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Nsa2 Is an Unstable, Conserved Factor Required for the Maturation of 27 SB Pre-rRNAs*

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In *Saccharomyces cerevisiae*, a large variety of pre-ribosomal factors have been identified recently, a number of which are still of unknown function. The essential pre-ribosomal 30-kDa protein, Nsa2, was characterized as one of the most conserved proteins from yeast to human. We show here that the expression of the human orthologue *TINP1* complements the repression of *NSA2* in yeast. Nsa2 was co-purified in several pre-ribosomal complexes and found to be essential for the large ribosomal subunit biogenesis. Like several other factors of the pre-60 S particles, the absence of Nsa2 correlated with a decrease in the 25 S and 5.8 S ribosomal RNA levels, and with an accumulation of 27 SB pre-ribosomal RNA intermediates. We show that Nsa2 is a functional partner of the putative GTPase Nog1. In the absence of Nsa2, Nog1 was still able to associate with pre-ribosomal complexes blocked in maturation. In contrast, in the absence of Nog1, Nsa2 disappeared from pre-60 S complexes. Indeed, when ribosome biogenesis was blocked upstream of Nsa2, this short half-lived protein was largely depleted, suggesting that its cellular levels are tightly regulated.

Ribosome biogenesis is a highly conserved process among eukaryotes and results in the synthesis of functional small and large ribosomal subunits, necessary for the translation of mRNAs into proteins in the cytoplasm. This essential process is tightly regulated; indeed, in exponentially growing *Saccharomyces cerevisiae* cells, it accounts for about 60% of the metabolic effort (1), whereas it is almost completely turned off during the stationary phase.

The pathway begins with the transcription by RNA polymerase I of a 35 S ribosomal RNA (rRNA) precursor and of the 5 S rRNA by RNA polymerase III. This transcription, together with the nuclear import of ribosomal proteins, pre-ribosomal factors, and small nucleolar RNAs, is responsible for the self-assembly of the nucleolus (reviewed in Ref. 2), a region of the nucleus specialized in the production of ribosomes. Association of ribosomal proteins and pre-ribosomal factors with nascent pre-rRNAs gives birth to a 90 S pre-ribosomal complex, which undergoes various steps of maturation, first in the nucleolus, then in the nucleoplasm, and finally in the cytoplasm after export through the pores of the nuclear envelope (for a review of the whole pathway, see Refs. 3–5). Along this maturation, the 90 S complex separates into a pre-60 S complex, which will generate the large ribosomal subunit containing mature 25 S, 5.8 S, and 5 S rRNAs, and a pre-40 S complex, which will generate the small ribosomal subunit containing 18 S rRNA. A large number of factors are necessary for the correct modification, cleavage, and processing of pre-rRNAs, the positioning of ribosomal proteins, and the export of the pre-60 S and pre-40 S particles toward the cytoplasm.

More than 100 factors associated with pre-60 S complexes (pre-60 S factors) have been identified to date, a number of which remain to be characterized (4, 6–9). Some of them display obvious enzymatic functions (RNases, helicases, methylases, etc.). Others are annotated as putative enzymes based on sequence similarities, but their exact role in the biogenesis is still unclear. Among these factors, a set of putative GTPases is required at various steps of the maturation of the large subunit, namely nuclear-nucleolar Nog1, Nog2, Nug1, and cytoplasmic Lsg1 (10–12). As putative GTPases, these proteins are believed to participate in the control of biogenesis; they may play a part as proofreaders of the correct maturation of pre-ribosomal particles at a precise step. In a previous work, we demonstrated that the physical and functional partners Nog1 and Rlp24 are required for adequate processing of the internal transcribed spacer 2 (ITS2)[3] in 27 S pre-rRNAs, located between the 5.8 S and 25 S mature rRNAs (13).

Here, we have focused on a partner of Nog1, the essential pre-60 S factor, Nsa2 (for Nop seven-associated 2), which was found associated with pre-60 S complexes in tandem affinity purifications (6, 7, 13, 14). It contains an S8 domain, named after the Rps8 archaeal and eukaryotic ribosomal proteins, which could be involved in protein-RNA interactions. This factor appears to be one of the most conserved proteins in the eukaryotic kingdom (15). Sequence conservation correlates with function conservation, as we show here that the human orthologue *TINP1* is able to complement the repression of *NSA2* in yeast.

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[3] The abbreviations used are: ITS2, internal transcribed spacer 2; 3-AT, 3-aminotriazol; G6PDH, glucose-6-phosphate dehydrogenase; ORF, open reading frame; TAP, tandem affinity purification; TEV, nuclear inclusion A protein of tobacco etch virus; YPD, yeast extract-peptone-D-glucose; snRNA, small nuclear RNA.

TABLE 1
Yeast strains used in this study

Strain	Genotype	Ref./source
MGD353-13D	*MATa, trp1-289, ura3-52, ade2, leu2-3,-112, arg4*	19
BY4742	*MATα, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0*	17
BMA64	*MATa/α, ura3-1/ura3-1, trp1Δ1/trp1Δ1, ade2-1/ade2-1, his3-11,-15/his3-11,-15, leu2-3,-112/leu2-3,-112*	23
SC0935	*MATa, trp1-289, ura3-52, ade2, leu2-3,-112, arg4, NSA2-TAP:URA*	6
LMA158	*MATa, trp1-289, ura3-52, ade2, leu2-3,-112, arg4, NOG1-TAP:TRP1*	11
LMA240	*MATa, ura3-1, trp1Δ1, ade2-1, his3-11,-15, leu2-3,-112, Ypl093wΔ::KanMX6* [pFL36-*NOG1*]	This work
LMA241	*MATα, ura3-1, trp1Δ1, ade2-1, his3-11,-15, leu2-3,-112, Ypl093wΔ::KanMX6* [pFL36-*nog1-2*]	This work
LMA242	*MATα, ura3-1, trp1Δ1, ade2-1, his3-11,-15, leu2-3,-112, Ypl093wΔ::KanMX6* [pFL36-*nog1-3*]	This work
LMA244	*MATa, ura3-1, trp1Δ1, ade2-1, his3-11,-15, leu2-3,-112, Ypl093wΔ::KanMX6* [pFL36-*nog1-4*]	This work
LMA245	*MATα, ura3-1, trp1Δ1, ade2-1, his3-11,-15, leu2-3,-112, Ypl093wΔ::KanMX6* [pFL36-*nog1-5*]	This work
LMA271	*MATa, trp1-289, ura3-52, ade2, leu2-3,-112, arg4, KanMX6:*P_{GAL1}*-NSA2*	This work
LMA272	*MATa, trp1-289, ura3-52, ade2, leu2-3,-112, arg4, KanMX6:*P_{GAL1}*-NSA2, NOG1-TAP:TRP1*	This work
LMA275	*MATa, trp1-289, ura3-52, ade2, leu2-3,-112, arg4, KanMX6:*P_{GAL1}*-NOG1, NSA2-TAPI:TRP1*	This work
LMA276	*MATa, trp1-289, ura3-52, ade2, leu2-3,-112, arg4, KanMX6:*P_{GAL1}*-RLP24, NSA2-TAP:TRP1*	This work
LMA277	*MATa, trp1-289, ura3-52, ade2, leu2-3,-112, arg4, KanMX6:*P_{GAL1}*-SSF1, SSF2Δ::NAT, NSA2-TAP:TRP1*	This work
LMA344	*MATa, trp1-289, ura3-52, ade2, leu2-3,-112, arg4, KanMX6:*P_{GAL1}*-NOG2, NSA2-TAP:URA3*	This work
LMA392	*MATα, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0, NSA2-TAP:His3MX6*	This work
LMA393	*MATα, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0, NOG1-TAP:His3MX6*	This work
LMA402	*MATα, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0, KanMX6:*P_{GAL1}*-NOG1, NSA2-TAP:His3MX6*	This work
LMA403	*MATα, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0, KanMX6:*P_{GAL1}*-NOG1-TAP:His3MX6*	This work
LMA404	*MATα, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0, KanMX6:*P_{GAL1}*-NSA2-TAP:His3MX6*	This work
LMA405	*MATα, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0, KanMX6:*P_{GAL1}*-NSA2, NOG1-TAP:His3MX6*	This work

