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Nog2p, a putative GTPase associated with pre-60S subunits and required for late 60S maturation steps

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Eukaryotic ribosome maturation depends on a set of well ordered processing steps. Here we describe the functional characterization of yeast Nog2p (Ynr053cp), a highly conserved nuclear protein. Nog2p contains a putative GTP-binding site, which is essential *in vivo*. Kinetic and steady-state measurements of the levels of pre-rRNAs in Nog2p-depleted cells showed a defect in 5.8S and 25S maturation and a concomitant increase in the levels of both $27SB_S$ and $7S_S$ precursors. We found Nog2p physically associated with large pre-60S complexes highly enriched in the 27SB and 7S rRNA precursors. These complexes contained, besides a subset of ribosomal proteins, at least two additional factors, Nog1p, another putative GTP-binding protein, and Rlp24p (Ylr009wp), which belongs to the Rpl24e family of archaeal and eukaryotic ribosomal proteins. In the absence of Nog2p, the pre-60S ribosomal complexes left the nucleolus, but were retained in the nucleoplasm. These results suggest that transient, possibly GTP-dependent association of Nog2p with the pre-ribosomes might trigger late rRNA maturation steps in ribosomal large subunit biogenesis.

Keywords: GTPase/nucleolus/nucleus/pre-rRNA processing/ribosome

Introduction

The yeast ribosome is a large ribonucleoprotein particle dynamically assembled during translation initiation from a small (40S) and a large (60S) subunit. The 40S particle is structured around an 18S rRNA whereas the mature 60S subunit contains the 25S, 5.8S and 5S rRNAs. Three of the four rRNAs (18S, 5.8S and 25S) are processed from a large single 35S pre-rRNA precursor transcribed by RNA polymerase I, whereas the 5S rRNA is synthesized by RNA polymerase III from another gene within the same rDNA unit. The 35S pre-rRNA includes two external transcribed sequences (5′-ETS and 3′-ETS) and two internal transcribed spacers (ITS1 and ITS2). The maturation of this precursor involves numerous processing steps, including nucleotide modification steps as well as different endo- and exonucleolytic cleavages that sequentially remove the ETS and ITS sequences. This maturation pathway is summarized in Figure 1 and has been reviewed extensively (Kressler *et al*., 1999b; Venema and Tollervey, 1999; Geerlings *et al*., 2000).

In addition to early associating ribosomal proteins, endonucleases, exonucleases and putative RNA helicases, other *trans*-acting factors such as Nip7p, Nop8p, Nop2p, Rsa1p (see Kressler *et al*., 1999b and references therein), Ebp2p (Huber *et al*., 2000) and Rlp7p (Dunbar *et al*., 2000) preferentially impair the biogenesis of 60S subunits. While most of the factors involved in the biogenesis of 60S subunits are localized mainly to the nucleolus, nucleoplasmic maturation steps of pre-60S particles are likely. The late steps of rRNA processing are followed by export of the nascent ribosomal particles into the cytoplasm. The export of pre-60S particles is dependent on the abundant shuttling protein Nmd3p, which associates with the ribosomal protein Rpl10p and plays an adaptor role for the karyopherin Crm1p (Ho *et al*., 2000; Gadal *et al*., 2001). The association of Rpl10 with the ribosome might be a relatively late event in the nuclear maturation of the large ribosomal particle since depletion of the exclusively nucleoplasmic protein Rsa1p affects association of Rpl10 with 60S particles (Kressler *et al*., 1999a). Little information about the nature of the stable components associated with pre-ribosomal subunits, the dynamics of the assembly, the compartments where successive steps are taking place and the transport of these particles is available currently.

Here we describe the analysis of the previously uncharacterized *YNR053C* gene, which hereafter is referred to as *NOG2* (nuclear–nucleolar GTP-binding protein 2). The Nog2p protein is highly conserved and its human homologue was described previously as the nucleolar breast tumour-associated autoantigen NGP-1 (Racevskis *et al*., 1996). We show that Nog2p is an essential nuclear protein required for the processing of the 27S ribosomal precursor to mature 25S and 5.8S rRNAs. To explore further the role of this protein, we have purified and characterized its associated complex. In addition to components of the large ribosomal subunit, we identified new essential factors that are likely to participate in ribosomal biogenesis: Rlp24p (Ylr009wp), a conserved protein similar to archaeal and eukaryotic Rpl24e proteins; and Nog1p, a nucleolar putative GTP-binding protein conserved in eukaryotes (Park *et al*., 2001).

Results

Nog2p is an essential protein required for the maintenance of normal rRNA levels

The *NOG2* gene is essential for yeast viability (Fromont-Racine *et al*., 1997). We constructed a yeast

strain (LMA148) in which the galactose-inducible promoter *GAL1* replaced the endogenous *NOG2* promoter. In the LMA159 strain, the protein A-containing TAP tag (Rigaut *et al*., 1999) was inserted in-frame at the 3′ end of the *GAL1-NOG2* coding sequence (Table I). In order to deplete the Nog2p protein, the cells were shifted from galactose medium (YPGal) to glucose medium (YPGlu). We observed a severe reduction in the growth rate of the LMA148/159 strains after 10 h of culture in the presence of glucose (Figure 2A). An immunoblot, which revealed the protein A tag, confirmed that Nog2-TAP rapidly decreases and becomes undetectable in the LMA159 strain after 24 h in YPGlu (data not shown). However, by comparison with the LMA78 strain, which expressed Nog2-TAP under its own promoter (Table I), Nog2-TAP was overexpressed in the LMA159 strain in YPGal and its level remained higher than or equal to the natural level of Nog2-TAP for ~6 h after the shift to glucose (Figure 2B).

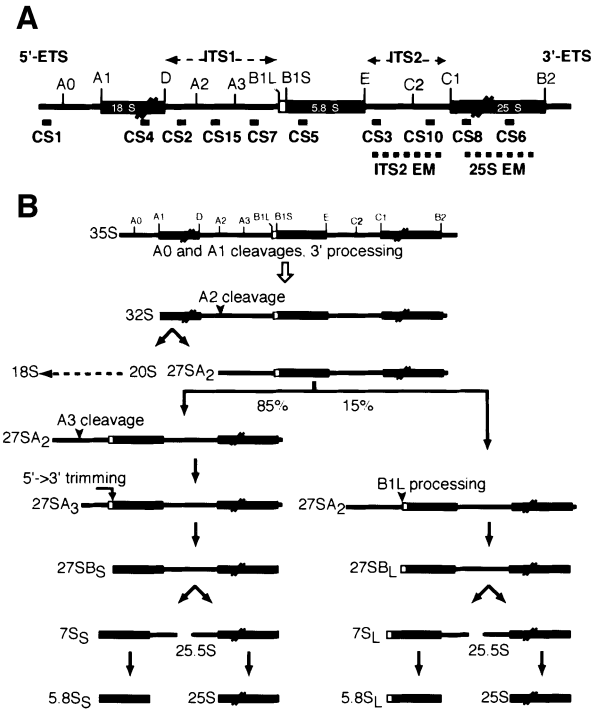

Fig. 1. Simplified maturation scheme for rRNA in yeast, adapted from Venema and Tollervey (1999). (**A**) Schematic representation of the large 35S primary pre-rRNA transcript. The relative position of the oligonucleotides used as probes in the study is also shown. (**B**) Processing of the 35S precursor to the mature rRNA species in yeast.

