenotypes, there is a defective synthesis of mature 25S and 5.8S rRNAs. In agreement with the fact that 5S rRNA forms a stable RNP with the 60S r-protein Rpl5p (Deshmukh et al. 1993), levels of 5S rRNA were almost unaffected in the *npa1-1* strain. Similar pre-rRNA processing defects have been described on mutation in or depletion of several factors involved in 60S r-subunit synthesis, among them Dbp6p, Dbp7p, Dbp9p, and Nop8p (Daugeron and Linder 1998; Kressler et al. 1998; Zanchin and Goldfarb 1999; Daugeron et al. 2001). Pre-rRNA processing analysis also suggests that the *rsa3* null allele leads to a mild reduction in both 27SB pre-rRNAs. In addition, we found that the *npa1-1* mutation affects the levels of mature 18S rRNA and its 20S precursor at 37°C. This is likely due to delayed processing at site A_2 and to a lesser extent at sites A_0 and A_1. As a consequence, both a 21S and, more abundantly, a 23S aberrant pre-rRNA accumulated. This type of defect in 18S rRNA synthesis is a general feature of mutations that interfere with the synthesis of mature 25S and 5.8S rRNAs (Venema and Tollervey 1999). Interestingly, an aberrant A_2-C_2 fragment also accumulated in the *npa1-1* strain at 37°C but not in the *rsa3* null mutant. This fragment has been previously observed in the Ssf1p-depleted strain and the temperature-sensitive *cic1-2/nsa3* mutant at 37°C (Fatica et al. 2002, 2003b). It seems possible that improper early assembly in the pathway to 60S r-subunits allows premature processing of the $27SA_2$ pre-rRNA at site C_2, leading to reduced levels of 27S and 7S pre-rRNAs and destabilization of 25.5S pre-rRNA (Fatica et al. 2002). It would be pertinent to assess the accumulation of the A_2-C_2 fragment on inactivation of other factors such as Dbp6p, Dbp7p, Dbp9p, or Nop8p, whose mutants show pre-rRNA processing defects very similar to the ones found in the *npa1-1* strain. We conclude that Npa1p is required for early pre-rRNA processing events in the pathway of 25S and 5.8S rRNA synthesis. In contrast to other factors, such as Cic1p (Fatica et al. 2003b), Npa1p seems not to be required for nuclear export of 60S r-subunits because we did not find nuclear accumulation of the Rpl25p-eGFP large subunit reporter in the *npa1-1* mutant at 30°C or following transfer to 37°C for 9 h (data not shown).

We found most of Npa1p associated with large complexes that are larger than mature 60S r-subunits and could correspond to pre-60S r-particles. Consistent with this pos-

sibility, Dez et al. (2004) have purified a preribosomal complex containing Npa1p. Interestingly, these authors demonstrated that Npa1p is predominantly associated with the $27SA_2$ pre-rRNA, suggesting that this complex corresponds to a very early pre-60S r-particle. In agreement with both this fact and our genetic data, Dbp6p, Dbp7p, Dbp9p, and Nop8p are also stably associated with the Npa1p-containing complex (Dez et al. 2004).

In conclusion, we have identified an additional factor required for early assembly of 60S r-subunits. However, because Npa1p lacks known enzymatic activity or obvious protein domains, we clearly still do not understand its mechanistic function in this pathway. Strikingly, Npa1p has been described to interact in the yeast two-hybrid system with Cdc28p, which is the main cell cycle cyclin-dependent protein kinase (Uetz et al. 2000). Several proteins have been implicated in both ribosome synthesis and cell cycle progression (see Introduction). However, our results suggest that there is no significant block of *npa1-1* cells at any stage of the cell cycle at 30°C or 37°C (I.V. Rosado, unpubl.). Further work is needed to understand the role of Npa1p in 60S r-subunits and to clarify its possible link to cell cycle progression.