The absence of Nsa2 results in impaired 60 S levels in yeast cells (14). We show in this work that this protein is required for the progression of the pre-60 S complexes through the ITS2 cleavage and processing steps. Additionally, the protein levels of Nsa2 appear to be tightly regulated and depend on proper 60 S biogenesis upstream in the metabolic pathway.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Oligonucleotides—The yeast strains used in the present study are listed in Table 1. Chromosomal deletions or gene fusions were generated by homologous recombination using PCR products to transform MGD353-13D, BMA64, or BY4742 strains (16). Details concerning these constructs may be provided upon request. Disrupted strains in the BY4742 background, with the KanMX4 marker, came from the Euroscarf collection of deletion strains (17). Plasmids for two-hybrid assays were obtained by Gateway cloning in pAS2ΔΔ and pACTIIst destination vectors. Thermosensitive alleles of *NOG1* (*nog1ts*) were generated by mutagenic PCR (18). They were expressed in the centromeric vector pFL36cII under control of the natural *NOG1* promoter and terminator sequences. The plasmid pFL46sII-P_{NSA2}-*TINP1* was obtained by cloning the complete *TINP1* open reading frame (ORF) from the *TINP1* cDNA in the multicopy plasmid pFL46sII, under control of the natural *NSA2* promoter and terminator sequences. The sequence of oligonucleotides used for Northern hybridization and primer extension analyses were as described previously (13).

High-copy-number Suppressor Genetic Screen—The LMA241, -242, -244, and -245 (*nog1ts*) strains were transformed with a yeast genomic high-copy-number vector library constructed in pFL44L. The transformants were grown on solid synthetic minimal medium lacking uracil at 37 °C. Colonies that had lost their thermosensitive phenotype when compared with the same strains transformed with an empty vector were selected. Plasmidic DNA was recovered and DNA inserts were sequenced. The suppressor plasmids were checked by retransformation of the *nog1ts* and the corresponding wild type LMA240 strains. Their growth phenotypes were compared by spotting transformants in 10^{-1} dilution steps on minimal medium without uracil at 25, 30, and 37 °C.

Matrix Two-hybrid Test—The strain CG1945, transformed with pAS2ΔΔ-*NOG1*, *NSA2*, or *RLP24* baits, was mated with the strain Y187 transformed with the same genes cloned in pACTIIst. Diploids were spotted on minimal medium without leucine and tryptophan (−LW) and then replicated on minimal medium without leucine, tryptophan, and histidine (−LWH) for the selection of positive clones. The strength of the interactions was estimated by replicating on −LWH medium containing 1, 5, 10, or 25 mM 3-aminotriazol (3-AT).

RNA Extraction, Northern Blotting, and Primer Extension—Cells were broken with glass beads, and total RNAs were subjected to phenol-chloroform extraction. RNAs were resolved on 6% polyacrylamide-urea gels or on 1% agarose gels and then transferred to Hybond-N+ membranes and probed with various ^{32}P-labeled oligonucleotides or with random PCR probes complementary to specific mRNAs. Primer extensions were performed with ^{32}P-labeled oligonucleotides, and the products were then resolved on 5% polyacrylamide-urea gels. Quantifications were performed with ImageQuant software.

Sucrose Gradient and Protein Analysis—Total protein extracts were prepared from exponentially growing yeast cells as described previously (11) and separated on 10–50% sucrose gradients by centrifugation for 3 h at 190,000 × *g* in a SW41 rotor (Beckman). In each fraction of the gradient, the proteins were precipitated with 10% trichloroacetic acid, separated on 10% polyacrylamide-SDS gels, and transferred to nitrocellulose membranes. Tandem affinity purification (TAP)-tagged proteins were detected with a 1:10,000 dilution of the peroxidase-anti-peroxidase-soluble complex (Sigma). Native proteins were detected by indirect immunoblotting, using as primary antibodies specific polyclonal rabbit antibodies at 1:2,000 to 1:10,000 dilutions. Anti-Nog1, anti-Rlp24, and anti-Nog2 antibodies were as described by Saveanu *et al.* (13). Anti-Nsa2 antibodies were produced by immunization of rabbits with

recombinant GST-Nsa2 fusion protein produced in *Escherichia coli* BL21. Anti-Arx1 and anti-Alb1 antibodies were produced by immunization of rabbits with specific immunogenic peptides and were affinity-purified (Covalab). Antibodies against Nop7, Rpl1, and Tif6 were obtained from J. L. Woolford (Dept. of Biological Sciences, Carnegie Mellon University, Pittsburgh), F. Lacroute (Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France), and F. Fasiolo (Institut de Biologie Moléculaire et Cellulaire, CNRS, Strasbourg, France), respectively. Rabbit anti-glucose-6-phosphate Dehydrogenase (G6PDH) antibody was from Sigma.

Secondary antibodies (goat anti-rabbit-horseradish peroxidase conjugate from Bio-Rad) were used at a 1:10,000 dilution. Visualization of the peroxidase activity was performed with the ECL+ chemiluminescence kit (Amersham Biosciences).

Tandem Affinity Purification of Complexes—Protein complexes associated with Nog1-TAP were purified according to the standard TAP protocol (19), starting from 4 liters of yeast culture. The purified protein samples were separated by SDS-PAGE and analyzed by Western blot as described above. For the analysis of Nsa2-associated RNAs, only the first step of the TAP protocol was performed, with a lysis buffer containing 20 mM vanadyl ribonucleoside complex (New England Biolabs) and a TEV protease digestion buffer containing 0.1 unit/μl recombinant RNasin (Promega). The RNAs contained in the TEV eluates were extracted twice with phenol-chloroform, precipitated, and then analyzed by primer extension or Northern blot as described above.

Glucose Repression of Specific Genes—Strains expressing various pre-60 S genes under the control of a galactose-inducible promoter were shifted from galactose- to glucose-containing rich medium. The absorbance at 600 nm was measured for each mutant strain and the corresponding wild type strain over a 24-h time course. Measurements were standardized to $A_{600\ nm}$ at time 0. Total proteins or RNAs were extracted from these strains along the time course and consequently analyzed by Western blot or Northern blot as described above.

RESULTS

Nog1 and Nsa2 Are Functionally and Physically Linked—Nog1 is a putative GTPase involved in the biogenesis of the 60 S ribosomal subunit. In a previous work (13), we showed that Nog1 is required, together with Rlp24, for adequate cleavage of ITS2 in the rRNA maturation pathway, suggesting that it might be a regulator of the progression through this step.

To get more insights into the function of Nog1 in this process, we searched for functional partners of this factor. Several mutants of *NOG1* were obtained by random PCR mutagenesis on the whole ORF. Conditional alleles were selected in a *nog1*Δ strain, expressing the mutant alleles on a centromeric plasmid. *nog1-2* to *nog1-5* alleles conferred a thermosensitive phenotype as well as impaired 27 SB processing when strains were grown at 37 °C (data not shown). The most severe phenotype was obtained for *nog1-5*-containing strains, which were also strongly affected for growth at 25 and 30 °C. *nog1-3*-containing strains were less affected but displayed pre-60 S export defects when grown for 2 h at 37 °C (data not shown).