A severe decrease in the cellular levels of 18S and 25S rRNAs was observed from cells collected at different times after a shift to glucose (Figure 2C and D). The level of 25S rRNA was more affected than that of 18S rRNA (Figure 2D). Consistent with these findings, polysome fractionation by sucrose gradient ultracentrifugation revealed that, in Nog2p-depleted cells, the free 60S subunit level was more affected than that of the 40S subunit (Figure 3A). After 15 h of *NOG2* repression, the polysome level was severely reduced, consistent with the dramatic reduction of the growth rate (Figure 2A). Altogether, these data suggested that Nog2p is involved in pre-rRNA processing and specifically affects the biogenesis of the 60S subunit.

27S rRNA processing is affected in Nog2p-depleted cells

To identify the pre-rRNA processing steps at which Nog2p is involved, we first studied the kinetics of rRNA maturation using L-[methyl-^{3}H]methionine pulse–chase labelling *in vivo*. In galactose medium, the 35S precursor was processed into 27S and 20S intermediate species in <2 min (Figure 4) and these intermediates were then completely converted to mature rRNAs within 5 min after the chase. In the Nog2p-depleted strain (glucose medium), the 35S precursor is still visible after 5 min of chase and the 27S precursor remained present for >12 min after the chase. We also detected an aberrant 23S form. In contrast, the time course of conversion of the 20S into 18S rRNAs was not affected. Moreover, the accumulation of the 18S rRNA was only modestly inhibited by comparison with the appearance of the 25S rRNA, which was very much delayed. These early step effects are commonly observed for mutations impaired primarily in late steps of 5.8S/25S synthesis, and it has been proposed that they result from a feedback mechanism exerted on early processing steps at A_0–A_2 (Venema and Tollervey, 1999). Nog2p thus seems to be involved primarily in the processing of the 27S rRNA intermediate to 25S rRNA.

The 27S species consist of several distinct intermediates (see Figure 1). The $27SA_2$ intermediate that results from cleavage of the 32S molecule at site A_2 may follow two distinct routes to generate the mature 5.8S and 25S products. The minor pathway generates the $27SB_L$ intermediate, while, in the major pathway, the $27SA_2$ molecule is first cleaved by the RNase MRP at site A_3 and then trimmed by Rat1 to generate the $27SB_S$ RNA. This results in the minor pathway giving rise to the $5.8S_L$ mature RNA, while the major pathway produces the $5.8S_S$ transcript, which represents 85% of the mature 5.8S molecules

Table I. Yeast strains used in this study

Strain	Genotype	Reference
MGD353-13D	*MAT***a**, *trp1-289, ura3-52, ade2, leu2-3,-112, Arg4*	Rigaut *et al*. (1999)
BMA64	*MAT***a**/α, *ura3-1/ura3-1,Δtrp1/Δtrp1, ade2-1/ade2-1, leu2-3,-112/leu2-3,-112, his3-11,15/his3-11,15*	Baudin *et al*. (1993)
LMA51-2	*MATα, ura3-1, Δtrp1, ade2-1, leu2-3,-112, his3-11,15, Nog2-GFP::HIS*	this study
LMA78	*MAT***a**, *trp1-289, ura3-52, ade2, leu2-3,-112, Arg4, Nog2-TAP/TRP1*	this study
LMA148	*MAT***a**, *ura3-1, Δtrp1, ade2-1, leu2-3,-112, his3-11,15, GAL::NOG2/KanMX6*	this study
LMA158	*MAT***a**, *trp1-289, ura3-52, ade2, leu2-3,-112, Arg4, Nog1-TAP/TRP1*	this study
LMA159	*MAT***a**, *ura3-1, Δtrp1, ade2-1, leu2-3,-112, his3-11,15, GAL::Nog2-TAP/KanMX6/TRP1*	this study
LMA160	*MAT***a**, *trp1-289, ura3-52, ade2, leu2-3,-112, Arg4, Rlp24-TAP/TRP1*	this study
LMA173	*MAT***a**, *trp1-289, ura3-52, ade2, leu2-3,-112, Arg4, Rpl10-TAP/TRP1*	this study

(Henry *et al*., 1994). To pinpoint the precise steps at which Nog2p is involved, we analysed the steady-state levels of each pre-rRNA intermediate and mature rRNA by northern blotting and primer extension experiments using a set of specific oligonucleotide probes (Table II; the locations of the primers are shown in Figure 1). The main results are summarized in Figure 5. When Nog2p was depleted, we observed an accumulation of the $27SB_S$ intermediate, whereas the level of the $27SA_2$ intermediate remained constant. We also noted that the level of the $27SB_L$ intermediate-specific product (6–8 nucleotides longer than $27SB_S$) remained essentially unchanged (Figure 5A). We observed a similar accumulation of the reverse transcription products whose 5′ ends correspond to the B_S site both with primer CS8, which hybridizes within the 25S mature sequence region (see Figure 1), and with primer CS3, which hybridizes within the 7S intermediate region (data not shown). Figure 5B shows that the $7S_S$ intermediate accumulated in mutant cells shifted to glucose, whereas the level of the $7S_L$ intermediate remained unchanged. In contrast, northern blot analysis revealed that the $5.8S_S$ and $5.8S_L$ levels were equally affected upon *NOG2* repression (Figure 5C). The only additional major effects observed upon Nog2p depletion were the accumulation of the 35S species and the appearance of the 23S species (Figure 4 and data not shown).

We concluded that Nog2p is implicated both in the 27SB to 7S and the 7S to 5.8S processing steps (see Figure 1). More specifically, we observed that only the short forms of the 27SB and 7S intermediates accumulated. This might indicate that Nog2p is only involved in the major pathway. However, the $5.8S_S$ and $5.8S_L$ levels were equally affected upon *NOG2* repression (see Discussion).

Nog2p is associated with pre-60S particles

The presence of Nog2-TAP and large subunit ribosomal protein Rpl1p/Ssm1p was examined in the different fractions of sucrose gradients by immunoblotting (Figure 3B). Nog2-TAP was found associated with a complex that had a sedimentation rate similar to that of the 60S particles. As expected, the ribosomal Rpl1p protein, used as a control, co-sedimented with the 60S subunits, 80S ribosomes and the polysomes. We noted that after 15 h of *NOG2* repression, Nog2-TAP was still detectable in the gradient around the 60S position even if the total amount of the protein was reduced dramatically (Figure 2B). This was correlated with the presence at the top of the gradient of some free Rpl1p, suggesting that some of the ribosomal proteins became unbound. The detection of Nog2p around the position of the 60S subunit was consistent with this protein being part of a 66S pre-ribosomal subunit, a nuclear precursor of mature 60S particles (Trapman *et al*., 1975).