MATERIALS AND METHODS

Strains, media, and genetic manipulations

All yeast strains used in this study are derivatives of strain W303 (*MAT*a/*MAT*α *ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1*) with the exception of the derivatives of the strain Y24863, which is in the BY4743 background (*MAT*a/*MAT*α *his3Δ1/his3-Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 met15Δ0/MET15 ura3Δ0/ura3Δ0 YKL014C::kanMX4/YKL014C*) and was purchased from EUROSCARF (Table 1). The starting strain for the sl screen, IVY252, was constructed by crossing YDK11-5A and YMD3-2D. The resulting diploid was sporulated and the tetrads were dissected. Two spore clones of opposite mating types, IVY252 and IVY262, carrying the relevant markers were selected and transformed with pHT4467-RSA3. *RSA3* was cloned in pHT4467 (Venema and Tollervey 1996) as a 1.5-kb XbaI fragment from pFL44-RSA3 (de la Cruz et al. 2004b). Growth and handling of yeast were according to standard procedures (Burke et al. 2000). Antibiotic-containing plates were prepared by adding the drugs from stock solutions into YPD before pouring the plates. Effective concentrations having no apparent effects on the growth of W303-1B were 1 mg/mL paromomycin, 5 mg/mL neomycin, and 0.1 μg/mL cycloheximide. *Escherichia coli* strain DH5α was used for cloning and propagation of plasmids.

Isolation of sl mutants

Strain IVY252 (pHT4467-RSA3) was grown in YPD liquid medium until mid-log phase, and ~500,000 cells were spread on YPD plates at the density of about 800 cells/plate. The plates were then UV irradiated, resulting in a 40%–50% survival, and incubated for 5 d at 30°C in the dark. Red colonies were streaked twice onto fresh YPD plates to identify the nonsectoring ones. Then these were streaked on plates containing 5-FOA and incubated for 5 d at 30°C. Eighteen candidates were unable to grow on 5-FOA plates. To confirm that this phenotype was not due to genomic integration of the plasmid pHT4467-RSA3, we transformed candidates with pHAC111-RSA3 and pHAC111 (de la Cruz et al. 2004b). Thirteen candidates, named sl1-1, sl1-3, sl1-4, sl1-5, sl1-7, sl1-8, sl2-2, sl2-3, sl3-3, sl3-4, sl4-1, sl4-2, and sl4-3, showed restored sectoring and 5-FOA resistance on transformation with pHAC111-RSA3 but not with pHAC111. Candidates were crossed to IVY262, the resulting diploid strain was sporulated, and the tetrads were dissected. Two sl mutant segregants were recovered from 10 complete tetrads of each diploid strain analyzed, indicating the monogenic character of all sl mutations. The resulting diploid strains were also streaked on YPD and 5-FOA-containing plates. All diploids showed restored sectoring and 5-FOA resistance, indicating the recessive character of all sl mutations. The growth phenotype of the different sl mutants was assessed on YPD plates at 30°C, 18°C, and 37°C.

Cloning of *NPA1*

All sl mutants were transformed with plasmids YCplac111-DBP6, YCplac111-DBP7, YCplac111-DBP9, YCplac111-NOP8, YCplac111-RPL3, YCplac111-RSA1, and YCplac111 (de la Cruz et al. 2004b) and transformants were selected on SD plates lacking leucine (SD-Leu). Transformants were streaked on SD-Leu plates to identify sectoring colonies and then on 5-FOA-containing plates. The sl phenotype of the sl4-1 mutant was complemented by the *DBP6* gene. The sl phenotype of the sl4-2 mutant was complemented by the *DBP9* gene. The sl phenotype of the sl1-8 mutant was complemented by the *NOP8* gene. The sl phenotype of the sl1-1, sl1-3, sl1-5, sl1-7, sl2-2, sl3-3, and sl4-3 mutants was complemented by the *RPL3* gene. Strain sl1-4, which has an sg phenotype at any temperature tested, was transformed with a YCplac111-based yeast genomic library (Kressler et al. 1999a) and about 15,000 transformants were screened for wild-type growth on SD-Leu plates at 37°C. One plasmid, pIV222, containing an 8.4-kb insert, complemented both the sg and sl phenotypes of the sl1-4 mutant. The sequence of the terminal regions shows that the library insert contained YKL013C (*ARC19*) and YKL014C as sole complete ORFs. Further subcloning of YKL014C as a 5.9-kb ApaI-NsiI fragment, which was blunt ended, into SmaI-restricted YCplac111 (thereafter named YCplac111-NPA1) and YCplac33 (thereafter named YCplac33-NPA1) confirmed that YKL014C was sufficient to complement the sg and sl phenotype of the sl1-4 mutant.