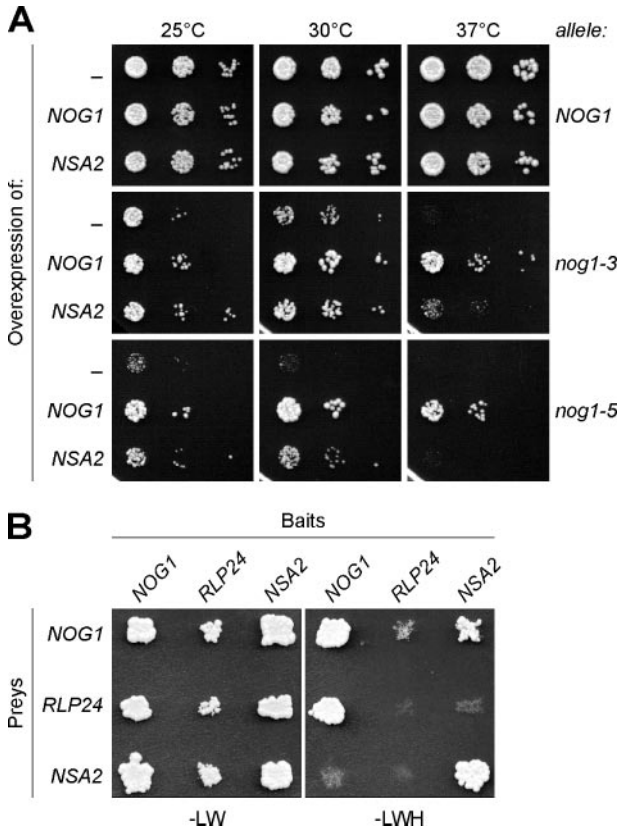

FIGURE 1. **Nog1 and Nsa2 interact genetically and physically.** *A*, *NSA2* is a high-copy-number suppressor of *nog1ts*. Wild type, *nog1-3*, or *nog1-5* mutant strains were transformed with high-copy-number plasmids (pFL44sII) carrying distinct DNA inserts (*NOG1*, *NSA2*, or no insert (−)) and plated in 10^{-1} dilution series on synthetic medium without uracil at 25, 30, or 37 °C for 2 days. *B*, Nsa2 is a two-hybrid partner of Nog1. *NOG1*, *RLP24*, and *NSA2* were cloned as complete ORFs in the two-hybrid pAS2ΔΔ and pActIIst vectors. Bait ORFs cloned in pAS2ΔΔ were transformed into the CG1945 strain; prey ORFs cloned in pActIIst were transformed into the Y187 strain. Both strains were mated, and diploids were selected on minimal medium without leucine and tryptophan (*−LW*). Then bait-prey pairs that displayed a two-hybrid interaction were selected on minimal medium without leucine, tryptophan, and histidine (*−LWH*).

We performed a high-copy-number suppressor screen with these *nog1ts* strains grown at 37 °C. In addition to *NOG1* itself, the *YER126C/NSA2* ORF was selected in eight distinct DNA inserts, seven of which also contained the adjacent ORF, *LCP5*. The smallest insert was constituted by a DNA fragment extending from 1,040 base pairs upstream of the *NSA2* initiation codon to 1,385 base pairs downstream of its stop codon. *NSA2* and *LCP5* were subcloned, along with their promoter and terminator regions, in 2 μ plasmids. *LCP5* alone had no effect on the growth of the mutant strains (data not shown). Fig. 1*A* shows the suppression of the phenotypes for *nog1-3* at 30 and 37 °C and *nog1-5* at 25 and 30 °C when either *NOG1* or *NSA2* was overexpressed. In contrast to *NOG1*, *NSA2* did not complement *nog1*Δ strains (data not shown). Hence, the genetic link between *NSA2* and *NOG1* is not due to a functional redundancy of the gene products.

Apart from this genetic link between both genes, we could identify, through a two-hybrid matrix analysis, a physical interaction between Nog1 as a prey and Nsa2 as a bait (Fig. 1*B*). This interaction supported growth of the diploid cells on minimal medium without leucine, tryptophan, or histidine containing

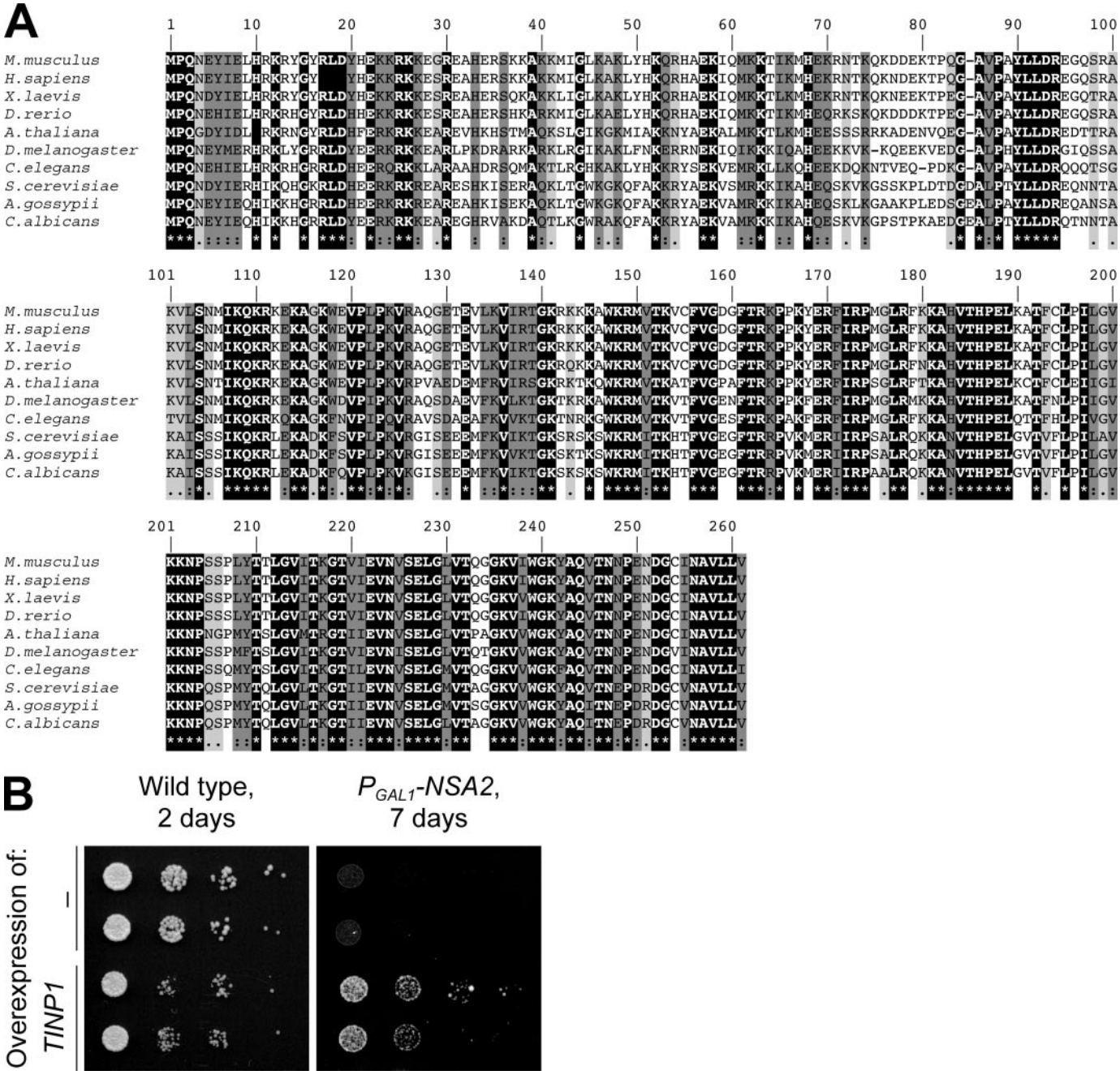

FIGURE 2. ***TINP1* is the human orthologue of *NSA2*.** *A*, Nsa2 is much conserved among eukaryotes. Sequences of Nsa2 homologues in 10 model organisms were aligned with ClustalX. The conserved residues between all species are *shaded* in *black*. The residues that are conserved between at least five species are *shaded* in *gray*. *Dark gray* indicates residues for which the chemical properties are conserved. *B*, overexpression of *TINP1* complements the absence of Nsa2. A strain expressing *NSA2* under the control of the P_{GAL1} promoter and the corresponding wild type strain were transformed with pFL46sII expressing *TINP1* under control of the *NSA2* promoter and terminator sequences or with the corresponding empty vector (−). Transformants were plated in 10^{-1} dilution steps on glucose-containing minimal medium without leucine and then incubated for 2–7 days at 30 °C.