In order to purify and characterize this complex, we used the LMA78 strain in which a TAP tag was introduced in-frame at the 3′ end of *NOG2* under the control of its own promoter (see above and Table I). We purified this TAP-tagged version of Nog2p under native conditions by two successive affinity purification steps according to Rigaut

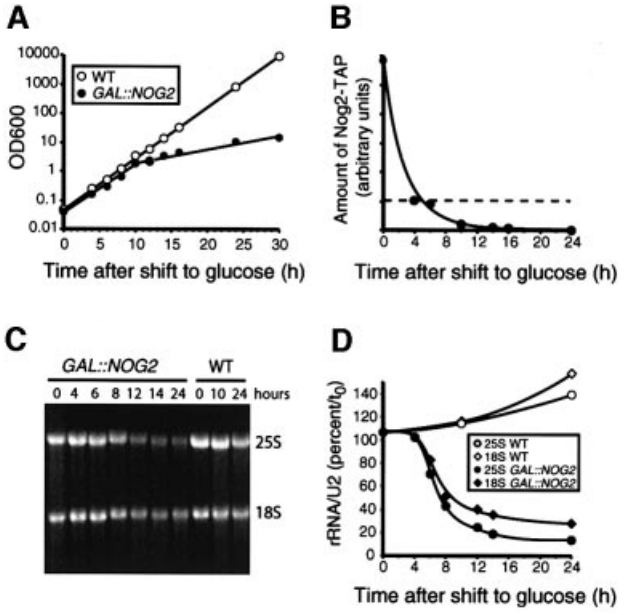

Fig. 2. Depletion of Nog2p (Ynr053cp) causes growth arrest and reduction of mature rRNA levels. (**A**) A conditionally lethal *GAL::NOG2-TAP* strain (LMA159) and a wild-type strain were grown at 30°C in YPGal and shifted to YPGlu medium. Optical densities were measured at various times of culture in glucose-containing medium. (**B**) Analysis of the Nog2p level in LMA159 cells at different times after the shift to glucose-containing medium. Proteins were extracted from the same number of cells and loaded on an 8% polyacrylamide gel. Immunoblotting with peroxidase–anti-peroxidase-soluble complexes revealed the protein A component of the tagged Nog2p. These levels were compared with the level measured for Nog2-TAP under the control of its own promoter (LMA78 strain) (horizontal dashed line). (**C**) Total RNAs were purified from either LMA159 or wild-type strains after growth in YPGlu for up to 40 h. RNAs were separated on a 1% agarose–formaldehyde gel and stained with ethidium bromide. (**D**) The levels of 25S and 18S mature rRNAs were normalized to the amount of U2 RNA; 100% corresponds to the galactose growth conditions.

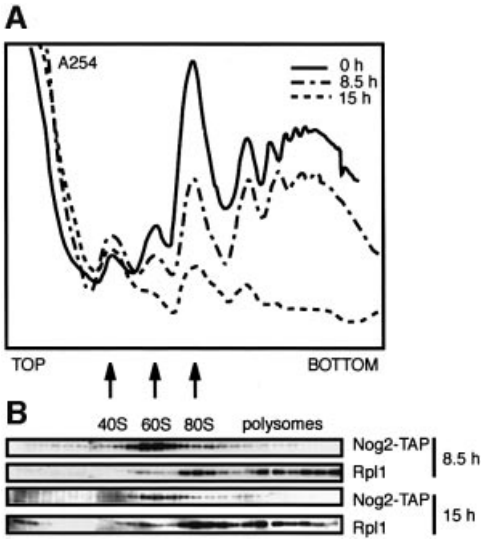

Fig. 3. Nog2p-depleted cells display a decrease in the abundance of free 60S ribosomal subunits. (**A**) Polysome profiles were analysed for the LMA159 strain by sedimentation through 10–50% sucrose gradients. Cells were either grown on YPGal (0 h) or shifted to glucose for 8.5 or 15 h. (**B**) Cellular proteins were extracted from various fractions of the sucrose gradient and analysed by immunoblotting using antibodies that revealed specifically either the Rpl1p (1:20 000 dilution) (Petitjean *et al*., 1995) or the Nog2-TAP proteins (peroxidase–anti-peroxidase 1:10 000 dilution).

et al. (1999). Figure 6 shows that Nog2p co-purified with a large complex, ~30 bands being visible after Coomassie Blue staining. Since Nog2p co-sedimented with the large ribosomal subunit in ultracentrifugation experiments, we compared the profile of the complex purified with Nog2-TAP with the protein profile of the purified large and small ribosomal subunits (Figure 6A). The protein profile of the TAP-purified complex revealed extensive similarities with the profile of the large ribosomal subunit. Individual bands were analysed by mass spectrometry and the corresponding proteins identified (Figure 6A). As expected, this analysis revealed that the Nog2p-associated complex contained a number of large ribosomal subunit proteins. In addition, two novel proteins, Nog1p and Ylr009wp, were also identified. Nog1p, like Nog2p, harbours a GTP-binding site motif (see Discussion), has been shown previously to be essential for yeast viability and has a nucleolar localization (Park *et al*., 2001). An essential uncharacterized gene, *YLR009W*, encodes the second novel protein. Because of its strong similarity to both the yeast cytoplasmic and the archaeal/eukaryotic Rpl24e ribosomal proteins (see Discussion), we now refer to this protein as Rlp24p (ribosomal-like protein 24).

To confirm that Nog2p, Nog1p and Rlp24p were part of the same complex, additional purifications were performed with both the Nog1-TAP and Rlp24-TAP tagged proteins. To this end, we constructed the LMA158 and LMA160 strains expressing Nog1-TAP and Rlp24-TAP under the control of their own promoters, respectively (Table I). The protein profiles of the three TAP-purified complexes were highly similar (see Figure 6B), with the exception of the bands that corresponded to the tagged versions of the proteins and which were identified by mass spectrometry. Their apparent mass was 5 kDa larger than that of the wild-type versions as a result of the remaining calmodulin-binding tag. We were able to identify both Nog1p and Rlp24p in the three complexes, whereas Nog2p was not detected in the Nog1p- and Rlp24-TAP-associated complexes (see Discussion). In addition, we observed that two ribosomal proteins, Rpl12p and Rpp0p, were absent from the three purified complexes (see Figure 6A and B). These results were consistent with other experiments showing that the Nog2p-associated complexes are confined to the nucleus (see Discussion).

To analyse the transcripts present within the Nog2p-associated complex, successive hybridizations were performed with various probes on RNAs extracted from complexes purified independently twice (experiments 1 and 2). We observed a strong hybridization signal with the CS3 and CS10 probes. Since other probes (CS2 and CS7) hybridizing to 35S, 33S, 32S, $27SA_2$ and $27SA_3$ revealed no enrichment for these species, we concluded that the signal generated by probe CS10 results from an enrichment of 27SB forms (50- to 150-fold relative to 18S; see Figure 6C). Likewise, probe CS3 revealed a 380- to 480-fold enrichment over 18S for the 27SB plus 7S intermediates, hence an enrichment of >200-fold for 7S if the contribution of 27SB (as estimated with probe CS10) is subtracted. The high enrichment of the 7S RNA species was confirmed by northern blotting of RNAs co-purified with Nog2-TAP (see Figure 6D). A significant enrichment was also observed with probes CS5 and CS6-8, which hybridize within the 5.8S and 25S sequences, respectively. These probes also hybridize to rRNA intermediates, but the contribution of these transcripts to the observed signal can be neglected, as they are ~500 times less abundant than the 25S mature transcripts. We thus concluded that the purified fractions contained significant amounts of mature 5.8S and 25S, which correspond to the products of the two reaction steps inhibited in the Nog2p-depleted cells. The same analyses performed on the RNAs present in the Nog1p- and Rlp24p-associated complexes revealed that these particles were also strongly enriched for the 27S and 7S intermediates (data not shown). In summary, Nog2p was found as part of large complexes involved in late steps of rRNA maturation that contain, in addition to nuclear associated 60S ribosomal proteins, two novel proteins, Nog1p and Rlp24p, as well as 27SB, 7S, 25S and 5.8S rRNAs.