Fluorescence microscopy

Strain Y24863 was first transformed with YCplac111-NPA1, it was subsequently sporulated, and the tetrads were dissected. A *npa1::KanMX4* haploid segregant harboring YCplac111-NPA1 (IVY507) was first transformed with YCplac33-NPA1-eGFP and subjected to plasmid segregation on SD-Ura plates. Plasmid YCplac33-NPA1-eGFP was constructed as follows: a 5.9-kb ApaI-NsiI fragment from pIV222 was blunt ended and cloned into SmaI-restricted YCplac33-yeGFP/TCYC1 (a gift from M. Hall). One candidate in the appropriate orientation, pIV236, was selected. Then, a PCR was performed using pIV222 as a template and the

oligonucleotides NPA1StuIUP (5′-GTCCATTGACAGATACG-3′, placed 8 bp upstream of the sole StuI site present in *NPA1*) and NPA1StopLO (5′-CGCGGATCCTTTACGTAGCCTC-3′, complementary to the end of the YKL014C ORF but lacks the stop codon; a BamHI site is underlined). To generate YCplac33-NPA1-eGFP, we digested the PCR product with StuI and BamHI and cloned it into pIV236, which was partially restricted with StuI and totally digested with BamHI. The resulting YCplac33-NPA1-eGFP complemented the null *npa1* allele to the wild-type extent. For localization, strain IVY507 (YCplac33-NPA1-eGFP) was first transformed with pUN100-DsRedNOP1 (a generous gift from J. Bassler). Then, several transformants were grown to mid-log phase in SD-Leu-Ura liquid medium, washed, and resuspended in water. Acquisition was done in a Leica DMR microscope equipped with a DC camera following the instructions of the manufacturer. Digital images were processed with Adobe Photoshop 7.0. To stain nuclear DNA, we included DAPI in the resuspension step at the concentration of 1mg/mL.

To test pre-60S r-particle export, we studied the localization of the Rpl25-eGFP (expressed from plasmid pRS315-RPL25-eGFP, a gift from O. Gadal) in exponentially growing cells in SD-Leu liquid medium.

Sucrose gradient centrifugation

Polyribosome preparations, polysome analyses, and r-subunit preparations were done as described (de la Cruz et al. 1998a). Gradient analysis was performed with an ISCO UA-6 system with continuous monitoring at A_{254}. Fraction analyses of proteins were done as described (de la Cruz et al. 1998a). Antibodies against GFP were purchased from BD Biosciences and used at a 1:2000 dilution.

RNA analyses

RNA extraction, Northern hybridization, and primer extension analysis were carried out according to standard procedures (Venema et al. 1998). In all experiments, RNA was extracted from samples corresponding to 10 OD_{600} units of exponentially growing cells, and RNA from 0.4 OD_{600} units of cells were loaded in gels or used for primer extension reactions. The following oligonucleotides were used: 5′A_0 (5′-GGTCTCTCTGCTGCCGG-3′), 3-D/A_2 (5′-GACTCTCCATCTCTTGTCTTCTTG-3′), A_2/A_3 (5′-TGTTACCTCTGGGCCC-3′), E/C_2 (5′-GGCCAGCAATTTCAAGTTA-3′), C_1/C_2 (5′-GAACATTGTTCGCCTAGA-3′), 18S (5′-CATGGCTTAATCTTTGAGAC-3′), 5.8S (5′-TTTCGCTGCGTTCTTCATC-3′), and 5S (5′-GGTCACCCACTACACTACTCGG-3′).

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REFERENCES

Baßler, J., Grandi, P., Gadal, O., Lessmann, T., Petfalski, E., Tollervey, D., Lechner, J., and Hurt, E. 2001. Identification of a 60S preribosomal particle that is closely linked to nuclear export. *Mol. Cell* **8:** 517–529.

Benard, L., Carroll, K., Valle, R.C.P., and Wickner, R.B. 1998. Ski6p is a homolog of RNA-processing enzymes that affects translation of non-poly(A) mRNAs and 60S ribosomal subunit biogenesis. *Mol. Cell. Biol.* **18:** 2688–2696.