up to 5 mM 3-AT (data not shown). Moreover, both Nog1 and Nsa2 also interacted strongly with themselves in the two-hybrid test (Fig. 1*B*); for Nog1-Nog1 or Nsa2-Nsa2 interactions, diploid cells resisted 3-AT concentrations of 25 mM (data not shown). As a negative control, no interaction was observed between the Rlp24 bait and the Nsa2 prey, whereas this bait could interact with its partner Nog1 (Fig. 1*B*); this interaction resisted 25 mM 3-AT (data not shown). A two-hybrid interaction was also observed between Nsa2 and mutants of Nog1 blocked either in a GTP- or in a GDP-bound state, as well as with a N-terminal fragment of Nog1 reduced to its first 160 amino acids, a conserved domain located upstream of the hypothetical GTP binding site of the protein (data not shown).

TinP1 Is the Orthologue of Nsa2 in Human Cells—Nsa2 (Nop-seven-associated protein 2) is a 261-amino acid-long protein, which was previously identified in pre-60 S complexes isolated by tandem affinity purification (14). This protein is highly conserved among eukaryotes; its human homologue, TinP1 (TGFβ-inducible nuclear protein 1), displays 62.1% identity and 77.4% similarity with Nsa2, which makes it one of the most conserved proteins in

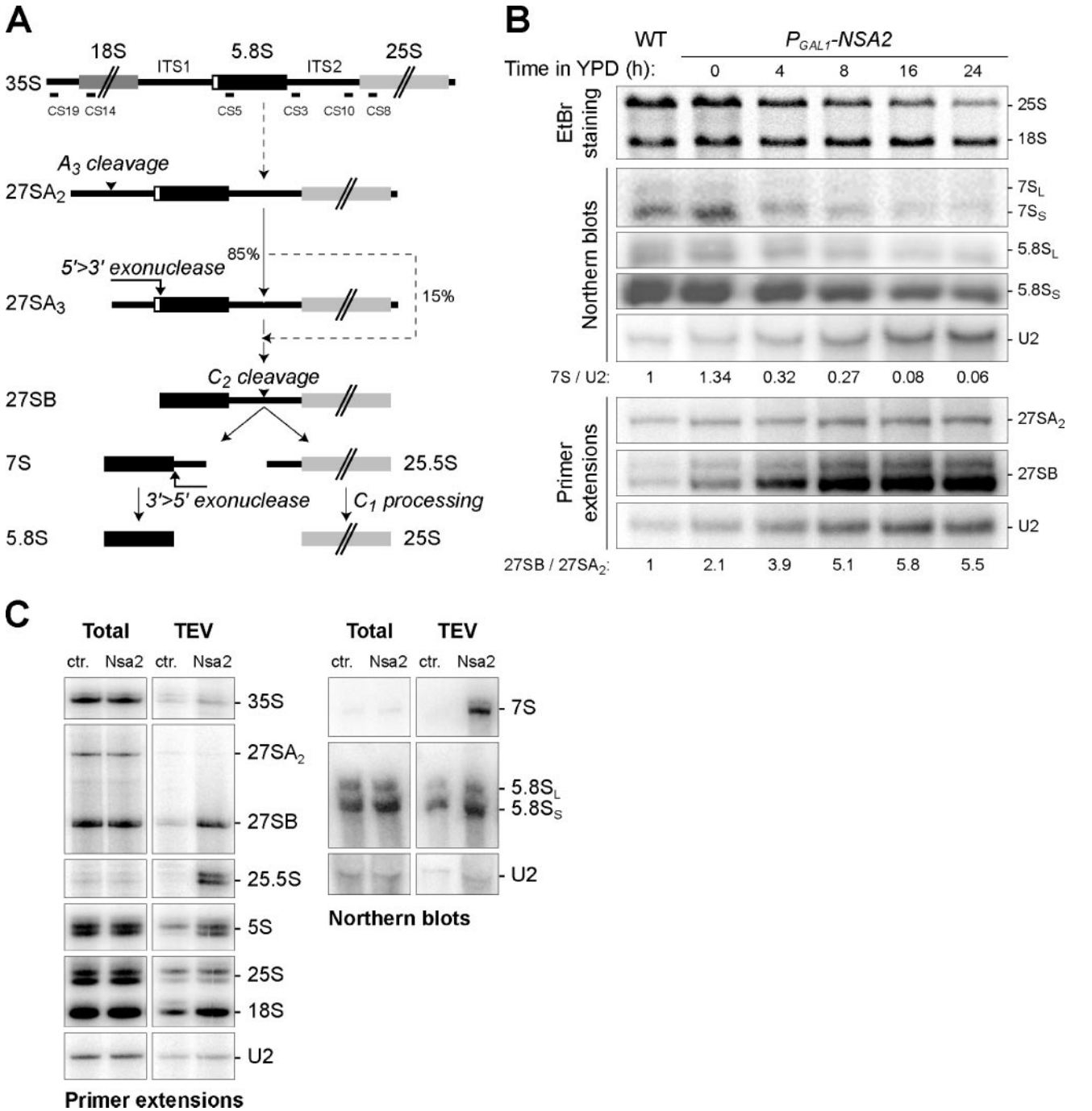

FIGURE 3. **Nsa2 is involved in the processing of ITS2.** *A*, schematic representation of the pre-rRNA processing steps involved in the formation of mature 5.8 S and 25 S rRNA in *S. cerevisiae*. The relative positions of the oligonucleotides used in this study are indicated. *B*, Nsa2 is required for adequate processing of 27 SB pre-rRNAs. Total RNAs were extracted from the wild type BY4742 strain (*WT*) grown in YPD medium, as well as of the LMA392 strain in which *NSA2* was under control of a P_{GAL1} promoter, at several time points after a shift to YPD medium. Mature rRNAs were analyzed by agarose gel electrophoresis and EtBr staining. 27 SB and 27 SA_2 intermediates were detected by primer extension with oligonucleotide CS10. 7 S and 5.8 S were analyzed by Northern blot, using the CS3 and CS5 probes, respectively. Detection of snRNA U2 was used as a loading control. The 27 SB/27 SA_2 ratio in the P_{GAL1}-*NSA2* strain was normalized to the wild type ratio, set arbitrarily as 1. *C*, Nsa2-TAP co-purifies with the substrate and products of the ITS2 cleavage. Extracts from a non-tagged or *NSA2-TAP* strains were affinity-purified on IgG-Sepharose. RNAs contained in the crude extract or in the TEV-eluted fraction were extracted and analyzed by primer extension (*left panel*) and Northern blot (*right panel*). The 35 S pre-rRNA was detected with oligonucleotide CS19 and 25 S, 18 S, and 5 S pre-rRNA with oligonucleotides CS8, CS14, and MFR422, respectively. *ctr.*, control.

the yeast proteome (15). The alignment of Nsa2 homologues in model organisms (Fig. 2*A*) illustrates this strong conservation.