Nog2p is localized to the nucleolus and the nucleoplasm

Most of the proteins involved in pre-rRNA processing are localized in the nucleolus. To obtain insight into the

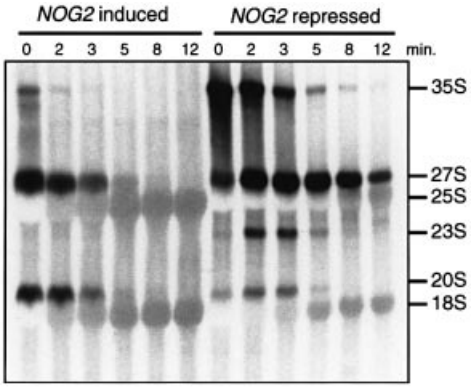

Fig. 4. Nog2p depletion leads to reduced synthesis of 25S rRNA. Pulse–chase labelling with [methyl-^{3}H]methionine was performed with the LMA159 strain (*GAL::NOG2*) grown in galactose medium (*NOG2* induced) or shifted to glucose medium (*NOG2* repressed) for 16 h. Cells were pulse-labelled for 2 min and then chased for the indicated times with an excess of cold methionine. Total labelled RNAs were purified, separated on a denaturing agarose gel and autoradiographed. The position of the intermediate and mature rRNAs is indicated.

Table II. Oligonucleotides used in this study

Probe name	Sequence
CS1	TCGGGTCTCTCTGCTGC
CS2	CGGTTTTAATTGTCCTA
CS3	GGCCAGCAATTTCAAGTTA
CS4	AATGATCCTTCCGCAGGTTCAC
CS5	CGGAATTCTGCAATTCACATTACG
CS6	ACGAGCCTCCACCAGAGTTTCC
CS7	CCAGTTACGAAAATTCTTG
CS8	CTCCGCTTATTGATATGC
CS10	CGCCTAGACGCTCTCTTCTTA
CS15	TGTTACCTCTGGGCCC
MFR398-5′	GGTCAATCCAAACGTATTTGGAACG
MFR399-3′	TCTCGTCTCGATAGCCGATAAACCCTACA
MFR400-5′	TCTCGTCTCGCTAT**GT**AAACACTGGTAAATCGTCC
MFR402-3′	TCTCGTCTCATTTACCAGTGTTTGGATAGCC
MFR403-5′	TCTCGTCTCGTAAA**AAT**TCCATCATTAACACATTGAG
MFR407-3′	TCCATCCTGAGATCTCGTAAGTTC

Mutated bases are indicated in bold.

subcellular localization of Nog2p, we generated a strain where the sequence encoding the green fluorescent protein (GFP) was fused at the 3′ end of *NOG2* on the chromosome (LMA51-2) (see Table I). We observed that Nog2–GFP was located to the nucleolus and to the nucleoplasm (Figure 7) while no cytoplasmic signal was detected. The subcellular localization of Nog2p is fully consistent with a role in pre-rRNA processing and ribosome biogenesis.

Depletion of Nog2p results in the nuclear retention of pre-60S particles

We next examined the effect of Nog2p depletion on the localization of the pre-60S ribosomal particles *in situ*. BMA64 (wild type) and LMA148 (*GAL::NOG2*) cells were first transformed with a plasmid encoding Rpl25p, a protein of the large ribosomal subunit, fused to GFP. This chimeric protein binds to pre-rRNA and allows monitoring of the localization of pre-60S and 60S particles by fluorescence microscopy (Hurt *et al*., 1999). As shown in

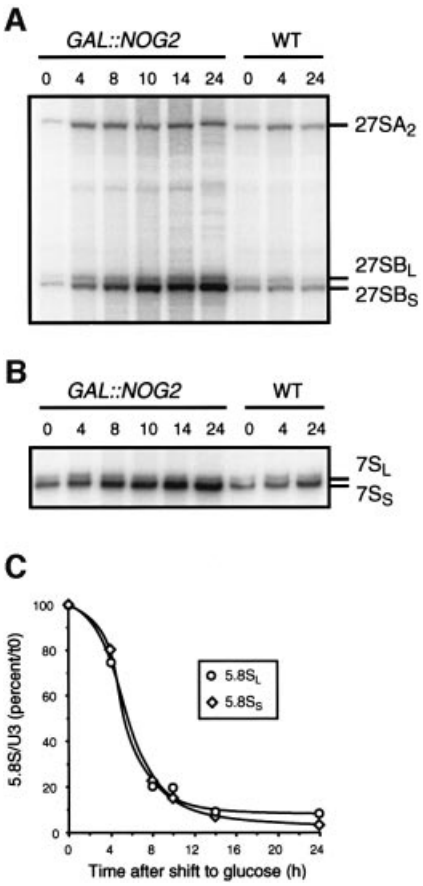

Fig. 5. Absence of Nog2p leads to an accumulation of the $27SB_S$ and $7S_S$ pre-rRNA. Total RNAs were extracted from the LMA159 strain after growth in YPGal or after shift to YPGlu for various times, as indicated. (**A**) Primer extensions were performed using primer CS10 (specific for the different 27S forms). The reaction products were resolved on a 5% acrylamide denaturing gel. (**B**) Northern blot analysis of 7S rRNA levels. The RNAs were separated on a 5% acrylamide denaturing gel, transferred to a nylon membrane and probed with oligonucleotide CS3, which hybridizes within the 7S region. (**C**) Quantification of the hybridization signals for the $5.8S_S$ and $5.8S_L$ mature rRNAs. The levels were normalized to the hybridization signals obtained with a U3 RNA-specific probe; 100% corresponds to the galactose-induced conditions.