Burke, D., Dawson, D., and Stearns, T. 2000. *Methods in yeast genetics: A Cold Spring Harbor Laboratory course manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Christie, K.R., Weng, S., Balakrishnan, R., Costanzo, M.C., Dolinski, K., Dwight, S.S., Engel, S.R., Feierbach, B., Fisk, D.G., Hirschman, J.E., et al. 2004. *Saccharomyces Genome Database* (SGD) provides tools to identify and analyze sequences from *Saccharomyces cerevisiae* and related sequences from other organisms. *Nucleic Acids Res.* **32:** D311–D314.

Daugeron, M.-C. and Linder, P. 1998. Dbp7p, a putative ATP-dependent RNA helicase from *Saccharomyces cerevisiae*, is required for 60S ribosomal subunit assembly. *RNA* **4:** 566–581.

Daugeron, M.-C., Kressler, D., and Linder, P. 2001. Dbp9p, a putative ATP-dependent RNA helicase involved in 60S-ribosomal-subunit biogenesis, functionally interacts with Dbp6p. *RNA* **7:** 1317–1334.

de la Cruz, J., Kressler, D., Rojo, M., Tollervey, D., and Linder, P. 1998a. Spb4p, an essential putative RNA helicase, is required for a late step in the assembly of 60S ribosomal subunits in *Saccharomyces cerevisiae*. *RNA* **4:** 1268–1281.

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

de la Cruz, J., Kressler, D., and Linder, P. 2004a. Ribosomal subunit assembly. In *Nucleolus* (ed. M.O.J. Olson). Landes Bioscience/eurekah.com, Georgetown, TX.

de la Cruz, J., Lacombe, T., Deloche, O., Linder, P., and Kressler, D. 2004b. The putative RNA helicase Dbp6p functionally interacts with Rpl3p, Nop8p and the novel trans-acting factor Rsa3p during biogenesis of 60S ribosomal subunits in *Saccharomyces cerevisiae*. *Genetics* (in press).

Decatur, W.A. and Fournier, M.J. 2002. rRNA modifications and ribosome function. *Trends Biochem. Sci.* **27:** 344–351.

Deshmukh, M., Tsay, Y.-F., Paulovich, A.G., and Woolford Jr., J.L. 1993. Yeast ribosomal protein L1 is required for the stability of newly synthesized 5S rRNA and the assembly of 60S ribosomal subunits. *Mol. Cell. Biol.* **13:** 2835–2845.

Dez, C., Froment, C., Noaillac-Depeyre, J., Monsarrat, B., Caizergues-Ferrer, M., and Henry, Y. 2004. Npa1p, a component of very early pre-60S ribosomal particles, associates with a subset of snoRNPs required for peptidyl transferase center modification. *Mol. Cell. Biol.* (in press).

Doye, V. and Hurt, E.C. 1995. Genetic approaches to nuclear pore structure and function. *Trends Genet.* **11:** 235–241.

Dragon, F., Gallagher, J.E., Compagnone-Post, P.A., Mitchell, B.M., Porwancher, K.A., Wehner, K.A., Wormsley, S., Settlage, R.E., Shabanowitz, J., Osheim, Y., et al. 2002. A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. *Nature*