We tested whether TinP1 was the orthologue of Nsa2 by complementation experiments. A 2 μ plasmid expressing *TINP1* under the control of the P_{NSA2} promoter was able to complement a strain in which *NSA2* was repressed (*i.e.* a P_{GAL1}-*NSA2* strain grown in glucose-containing medium) compared with the same strain transformed with the corresponding empty vector (Fig. 2*B*). In contrast, in a wild type strain, the overexpression of *TINP1* was slightly toxic. Our

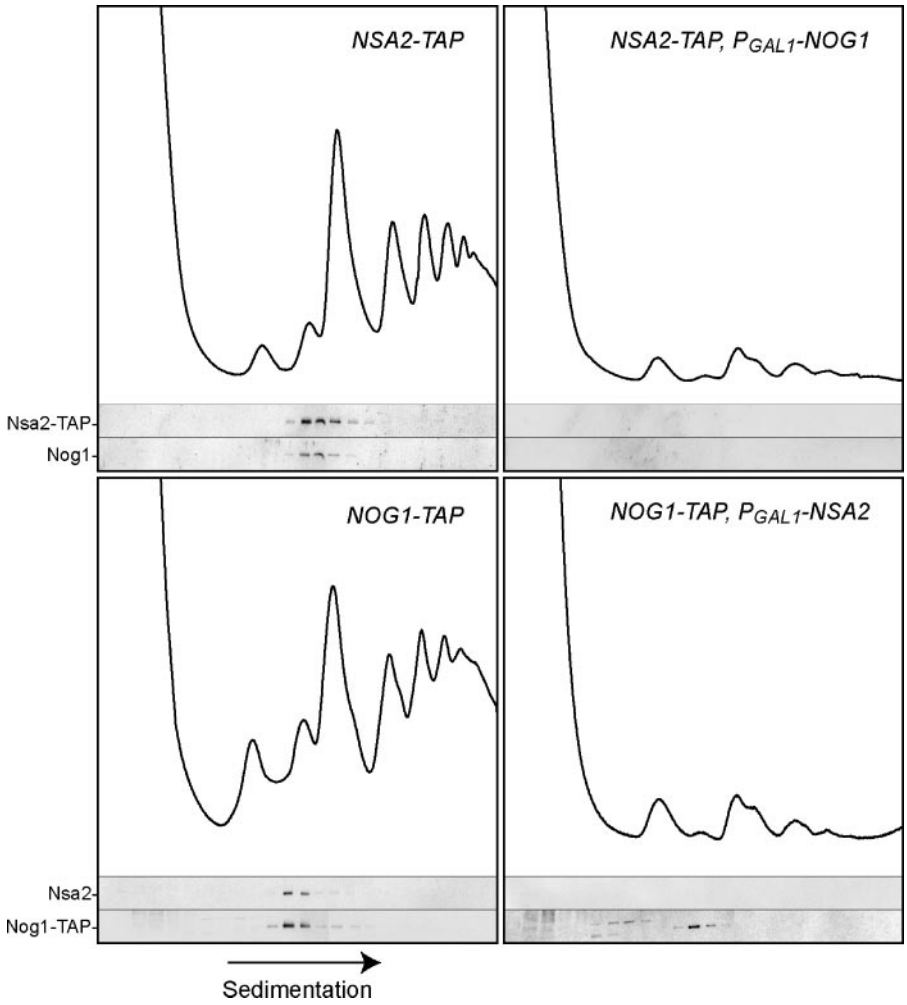

FIGURE 4. **Nog1 precedes Nsa2 in the pre-60 S assembly scenario.** Whole cell extracts were prepared from LMA392, LMA393, LMA402, and LMA405 producing Nsa2-TAP or Nog1-TAP in wild type, P_{GAL1}-*NOG1*, or P_{GAL1}-*NSA2* contexts and were grown for 16 h in YPD. Complexes were separated on sucrose gradients by ultracentrifugation. Absorbance at 254 nm was measured, and 0.5-ml fractions of the gradient were collected. Peaks corresponding to the 40 S, 60 S, 80 S particles or to the polysomes are indicated. The proteins of each fraction were precipitated with trichloroacetic acid and analyzed by Western blot, with peroxidase-anti-peroxidase complex for the detection of the TAP fusion proteins or with antibodies directed against non-tagged Nsa2 or Nog1.

data strongly suggest that Human *TINP1* is thus the orthologue of *NSA2*.

27 SB rRNA Intermediates Accumulate in the Absence of Nsa2—The functional links between Nog1 and Nsa2 suggested that both factors might be involved in the same cellular pathway. Additionally, previous work (14) had shown that Nsa2 is required for maintenance of adequate cellular 60 S ribosomal subunit amounts. Altogether, these data suggested that Nsa2 might be required for the completion of the large ribosomal subunit biogenesis. To identify this step, a strain in which NSA2 was under control of a P_{GAL1} promoter was shifted to glucose from 0 to 24 h. Mature rRNAs and pre-rRNA species were analyzed and quantified along this repression time course (Fig. 3*B*). This experiment revealed a decrease in the levels of mature 5.8 S and 25 S rRNA species in the mutant compared with the wild type strain grown in glucose-containing medium. Within the same period of time, 18 S rRNA levels were not affected.

As far as rRNA intermediates are concerned, we detected a drop in the 7 S levels (standardized to U2 snRNA), which correlated with an increase in the 27 SB/27 SA_2 ratio. We concluded that Nsa2 is required for appropriate C_1/C_2 cleavage and processing steps of ITS2 during the maturation of the large ribosomal subunit (Fig. 3*A*).

27 SB and 7 S rRNA Intermediates Accumulate in Nsa2-TAP Complexes—To confirm the involvement of Nsa2 in the processing of ITS2, we isolated rRNA intermediates contained in Nsa2-TAP-associated complexes. After affinity purification on IgG-Sepharose of either a strain expressing NSA2-TAP or a wild type, non-tagged strain as a negative control, primer extension and Northern blot analysis were performed on the TEV eluates (Fig. 3*C*). 27 SB, 25.5 S, and 7 S rRNA intermediates were strongly enriched in the Nsa2-TAP-associated complexes when compared with the non-tagged strain. Hence, both the substrate and the products of the C_2 cleavage step can be found in Nsa2-TAP-containing complexes (Fig. 3*A*). We could also detect a slight enrichment of 5 S rRNA in the complexes, consistent with this RNA polymerase III transcript being already associated with pre-60 S particles when cleavage of ITS2 occurs. In contrast, no major difference between TEV eluates was detected in the levels of the other pre-rRNAs (27 SA2, 35 S) or mature RNA polymerase I transcripts (25 S, 5.8 S, or 18 S). The observed signals, as well as the observed U2 snRNA levels, thus probably correspond to background contamination. These data indicate that Nsa2 is present in pre-60 S complexes during the ITS2 cleavage.

Nog1 Is Involved in Pre-60 S Assembly before Nsa2—To better define the biological processes in which Nog1 and Nsa2 are involved, we attempted to place them in the assembly sketch of the pre-ribosome.

We first checked that both proteins were present in macromolecular particles in sucrose gradient experiments followed by Western blot analysis (Fig. 4). Both Nsa2-TAP and Nog1-TAP could be detected in fractions corresponding to the 60 S particles. As a control, the ribosomal protein Rpl1 was found to sediment in the 60 S peak, the 80 S peak, and polysomes (data not shown).