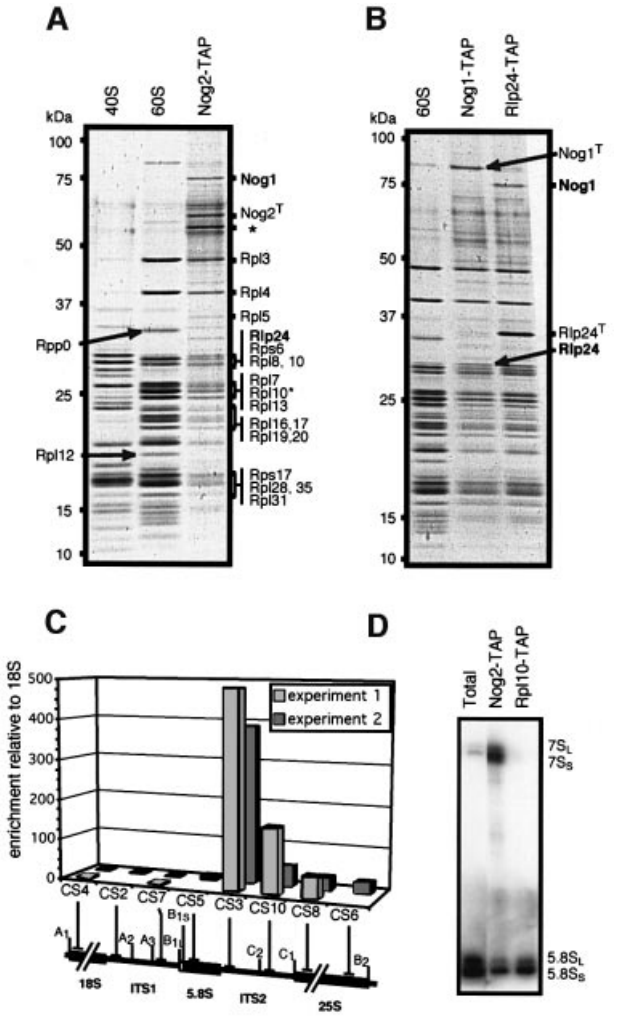

Fig. 6. Nog2p, Nog1p and Rlp24p are associated with similar pre-ribosomal complexes. (**A**) The protein composition of the affinity-purified Nog2p-associated complex (lane Nog2-TAP) was determined after electrophoresis on a gradient gel and Coomassie Blue staining. Proteins were identified using mass spectrometry and the pattern of protein bands was compared with the pattern of bands obtained with purified large (lane 60S) and small (lane 40S) ribosomal subunits. The tagged protein is marked $Nog2^T$; a presumed Nog2p fragment is marked with *; a fragment of Rpl10p protein is marked Rpl10*. (**B**) Protein composition of the complexes obtained by tandem affinity purification using tagged versions of Nog1p (Nog1-TAP) and Rlp24p (Rlp24-TAP). Both Nog1p and Rlp24p were identified by mass spectrometry as components of the Rlp24p- and Nog1p-associated complexes, respectively. The tagged versions of the proteins are marked $Nog1^T$ and $Rlp24^T$. For comparison, the proteins of the large ribosomal subunit were separated on the same gel (lane 60S). (**C**) Dot-blot quantitation of the different precursors of rRNAs in the Nog2p-associated complex. The RNAs were extracted from the purified complex, heat denatured, spotted on Hybond N+ membranes and probed with radiolabelled oligonucleotides specific for different rRNA precursors. The signal was quantified on a PhosphorImager system. The ratio of the signal obtained with the purified RNAs over the signal obtained with the total RNAs was calculated; these values were normalized by arbitrarily taking the ratio obtained with the 18S RNA probe (CS4) as a reference (equal to one on the figure). (**D**) Northern blot hybridization after polyacrylamide gel electrophoresis of total RNAs and RNAs purified in one step using TAP-tagged versions of Nog2p or ribosomal protein Rpl10-TAP used as a control. Radiolabelled oligonucleotide CS5, complementary to a region of the 5.8S mature rRNA, was used to probe the membrane. The positions of 5.8S and 7S RNAs are indicated. The enrichment for the 7S species in the Nog2p-associated RNAs can be observed by comparison with total RNAs or RNAs associated with Rpl10-TAP.

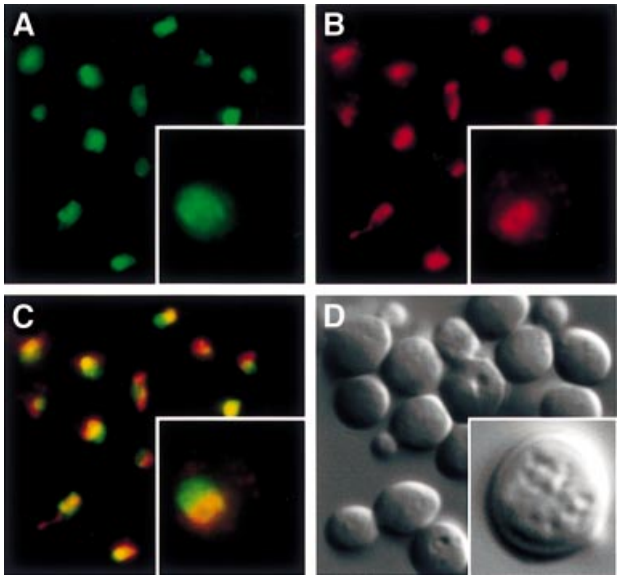

Fig. 7. Nog2p is located in the nucleolus and nucleoplasm. The LMA51-2 strain, containing the Nog2–GFP fusion under the control of its own promoter, was grown in SD-His medium. Cells were stained with Hoechst 33342 and photographed with filters specific for GFP fluorescence (**A**), Hoechst (**B**) or Nomarski (**D**). A merged image of (A) and (B) is shown in (**C**).

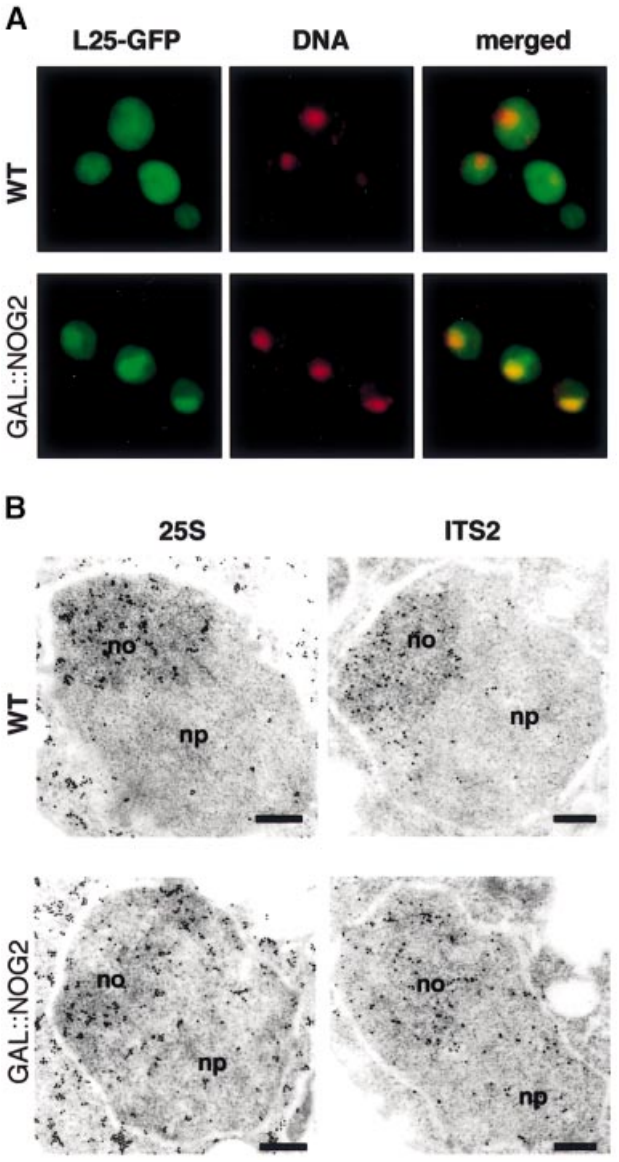

Fig. 8. Effect of Nog2p depletion on the localization of the pre-60S ribosomal particles. (**A**) Wild-type and *GAL::NOG2* cells expressing Rpl25p–GFP were grown for 16 h in glucose-containing medium, stained with DAPI and photographed with filters for GFP fluorescence (L25–GFP) or DAPI (DNA); merge = images of the GFP and DAPI fluorescence merged. (**B**) *In situ* hybridization and electron microscopy were applied to wild-type and *GAL::NOG2* cells grown for 16 h in glucose-containing medium using probes directed against the 25S rRNA sequence (25S) or with a probe complementary to ITS2 (ITS2).

Figure 8A, Rpl25–GFP was detected primarily in the cytoplasm of wild-type cells. In contrast, Rpl25–GFP was found to accumulate in the whole nucleus of *GAL::NOG2* cells depleted of Nog2p after 16 h of shift to glucose. To refine these data, we directly localized pre-rRNA by *in situ* hybridization and electron microscopy (Figure 8B). As expected, in wild-type cells, a probe hybridizing to the 25S rRNA sequence labelled the nucleolus, the main site of ribosome biogenesis, and the cytoplasm, where ribosomes ensure translation. A lower signal was also observed in the nucleoplasm. In contrast, the whole nucleus of Nog2p-depleted *GAL::NOG2* cells was found to be labelled with this probe, indicating the nuclear accumulation of pre-25S rRNA. In these cells, the nucleolus appeared to be dilated and loose. In addition, the strong labelling in the nucleoplasm was often associated with electron-dense material, consistent with the accumulation of pre-ribosomes in this nuclear compartment. That this material corresponded to accumulating pre-ribosomal particles was confirmed using a probe complementary to ITS2. Like the 25S probe, this probe was detected mostly in the nucleolus of wild-type cells, whereas it also labelled electron-dense domains in the nucleoplasm in *GAL::NOG2* cells after 16 h in glucose (Figure 8B). These observations are consistent with the biochemical data presented above showing the accumulation of 27S and 7S pre-rRNAs upon depletion of Nog2p, and indicate that the pre-ribosomal complexes containing these precursors leave the nucleolus, but are retained in the nucleoplasm in the absence of Nog2p.