417: 967–970.
Du, Y.C. and Stillman, B. 2002. Yph1p, an ORC-interacting protein: Potential links between cell proliferation control, DNA replication, and ribosome biogenesis. *Cell* **109:** 835–848.
Fatica, A. and Tollervey, D. 2002. Making ribosomes. *Curr. Opin. Cell Biol.* **14:** 313–318.
Fatica, A., Cronshaw, A.D., Dlakic, M., and Tollervey, D. 2002. Ssf1p prevents premature processing of an early pre-60S ribosomal particle. *Mol. Cell* **9:** 341–351.
Fatica, A., Oeffinger, M., Dlakic, M., and Tollervey, D. 2003a. Nob1p is required for cleavage of the 3′ end of 18S rRNA. *Mol. Cell. Biol.* **23:** 1798–1807.
Fatica, A., Oeffinger, M., Tollervey, D., and Bozzoni, I. 2003b. Cic1p/Nsa3p is required for synthesis and nuclear export of 60S ribosomal subunits. *RNA* **9:** 1431–1436.
Fromont-Racine, M., Senger, B., Saveanu, C., and Fasiolo, F. 2003. Ribosome assembly in eukaryotes. *Gene* **313:** 17–42.
Gerbi, S.A. and Borovjagin, A.V. 2004. Pre-ribosomal RNA processing in multicellular organisms. In *Nucleolus* (ed. M.O.J. Olson). Landes Biosciences/Eurekah.com, Georgetown, TX.
Grandi, P., Rybin, V., Bassler, J., Petfalski, E., Strauss, D., Marzioch, M., Schafer, T., Kuster, B., Tschochner, H., Tollervey, D., et al. 2002. 90S pre-ribosomes include the 35S pre-rRNA, the U3 snoRNP, and 40S subunit processing factors but predominantly lack 60S synthesis factors. *Mol. Cell* **10:** 105–115.
Hazbun, T.R., Malmstrom, L., Anderson, S., Graczyk, B.J., Fox, B., Riffle, M., Sundin, B.A., Aranda, J.D., McDonald, W.H., Chiu, C.H., et al. 2003. Assigning function to yeast proteins by integration of technologies. *Mol. Cell* **12:** 1353–1365.
Hess, S.M., Stanford, D.R., and Hopper, A.K. 1994. SRD1, *S. cerevisiae* gene affecting pre-rRNA processing contains a C_2/C_2 zinc finger motif. *Nucleic Acids Res.* **22:** 1265–1271.
Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., and O'Shea, E.K. 2003. Global analysis of protein localization in budding yeast. *Nature* **425:** 686–691.
Kallstrom, G., Hedges, J., and Johnson, A. 2003. The putative GTPases Nog1p and Lsg1p are required for 60S ribosomal subunit biogenesis and are localized to the nucleus and cytoplasm, respectively. *Mol. Cell. Biol.* **23:** 4344–4355.
Kiss, T. 2002. Small nucleolar RNAs: An abundant group of noncoding RNAs with diverse cellular functions. *Cell* **109:** 145–148.
Kressler, D., de la Cruz, J., Rojo, M., and Linder, P. 1998. Dbp6p is an essential putative ATP-dependent RNA helicase required for 60S-ribosomal-subunit assembly in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **18:** 1855–1865.
Kressler, D., Doère, M., Rojo, M., and Linder, P. 1999a. Synthetic lethality with conditional *dbp6* alleles identifies the previously uncharacterized *RSA1* gene, encoding a protein involved in a late nucleoplasmic step of 60S-ribosomal-subunit assembly. *Mol. Cell. Biol.* **19:** 8633–8645.
Kressler, D., Linder, P., and de la Cruz, J. 1999b. Protein *trans*-acting factors involved in ribosome biogenesis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **19:** 7897–7912.
Kruiswijk, T., Planta, R.J., and Krop, J.M. 1978. The course of the assembly of ribosomal subunits in yeast. *Biochim. Biophys. Acta* **517:** 378–389.
Leary, D.J. and Huang, S. 2001. Regulation of ribosome biogenesis within the nucleolus. *FEBS Lett.* **209:** 145–150.
Milkereit, P., Kuhn, H., Gas, N., and Tschochner, H. 2003. The pre-ribosomal network. *Nucleic Acids Res.* **31:** 799–804.
Nissan, T.A., Bassler, J., Petfalski, E., Tollervey, D., and Hurt, E. 2002. 60S pre-ribosome formation viewed from assembly in the nucleolus until export to the cytoplasm. *EMBO J.* **21:** 5539–5547.
Nomura, M., Nogi, Y., and Oakes, M. 2004. Transcription of rDNA in the yeast *Saccharomyces cerevisiae*. In *Nucleolus* (ed. M.O.J. Olson). Landes Bioscience/Eurekah.com, Georgetown, TX (in press).