To go further with the investigation of the assembly order, we tried to define whether one of these factors was essential for the loading of the other one on the particle. Analysis of Nsa2-TAP-associated complexes was performed in a P_{GAL1}-*NOG1* strain

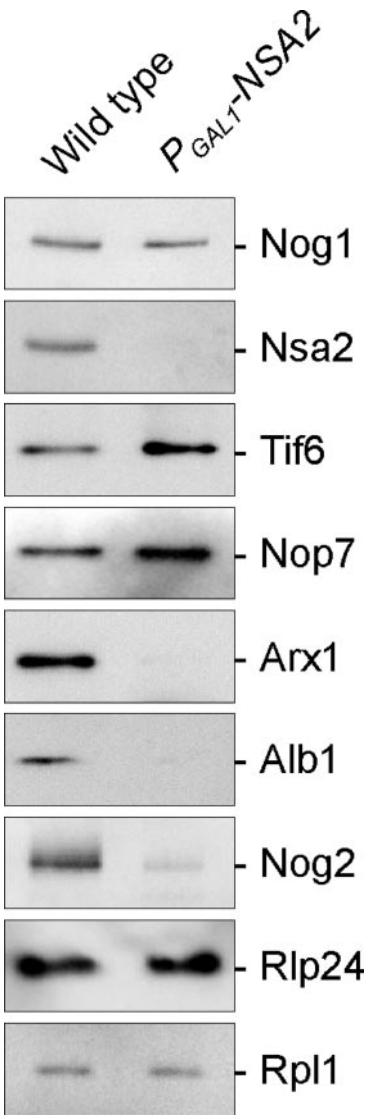

FIGURE 5. **Nog1-associated complexes in the absence of Nsa2.** Nog1-TAP-associated complexes were purified using the TAP method, either under wild type conditions or in a P_{GAL1}-*NSA2* strain shifted to glucose-containing medium for 16 h. The eluates were separated by SDS-PAGE and then analyzed by Western blot with specific antibodies.

shifted to YPD medium for 16 h in order to repress *NOG1*. Under these conditions, Nsa2 was no longer observed in the 60 S fractions and was undetectable in sucrose gradients (Fig. 4). This correlated with a complete disappearance of the Nsa2-TAP-purified complex (data not shown). Meanwhile, the ribosomal protein Rpl1 was still detected in the 60 S peak, 80 S peak, and polysomes on sucrose gradients (data not shown). Hence we conclude that Nog1 is essential for the presence of Nsa2 in pre-60 S particles.

In contrast, Nog1-TAP still sedimented with 60 S particles when *NSA2* was repressed for 16 h (Fig. 4). Hence, the absence of Nsa2 did not impair the loading of Nog1 to pre-60 S complexes. However, biogenesis of the large ribosomal subunit was blocked, because rRNA maturation was impaired (Fig. 2*B*) and a strong decrease in the 60 S levels was observed on sucrose gradients. Together, these data suggest that Nog1 is involved upstream of Nsa2 in the process of ribosome biogenesis. Because the 60 S biogenesis was blocked, we inferred that the purification of Nog1-TAP-associated complexes in absence of Nsa2 could provide a snapshot of the large ribosomal subunit at a precise step in the assembly.

Composition of the Pre-60 S Particles before the Action of Nsa2—Because Nsa2 seemed to be involved in pre-ribosomal complexes later than Nog1, we thought that we might block the biogenesis downstream of Nog1 by repressing *NSA2* and in this way enrich yeast cells in pre-ribosomal complexes characteristic of the ITS2-processing step. Nog1-TAP-associated particles were purified by the TAP method from a strain wild type for Nsa2 or a strain in which *NSA2* was repressed. Differences in composition between both complexes were assessed by immunoblotting for distinct known pre-60 S factors or proteins of the large ribosomal subunit (Fig. 5). When *NSA2* was repressed, we detected in the Nog1-TAP-co-purified complexes a slight increase in the amounts of Tif6 and Nop7 and a disappearance of Arx1, Alb1, and Nog2. Rlp24 levels appeared unchanged between the wild type and the mutant complexes. As loading controls, the Nog1 bait and the large ribosomal protein Rpl1 were found in similar amounts in both complexes. Together, these data suggest that Nsa2 effects on the large ribosomal subunit biogenesis occur after the loading of Nog1, Tif6, and Nop7 to pre-60 S particles and before the loading of Nog2, Arx1, and Alb1. Unchanged levels for Rlp24 might indicate that this factor, like Rpl1, is present in stoichiometric amounts in all Nog1-associated complexes from the beginning to the end. This is consistent with previous results showing that Rlp24 precedes Nog1 and dissociates from pre-60 S particles at late cytoplasmic steps of biogenesis (13).

Presence of Nsa2 in Yeast Cells Is Dependent upon the Presence of Pre-60 S Complexes—As noted previously, when *NOG1* was repressed, not only was Nsa2-TAP absent from the 60 S fraction of sucrose gradient, but it also could not be detected anywhere else in the gradient (Fig. 4), whereas Rpl1 remained bound to 60 S complexes. This disappearance of Nsa2-TAP in Nog1-depleted cells was also observed by immunofluorescence (data not shown). We repeated the experiment on whole cell extracts depleted for pre-ribosomal factors Nog1, Ssf1, Rlp24, and Nog2, with G6PDH used as a loading control (Fig. 6*A*).

In the absence of factors involved in the early steps of the 60 S subunit maturation (*e.g.* Ssf1, Rlp24, Nog1), Nsa2-TAP vanished from the yeast cell extracts. Conversely, in the absence of the later acting factor, Nog2, Nsa2 was still present and even tended to accumulate.

This experiment was reproduced, detecting the wild type form of Nsa2 in strains in which various known pre-60 S factors were repressed. Nsa2 levels decreased when upstream factors such as Ebp2, Mak11, Rlp24, Pop3, or Nog1 were depleted but not when downstream factors such as Nog2 or Lsg1 were depleted (data not shown).

To exclude possible effects of the long-term glucose shift, we also looked for Nsa2 in the *nog1-3* and *nog1-5* strains, grown for 2 h at 25 or 37 °C (Fig. 6*B*). Nsa2 levels were lower in extracts from the *nog1-3* thermosensitive strain at restrictive temperature than at permissive temperature. In the *nog1-5* slow growing strain, Nsa2 levels were affected at both temperatures. As a control, no change was observed in Nsa2 levels in the wild type