Nog2p belongs to a subfamily of putative GTP-binding proteins

Sequence alignments revealed that Nog2p is highly conserved. We observed a strong similarity over the full length of Nog2p to proteins of as yet unknown function in higher eukaryotes, including one of human origin (50% identity between human NGP-1 and yeast Nog2p). In archaea and bacteria, Nog2p-related sequences are shorter and do not contain the equivalent of the Nog2p N-terminal domain. This region is, however, clearly conserved between diverse eukaryotic species. The Nog2p domain conserved in eukaryotes, archaea and bacteria contains the G1–G5 GTP-binding motifs previously described (Bourne *et al.*, 1991), but the order of the G-motifs is permutated in the Nog2p family, as previously observed for the HSR1/MMR1 GTP-binding protein subfamily (Vernet *et al.*, 1994) (Figure 9A). Three other yeast proteins share this specific arrangement of the G-motifs with Nog2p (Figure 9B). The corresponding Nog1p G-domain did

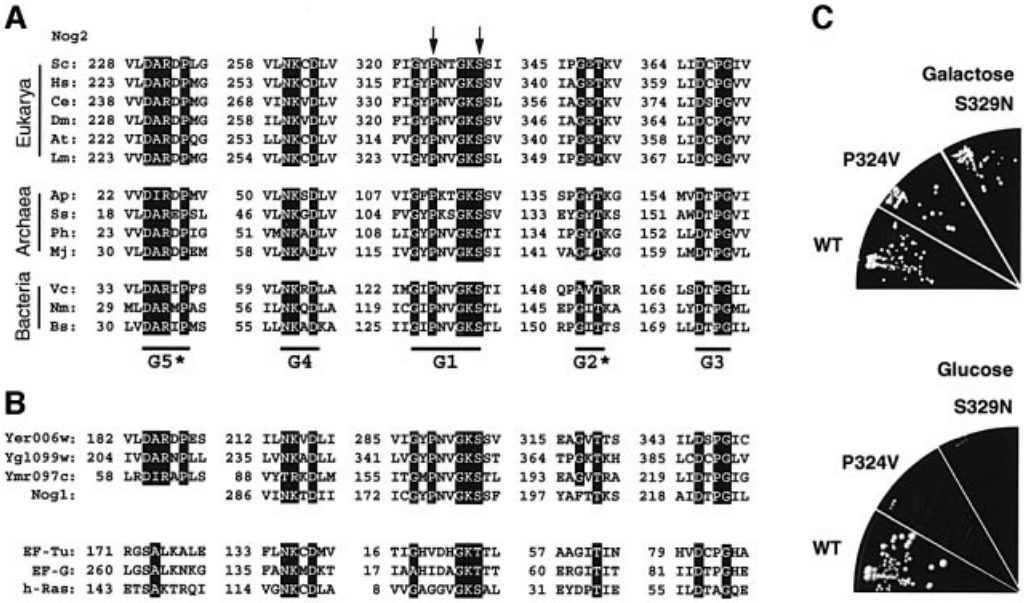

Fig. 9. Conserved residues within the GTP-binding signature of Nog2p are essential for viability. (**A**) Sequences of predicted proteins from eukaryotes, archaea and bacteria that are similar to yeast Nog2p. Only the conserved G-regions are shown, with the position of the first residue marked. G2- and G5-like motifs (G2* and G5*) were detected in the Nog2p protein family by multiple alignments and secondary structure predictions (Materials and methods). Arrows point to the residues that have been substituted in the mutants (see text and C). Species origins of sequences are designated as follows, with SwissProt or TrEMBL accession numbers in parentheses: Sc, *Saccharomyces cerevisiae* (P53742); Hs, *Homo sapiens* (Q13823); Ce, *Caenorhabditis elegans* (Q9XXN4); Dm, *Drosophila melanogaster* (Q9V844); At, *Arabidopsis thaliana* (Q9C923); Lm, *Leishmania major* (GenPept gi:13122225); Ap, *Aeropyrum pernix* (Q9YFZ6); Ss, *Sulfolobus sulfataricus* (GenPept gi:6015892); Ph, *Pyrococcus horikoshii* (O58379); Mj, *Methanococcus jannaschii* (Q58859); Vc, *Vibrio cholerae* (Q9KM60); Nm, *Neisseria meningitidis* (Q9JV89); Bs, *Bacillus subtilis* (ylqF, O31743). (**B**) Conserved regions G1–G5 in *S.cerevisiae* sequences that have the same permuted organization of G-motifs as Nog2p (G4 preceding G1) and corresponding motifs in Nog1p and three GTPases of known structure for comparison: *Escherichia coli* EF-Tu, *Thermus thermophilus* EF-G and *H.sapiens* H-ras p21. (**C**) Wild-type, P324N and S329D Nog2p variants, cloned into the pCM190 vector, were transformed into the *GAL::NOG2* strain (LMA159). The empty vector could not support growth of the LMA159 strain under restrictive conditions (glucose medium; data not shown). Transformed cells were streaked on either galactose- or glucose-containing medium.

not display the specific arrangement of the Nog2p family and no obvious homologies were found between Nog1p and Nog2p outside the G-domain, suggesting that these two proteins are members of different subfamilies of putative GTPases (Figure 9B).

To assess the functional importance of the Nog2p GTP-binding motifs, we took advantage of the functional and structural data available on known GTPases. In the Ras family, some of the most studied residues (Seeburg *et al*., 1984; Feig and Cooper, 1988) correspond in Nog2p to Pro324, which we changed to a valine, and Ser329, which we substituted by an asparagine (Figure 9A, arrows). Figure 9C shows that, in strain LMA159, the depletion of the chromosome-encoded Nog2p upon shift to glucose could be complemented by the wild type but not by the mutant genes expressed from a plasmid.

Discussion

Nog2p has previously been predicted to be involved in mRNA splicing (Schwikowski *et al*., 2000) based upon two-hybrid results (Fromont-Racine *et al*., 1997). Here we show that Nog2p is in fact a component of nuclear complexes involved in rRNA maturation. This conclusion is based on the observations discussed below.

Nature of the Nog2p-associated complexes

We observed that Nog2p was associated with particles sedimenting in the 60S region of the gradient but not with 40S subunits, 80S ribosomes or the polysomes (Figure 3). Characterization of the Nog2p-containing complexes revealed that they are highly enriched in the 27S and 7S intermediates and contain a number of components of the large ribosomal subunit. Strikingly, Rpp0p and Rpl12p are not visible and, at least, not stoichiometrically present in the Nog2p-associated complexes (Figure 6A). This is consistent with the proposal that Rpp0p associates with the ribosomes only in the cytoplasm (Santos and Ballesta, 1994) and that Rpl12p associates late with the pre-ribosomes (Kruiswijk *et al*., 1978). In contrast, we found the Rpl10p ribosomal protein in the Nog2p-associated complex. Rpl10p was initially described as a protein that associates with the 60S subunit in the cytoplasm to promote its association with the 40S subunit (Eisinger *et al*., 1997). However, a mutation in *RPL10* was recently found to determine 60S nuclear export defects, probably as a result of Rpl10p being the ribosome anchor site for the 60S export factor Nmd3p (Gadal *et al*., 2001). It was thus proposed that Rpl10p is a shuttling ribosomal protein involved in late 60S assembly steps and in its nuclear export. Our finding of Rpl10p as a component of a nuclear complex involved in the late steps of 60S maturation is thus fully consistent with this report.