Oeffinger, M. and Tollervey, D. 2003. Yeast Nop15p is an RNA-binding protein required for pre-rRNA processing and cytokinesis. *EMBO J.* **22:** 6573–6583.
Olson, M.O., Dundr, M., and Szebeni, A. 2000. The nucleolus: An old factory with unexpected capabilities. *Trends Cell Biol.* **10:** 189–196.
Pederson, T. and Politz, J.C. 2000. The nucleolus and the four ribonucleoproteins of translation. *J. Cell Biol.* **148:** 1091–1095.
Pestov, D.G., Strezoska, Z., and Lau, L.F. 2001. Evidence of p53-dependent cross-talk between ribosome biogenesis and the cell cycle: Effects of nucleolar protein Bop1 on G(1)/S transition. *Mol. Cell. Biol.* **21:** 4246–4255.
Planta, R.J. 1997. Regulation of ribosome synthesis in yeast. *Yeast* **13:** 1505–1518.
Planta, R.J., Goncalves, P.M., and Mager, W.H. 1995. Global regulators of ribosome biosynthesis in yeast. *Biochem. Cell Biol.* **73:** 825–834.
Raué, H.A. 2004. Pre-ribosomal RNA processing and assembly in *Saccharomyces cerevisiae:* The machine that makes the machine. In *Nucleolus* (ed. M.O.J. Olson). Landes Biosciences/Eurekah.com, Georgetown, TX (in press).
Ruggero, D. and Pandolfi, P.P. 2003. Does the ribosome translate cancer? *Nat. Rev. Cancer* **3:** 179–192.
Saveanu, C., Namane, A., Gleizes, P.E., Lebreton, A., Rousselle, J.C., Noaillac-Depeyre, J., Gas, N., Jacquier, A., and Fromont-Racine, M. 2003. Sequential protein association with nascent 60S ribosomal particles. *Mol. Cell. Biol.* **23:** 4449–4460.
Schäfer, T., Strauss, D., Petfalski, E., Tollervey, D., and Hurt, E. 2002. The path from nucleolar 90S to cytoplasmic 40S pre-ribosomes. *EMBO J.* **22:** 1370–1380.
Senger, B., Lafontaine, D.L., Graindorge, J.S., Gadal, O., Camasses, A., Sanni, A., Garnier, J.M., Breitenbach, M., Hurt, E., and Fasiolo, F. 2001. The nucle(ol)ar Tif6p and Efl1p are required for a late cytoplasmic step of ribosome synthesis. *Mol. Cell* **8:** 1363–1373.
Takahashi, N., Yanagida, M., Fujiyama, S., Hayano, T., and Isobe, T. 2003. Proteomic snapshot analyses of preribosomal ribonucleoprotein complexes formed at various stages of ribosome biogenesis in yeast and mammalian cells. *Mass Spectrom. Rev.* **22:** 287–317.
Tschochner, H. and Hurt, E. 2003. Pre-ribosomes on the road from the nucleolus to the cytoplasm. *Trends Cell Biol.* **13:** 255–263.
Udem, S.A. and Warner, J.R. 1973. The cytoplasmic maturation of a ribosomal precursor ribonucleic acid in yeast. *J. Biol. Chem.* **248:** 1412–1416.
Uetz, P., Giot, L., Cagney, G., Mansfield, T.A., Judson, R.S., Knight, J.R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., et al. 2000. A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. *Nature* **403:** 623–627.
Vanrobays, E., Gelugne, J.P., Gleizes, P.E., and Caizergues-Ferrer, M. 2003. Late cytoplasmic maturation of the small ribosomal subunit requires RIO proteins in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **23:** 2083–2095.
Venema, J. and Tollervey, D. 1996. *RRP5* is required for formation of both 18S and 5.8S rRNA in yeast. *EMBO J.* **15:** 5701–5714.
———. Ribosome synthesis in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **33:** 261–311.
Venema, J., Planta, R.J., and Raué, H.A. 1998. In vivo mutational analysis of ribosomal RNA in *Saccharomyces cerevisiae*. In *Protein synthesis: Methods and protocols* (ed. R. Martin), pp. 257–270. Humana Press, Totowa, NJ.
Warner, J.R. 1999. The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.* **24:** 437–440.
Warner, J.R., Vilardell, J., and Sohn, J.H. 2001. Economics of ribosome biosynthesis. *Cold Spring Harb. Symp. Quant. Biol.* **66:** 567–574.
Zanchin, N.I.T. and Goldfarb, D.S. 1999. Nip7p interacts with Nop8p, an essential nucleolar protein required for 60S ribosome biogenesis, and the exosome subunit Rrp43p. *Mol. Cell. Biol.* **19:** 1518–1525.

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IVÁN V. ROSADO and JESÚS DE LA CRUZ

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