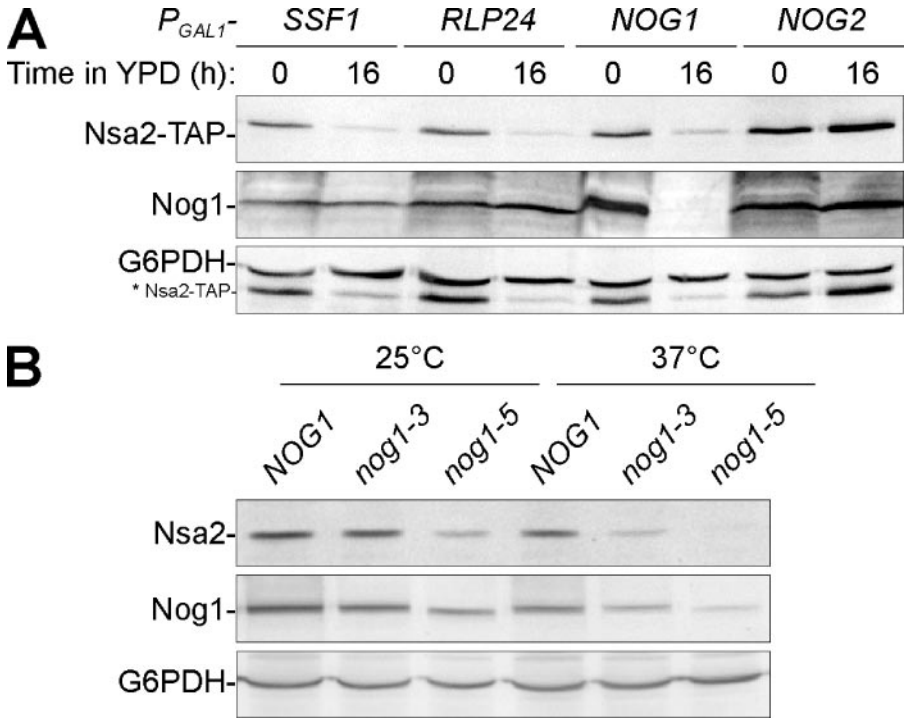

FIGURE 6. **Nsa2 is destabilized when ribosome biogenesis is blocked.** *A*, Nsa2 level decreases in cells depleted of upstream factors in the biogenesis of the large ribosomal subunit. The presence of Nsa2-TAP was assessed by Western blot on total extracts from strains expressing *SSF1*, *RLP24*, *NOG1*, or *NOG2* under the control of the P_{GAL1} promoter grown for 16 h in YPGal (yeast extract-peptone-galactose) or YPD liquid medium. As loading controls, Nog1 and G6PDH were detected with specific antibodies. The signal from Nsa2-TAP was still detectable on this blot, as indicated by an *asterisk*. *B*, Nsa2 is destabilized in *nog1ts* strains. The presence of Nsa2 or Nog1 was assessed by Western blot in wild type, *nog1-3*, or *nog1-5* strains grown for 2 h at 25 or 37 °C in YPD liquid medium. As a loading control, G6PDH were detected with specific antibodies.

strain, whatever the temperature. Hence, the above results are independent of the nature of the mutants used. Note that Nog1 was also hardly detectible with specific antibodies in the mutants, indicating that the selected mutations probably destabilize the protein at elevated temperature.

We wondered whether the observed loss of Nsa2 was because of effects at the protein or mRNA levels. In a Northern blot analysis of the strains tested above, we observed no change in the levels of *NSA2* mRNA when ribosome biogenesis was blocked (data not shown). This suggested that the Nsa2 protein itself, and not its mRNA, was destabilized.

Altogether, these results indicate that not only Nog1 is necessary for the presence of Nsa2 in pre-60 S particles, but also, when ribosome biogenesis is blocked upstream of Nsa2, this factor is no longer present in yeast cells. An efficient ribosome biogenesis upstream of the ITS2 cleavage thus seems necessary for adequate recruitment of Nsa2 to the particle, and whenever this pathway is impaired, Nsa2 is degraded. This phenomenon specifically affects Nsa2, because when either *SSF1* or *RLP24* are repressed, Nog1 can no longer anchor to pre-60 S particles (Ref. 13 and data not shown), but it is still present in equivalent amounts in the cells (Fig. 6*A*).

Nsa2 Is a Short-lived Factor—The disappearance of Nsa2 when ribosome biogenesis was blocked led us to study the stability of this protein independently of ribosomal biogenesis defects. Hence, we compared the clearance of Nsa2 after a transcriptional shut-off compared with that of other pre-60 S factors.

First, we compared the growth in liquid, glucose-containing medium for various strains in which a pre-60 S gene was under control of the P_{GAL1} promoter (Fig. 7*A*). After a shift to glucose, the P_{GAL1}-*NSA2* strain stopped growing earlier than strains depleted from other pre-60 S factors (*i.e.* Nog1, Nog2, or the very stable factor, Npa1). This is consistent with Nsa2 being a short half-lived, essential factor compared with other pre-60 S factors.

The clearance of Nsa2-TAP was followed for 16 h in a P_{GAL1}-*NSA2-TAP* strain shifted to YPD medium and compared with that of Nog1-TAP in a P_{GAL1}-*NOG1-TAP* strain. We observed a rapid clearance of Nsa2-TAP compared with that of Nog1-TAP (Fig. 7*B*). This was not due to a difference in the stability at the mRNA level, because both *NSA2-TAP* and *NOG1-TAP* mRNAs disappeared within minutes when the strains were shifted to glucose (Fig. 7*C*), whereas the proteins could still be detected for hours. The experiment was reproduced in P_{GAL1}-*NOG1* or P_{GAL1}-*NSA2* strains by detecting both proteins with specific antibodies (data not shown). The clearance profiles looked very similar under these conditions, which suggests that the observed difference between Nog1 and Nsa2 was not due to the presence of the TAP tag. We conclude that Nsa2 is naturally less stable than Nog1. A rapid degradation process could regulate levels of this factor in yeast, in correlation with the activity of ribosome biogenesis.

DISCUSSION

Nsa2 Is an Unstable Pre-60 S Factor—In this work, we describe Nsa2 as a pre-ribosomal factor required for adequate processing of the ITS2, which separates 25 S and 5.8 S rRNA. A number of pre-60 S factors, such as Nog1 or Rlp24, are necessary for this step of maturation. The key feature concerning Nsa2 is that its presence in cells appears to depend on the efficiency of ribosome biogenesis. Indeed, in mutants where the biogenesis of the large ribosomal subunit was blocked upstream of Nsa2, this protein was not detected in whole cell extracts (Fig. 6), whereas mRNA expression was not affected. This effect is not due to a destabilization of pre-60 S complexes, because they can still be purified in such mutants. Nsa2 is thus a good candidate for the coordination of regulation events affecting 60 S biogenesis at a rate-limiting step of the ITS2 processing.

Our data suggest that Nsa2 might play a part in the quality control of pre-60 S particles. Under normal, rapid growth conditions, high levels of Nsa2 would allow the progression of pre-60 S particles through the ITS2 processing; these particles would then give birth to wild type amounts of large ribosomal