In addition to 60S ribosomal proteins, we identified in the Nog2p-containing complexes two additional proteins of unknown function, Nog1p and Rlp24p (Ylr009wp). Nog1p is an essential, nuclear/nucleolar, putative GTP-binding protein (Rout *et al*., 2000; Park *et al*., 2001). The

human and trypanosomal Nog1p homologues are also nuclear/nucleolar (Park *et al*., 2001). Rlp24p belongs to the archaeal and eukaryotic Rpl24e family of ribosomal proteins and is similar to the yeast cytoplasmic ribosomal protein Rpl24p. Previous studies have shown that yeast Rpl24p is likely to associate with the large ribosomal subunit only after export from the nucleus to the cytoplasm (Kruiswijk *et al*., 1978). Moreover, disruption of both copies of the *RPL24* gene has little effect on the growth rate and apparently functional ribosomes may be assembled in the absence of Rpl24p (Baronas-Lowell and Warner, 1990). In contrast, disruption of the single copy of *RLP24* is lethal (Winzeler *et al*., 1999). It is thus an appealing hypothesis that Rlp24p is the conserved nuclear counterpart of the cytoplasmic ribosomal protein Rpl24p with an essential function in the biogenesis of the large subunit and that Rlp24p and Rpl24p successively occupy the same site of the rRNA.

Whereas Nog1p and Rlp24p were purified when Nog2p was used as bait in the affinity purification, we could not identify Nog2p in the complexes purified using tagged versions of Nog1p or Rlp24p. In addition, the complexes purified with Nog1-TAP and Rlp24-TAP were significantly more abundant than the complex purified with Nog2-TAP. These results suggest that either Nog2p is associated more weakly with the complex than Nog1p or Rlp24p or that Nog2p-associated complexes only represent a fraction of the complexes involving Nog1p and Rlp24p.

Role of Nog2p in rRNA maturation

The most obvious phenotype observed upon depletion of Nog2p was the accumulation of the pre-rRNA maturation intermediates $27SB_S$ and $7S_S$. Strikingly, the 27SB and 7S molecules were the intermediates found highly enriched within the Nog2p-associated complexes. This implicates Nog2p in two different steps of 60S maturation. The first one consists of the endonucleolytic cleavage at site C2, followed by a 5′ to 3′ degradation by exonucleases Rat1p and Xrn1p, to give rise to the 7S and the 25S rRNAs (Geerlings *et al*., 2000). The nuclease responsible for cleavage at site C2 has not yet been identified and few details are available for this step. The second step consists of the 3′ to 5′ degradation of the 7S precursors, which involves the exosome and gives rise to the mature 5.8S species (Allmang *et al*., 1999). The involvement of Nog2p in two different steps of a different catalytic nature suggests that Nog2p is not involved directly in catalysis. Nog2p could rather be a protein required, for example, for the structure of the complex, for conformational changes or for the control of the coupling between different processing steps. A similar 27SB and 7S pre-rRNA accumulation phenotype has been observed in cells depleted for the pseudouridine synthase, Cbf5p (Lafontaine *et al*., 1998). This was interpreted by the authors as a possible consequence of an alteration in the folding of the pre-rRNA or changes in the structure of the pre-ribosomal particles.

It is surprising to observe that the depletion of Nog2p led to the specific accumulation of only the short forms of the 27SB and 7S species. Indeed, the 27SB and 7S intermediates are present in both the major and the minor pathways. In addition, we observed a similar time course of depletion of both $5.8S_S$ and $5.8S_L$ upon *NOG2* repression (see Figure 5C). A similar situation has been observed for a *rlp7* mutant. This mutant impairs the synthesis of both the $5.8S_S$ and $5.8S_L$ products while only $27SA_3$ pre-rRNA, an intermediate specific for the major pathway, was found to accumulate (Dunbar *et al*., 2000). A possible explanation for these observations could be that the intermediates of the minor pathway are unstable and cannot accumulate. Alternatively, Nog2p depletion might affect specifically the major and not the minor pathway, but the feedback effects exerted on early steps, including transcription, could be more important quantitatively for the steady-state amounts of mature products. Indeed, the amounts of $27SB_S$ and $7S_S$ intermediates, which accumulate upon Nog2p depletion, remain comparatively small when compared with the amount of mature rRNAs in the wild type. Thus, specific inhibition of the major versus the minor pathway could, *in fine*, have marginal effects on the levels of the short or long forms of the 5.8S RNAs whose depletion would result mostly from the inhibition of early steps. At this point, it is thus difficult to conclude whether Nog2p or Rlp7p depletion specifically affects the major pathway or both pathways of $27SA_2$ processing.

Possible role of Nog2p in coupling 60S maturation and its nuclear export

Our data indicated that Nog2p is associated with a nuclear/nucleolar pre-60S particle. Nog2–GFP is located predominantly in the nucleoplasm and the nucleolus but is not detected in the cytoplasm (Figure 7). The pre-ribosomes formed in the absence of Nog2p are released from the nucleolus, but their export from the nucleoplasm is blocked (Figure 8). As shown by *in situ* hybridization and electron microscopy, and confirmed with an Rpl25–GFP fusion protein, depletion of Nog2p results in the accumulation in the nucleoplasm of pre-ribosomal particles containing 27S and/or 7S rRNAs. To our knowledge, the *in situ* hybridization data presented here, obtained with a probe complementary to ITS2, are the first to show directly the release of pre-25S rRNAs from the nucleolus. Similar results were observed in strains deficient in ribosome biogenesis, including mutants of *Gar1* and *Nop1* (P.-E.Gleizes, J.Noaillac-Depeyre, I.Léger-Silvestre and N.Gas, unpublished results). Thus, complete maturation of the precursors for the rRNAs of the large subunit does not constitute a prerequisite for their exit from the nucleolus. One may speculate that the last processing steps of these precursors take place in the nucleoplasm, as already suggested for some late steps of the assembly of the pre-60S particle (Kressler *et al*., 1999a). Nevertheless, the low amount of ITS2 detected in the nucleoplasm of wild-type cells indicates that, if the last steps of pre-rRNA processing occur in the nucleoplasm, they must do so rapidly after exit from the nucleolus. In addition, there must exist a mechanism controlling the proper degree of maturation of the 60S subunits before their transfer to the cytoplasm. Because we were not able to uncouple 60S nuclear export defects from rRNA maturation defects, we cannot prove that Nog2p is directly involved in the regulation of 60S nuclear export. Nevertheless, we note that Nog2p has many of the characteristics expected for a factor directly involved in this process: (i) in Nog2p-depleted cells, the nuclear

export of 60S particles is blocked; (ii) Nog2p is involved in the very last steps of nuclear rRNA maturation; (iii) a fraction of the Nog2p complexes was found associated with mature 5.8S and 25S rRNA; (iv) Nog2p is not found exclusively in the nucleolus but also occurs in the nucleoplasm; (v) Nog2p is found in a highly enriched nuclear pore complex fraction (Rout *et al*., 2000); and (vi) Nog2p is a putative GTPase. Indeed, cycles of GTP binding and hydrolysis regulate a number of fundamental cellular processes, and many essential GTPases have been described to date. We report here the presence of two putative GTPases tightly associated with pre-60S particles. The intimate mechanisms by which these proteins are essential during ribosome biogenesis remain to be described.

Materials and methods

Strains, plasmids and growth media

The genotypes of strains used in this study are listed in Table I. All the strains were obtained by homologous recombination using PCR products to transform the BMA64 or MGD353-13D strains (Baudin *et al*., 1993). PCR fragments used to generate the various strains were synthesized from either the plasmids of Longtine *et al*. (1998) or pBS1479 plasmid (Rigaut *et al*., 1999) using oligonucleotides designed as advised by the authors. Expression of the fusion proteins was checked by immunoblotting or by fluorescence microscopy. Yeast cells were grown either in standard rich medium or synthetic minimal medium containing either 2% glucose or galactose. For L-[methyl-^{3}H]methionine labelling, methionine was absent from the synthetic medium.

The mutated Nog2 constructs were produced as follows. The *NOG2* cDNA fragment from plasmid pASΔΔ/Ynr053c (Fromont-Racine *et al*., 1997) was cloned into a modified pGEX4-T vector (Stratagene) and then transferred into pCM190 (Gari *et al*., 1997). Mutant versions of this plasmid were obtained by subcloning PCR fragments between the *Cla*I and *Bgl*II restriction sites of *NOG2*. All sequences deriving from PCR amplification were entirely sequenced in the final constructs. The cDNA sequences are flanked by 5′ *Bam*HI and *Nco*I sites (containing ATG) and by 3′ *Sal*I, *Xho*I and *Not*I sites. The CCA to GTA (P324V) and the TCG to AAT (S329N) mutations were generated by subcloning PCR fragments generated with oligonucleotides MFR407, MFR400, MFR398 and MFR399, and oligonucleotides MFR407, MFR403, MFR398 and MFR402, respectively (Table II).

RNA extraction, northern blotting and primer extension

Total RNAs were extracted using glass beads and a phenol extraction procedure following standard protocols and RNA samples were separated on a 1% agarose–6% formaldehyde gel. To perform the analysis of the 5.8S rRNA, RNAs were separated on a 5% acrylamide–urea gel, transferred by electroblotting on Hybond N$^+$ membrane and probed with various ^{32}P-labelled oligonucleotides, which are listed in Table II. For primer extension experiments, 0.2 pmol of ^{32}P-labelled oligonucleotides complementary to specific regions of intermediate rRNA were mixed with 2.5 μg of total RNA in 7 μl of buffer. Extension was carried out using Superscript II reverse transcriptase (Gibco-BRL) for 30 min at 42°C in 1× buffer, 10 mM dithiothreitol, 0.5 μg actinomycin D and dNTPs (0.5 mM each). The reaction was stopped by addition of formamide-containing buffer.

Pulse–chase analysis

Cultures of 50 ml grown to an OD_{600} of 1 either in galactose or shifted to glucose-containing medium for 16 h were collected. Cells were resuspended in 6 ml of medium, labelled for 2 min with 250 μCi of L-[methyl-^{3}H]methionine and chased with cold methionine at 0.25 μg/ml. At various times, 1 ml of cells was collected after centrifugation for 15 s and the pellets were quickly frozen in a dry ice–ethanol bath. Total RNA was extracted and separated on a 1% agarose–6% formaldehyde gel. The RNAs were transferred on a Hybond N$^+$ membrane that was exposed to a BioMax film with a Transcreen LE intensifying screen (Kodak).

Polysome analysis

Monosomes and polyribosomes were analysed as described by Foiani *et al*. (1991). For each analysis, 150 ml of culture grown to an OD_{600} of 1 either on galactose or shifted to glucose-containing medium were collected. Lysates were obtained by glass bead vortexing and stored at –80°C until use. Eight to 12 A_{260} units of lysate were loaded on a 10–50% (w/v) sucrose gradient and centrifuged at 39 000 r.p.m. for 2 h 45 min at 4°C in an SW41-Ti rotor. Gradients were recovered using a Buchler Isodensiflow II fractionator and the 254 nm absorbance was measured. For immunodetection, the proteins were precipitated with trichloroacetic acid dissolved in SDS-containing sample buffer, separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes. A 1:5000 or 1:10 000 dilution of peroxidase–anti-peroxidase-soluble complexes (Sigma) allowed the detection of the protein A tag. Visualization of the peroxidase activity was performed with the ECL+ chemiluminescence kit (Amersham Pharmacia Biotech). The tagged Nog2p, Nog1p or Rlp24p was revealed by the same procedure.

Purification of complexes

Complexes were purified according to Rigaut *et al*. (1999) using 2–6 l of yeast culture. After two successive affinity purifications using an IgG–Sepharose column (Amersham Pharmacia Biotech) followed by an immobilized calmodulin column (Stratagene), the purified proteins were precipitated by the chloroform–methanol method (Wessel and Flugge, 1984), dissolved in SDS-containing sample buffer and separated on 5–20% gradient polyacrylamide gels. The proteins were visualized by colloidal Coomassie Blue staining (Neuhoff *et al*., 1988).

A rapid, partial purification of Nog2p- and Rpl10p-associated complexes for RNA analysis was performed identically to the first step of TAP purification in the presence of 1 U/μl recombinant RNasin (Promega). The RNAs were extracted directly from the IgG–Sepharose beads with phenol–chloroform.

Protein identification by mass spectrometry

Proteins were digested in-gel using bovine trypsin (Roche Biochemicals) and the peptides were used for mass spectrometric identification by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) and nanospray MS–MS tandem mass spectrometry as described (Saveanu *et al*., 2001).

Fluorescence microscopy

Cells transformed with a plasmid encoding an Rpl25–GFP fusion protein were cultured as indicated and fixed with 4% paraformaldehyde in the culture medium. The cells were mounted on glass slides in moviol containing 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) and observed with an epifluorescence microscope. Nog2–GFP cells were collected by centrifugation, washed once with deionized water and spread on the surface of a glass slide. After 1 min of drying at 65°C, 10 μl of 50% glycerol containing 1 μg/ml Hoechst 33342 (Molecular Probes) were added and the cells were observed using the appropriate fluorescence filters.

Electron microscopy in situ hybridization

To generate a probe against the 25S rRNA, an rDNA fragment encompassing a large portion of the 25S sequence (fragment 3378–6471 of *Saccharomyces cerevisiae* ribosomal transcribed unit) was inserted into pGEM4, and the resulting plasmid was nick-translated in the presence of dUTP-biotin (Roche Diagnostics). In addition, an antisense riboprobe complementary to ITS2 was made by *in vitro* transcription of the entire ITS2 reverse sequence (subcloned into pGEM4) with the Sp6 polymerase in the presence of UTP-digoxigenin (Roche Diagnostics). Processing of cells for electron microscopy and *in situ* hybridization on ultrathin sections were performed as previously described (Leger-Silvestre *et al*., 1997).

Sequence alignment

Nog2p-related sequences were retrieved from sequence databases using the FASTA3 and BLAST2 algorithms. The GTP-binding domain motifs G1, G3 and G4 (Bourne *et al*., 1991) were matched to the Nog2p sequence by using their minimal consensus sequences GxxxxGKS/T, DxxG and NxxD. To detect the G2 and G5 regions, we used multiple sequence alignments of Nog2p and its homologues obtained with ClustalX (Thompson *et al*., 1997) and secondary structure predictions obtained with the Pred2ary software (Chandonia and Karplus, 1999).

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