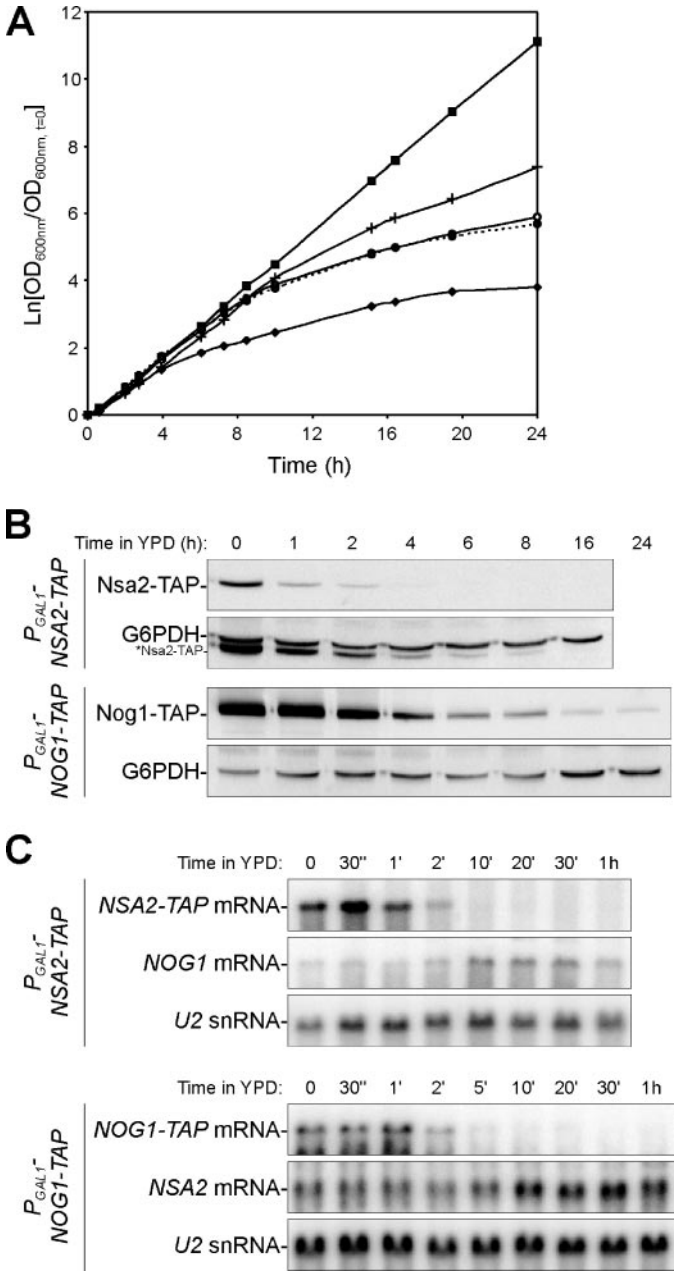

FIGURE 7. **Nsa2 is a short-lived pre-ribosomal factor.** *A*, strains in which *NSA2* was repressed stop growing within a few hours. Various strains in which a pre-60 S gene was under control of a P_{GAL1} promoter, as well as a wild type strain, were grown in glucose-containing medium for 24 h. The absorbance (optical density) at 600 nm ($OD_{600\ nm}$) was measured, normalized to the value at time 0, and drawn as the logarithmic coordinates relative to time. Strains: ■, wild type; +, P_{GAL1}-*NPA1*; ○, P_{GAL1}-*NOG1*; ●, P_{GAL1}-*NOG2*; ♦, P_{GAL1}-*NSA2*. *B*, the clearance of Nsa2-TAP is shorter than that of Nog1-TAP. The presence of Nsa2-TAP or Nog1-TAP in total extracts from P_{GAL1}-*NSA2-TAP* or P_{GAL1}-*NOG1-TAP* strains was assessed over a 16–24-h repression time course by Western blot. As a loading control, G6PDH was detected with specific antibodies. Nsa2-TAP corresponds to the detection of protein A from the TAP tag upon hybridization with G6PDH. The apparent increase of the signal in later fractions is an artifact of the successive hybridization with peroxidase-anti-peroxidase complex and G6PDH, which we consistently found to increase weaker signals relative to stronger ones and thus to equalize the observed levels. *C*, the difference observed between the clearances of Nsa2-TAP and Nog1-TAP is not due to the stability of the mRNAs. The presence of *NSA2-TAP* and *NOG1-TAP* mRNAs in total extracts from P_{GAL1}-*NSA2-TAP* and P_{GAL1}-*NOG1-TAP* strains was assessed over a 1-h repression time course by Northern blot. As loading controls, *NOG1* and *NSA2* mRNAs were detected.

subunits in the cytoplasm. When pre-60 S maturation is impaired upstream of Nsa2, a direct or indirect signal could account for a drop of Nsa2 levels. One interesting hypothesis would be that the absence of Nsa2 could block the ITS2 cleavage step, consequently preventing abnormal pre-60 S particles from being exported toward the cytoplasm. This hypothesis could explain why the absence of many pre-60 S factors results in defects in the ITS2 processing; some of these phenotypes might be an indirect consequence of the absence of upstream pre-60 S factors on Nsa2 cellular levels. It is also possible that Nsa2, as its human homologue, is involved in more physiological regulations in response to extracellular signals.

The low stability of Nsa2 in mutant conditions correlates with a short half-life of this protein in wild type cells (Fig. 7) compared with other pre-60 S factors. To date, we do not know whether this rapid turnover is inherent to the protein or involves specific degradation mechanisms. One interesting hint for this question is that the homologue of Nsa2 in *Caenorhabditis elegans* (Q9XTD3) interacts in two-hybrid with the Rpn10 subunit of the proteasome (20). Hence, when pre-60 S biogenesis is impaired, Nsa2 might be targeted to the proteasome degradation pathway.

Nsa2 Is Highly Conserved in the Eukaryotic Kingdom—Nsa2 is one of the most conserved proteins between *S. cerevisiae* and humans (15). Additionally, homologues of this protein can be found in all eukaryotic organisms sequenced thus far (Fig. 2*A*), with a high degree of similarity. We showed that expression of the human orthologue of the gene *TINP1* was able to restore growth in a strain in which Nsa2 was depleted (Fig. 2*B*). This indicates that not only the sequence but also the function of the protein was conserved during its evolution.

In higher eukaryotes, Nsa2 homologues seem to be tightly regulated. Indeed, *TINP1* stands for TGFβ-inducible Nuclear Protein 1 as its mRNA production was induced in response to stimulation with transforming growth factor β.[4] These features designate TinP1, like Nsa2 in yeast, as a good target for regulation, possibly as a limiting factor for ribosome biogenesis.

In addition to these expression features, *TINP1* is located in the human genome in the 5q13.3 region, which is compromised in patients with hairy cell leukemia (21). These authors suggest that *TINP1*, together with two other noncharacterized genes found in this region, constitute a candidate tumor suppressor gene (21). Moreover, recent evidence suggests that high levels of transforming growth factor-β are secreted by hairy cells and favor the progression of the pathology (22). An appealing hypothesis would therefore be that deregulation of *TINP1* expression could participate in the progression of the disease either because of chromosomal abnormalities or because of stimulation by transforming growth factor-β. The data provided here indicate that expression of *NSA2*, and possibly *TINP1*, is required for production of ribosomes and, consequently, for cell proliferation. A pathological overexpression of this normally tightly regulated gene might favor tumor progression.

Location of Nsa2 on Pre-60 S Particles—In the present work, we have shown that Nog1 binds to pre-60 S particles prior to Nsa2. Although we could not detect any *in vitro* interaction between the

[4] J. S. Zhang and D. I. Smith, as annotated in the NCBI data base.

two recombinant proteins expressed in *E. coli*, two-hybrid data suggest that Nog1 and Nsa2 interact. Even if this interaction was indirect, it would still indicate that the two proteins are positioned relatively close to each other on complexes (Fig. 1*B*). The first 160 amino acids of Nog1, which interact strongly with Nsa2 in two-hybrid assays, are more conserved between yeast and higher eukaryotes than the C-terminal part of the protein. It suggests that the N-terminal domain has undergone a higher selective pressure, possibly for the conservation of the interaction with Nsa2. Thus far, we could not determine whether the observed physical interaction was direct or needed the whole structure of pre-60 S particles to occur. If it occurred physiologically, a direct interaction between Nog1 and Nsa2 might require a specific conformation, which could be achieved within pre-60 S complexes but not when the proteins are free in solution.

An additional hint for the possible position of Nsa2 on pre-60 S complexes was provided by two-hybrid experiments. In a genomic two-hybrid screen performed with Nsa2 as bait, we found three distinct ribosomal proteins as preys: Rpl4, Rpl15, and Rpl18.[5] Interestingly, when looking at the position of the prokaryotic homologues in the structure of the mature large ribosomal subunit, all three proteins are grouped on the right shoulder, on the external side of the subunit. These data suggest that Nsa2 might anchor to the precursors of the large subunit in the vicinity of this site.

Conclusion—We have characterized Nsa2 as a pre-60 S factor required for the processing of the 27 SB pre-rRNAs. This physical and functional partner of Nog1 turns out to be an unstable factor, regulated in coordination with the activity of ribosome biogenesis. Together with its strong conservation during its evolution, these features make Nsa2 and its homologues interesting candidates for the understanding of how cell proliferation is coordinated to the biosynthesis of cellular components.

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