MOLECULAR AND CELLULAR BIOLOGY, Oct. 2011, p. 4156–4164 Vol. 31, No. 20
0270-7306/11/$12.00 doi:10.1128/MCB.05436-11

Dynamics of the Putative RNA Helicase Spb4 during Ribosome Assembly in *Saccharomyces cerevisiae*▽†

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Received 1 April 2011/Returned for modification 21 April 2011/Accepted 28 July 2011

Spb4 is a putative ATP-dependent RNA helicase that is required for proper processing of 27SB pre-rRNAs and therefore for 60S ribosomal subunit biogenesis. To define the timing of association of this protein with preribosomal particles, we have studied the composition of complexes that copurify with Spb4 tagged by tandem affinity purification (TAP-tagged Spb4). These complexes contain mainly the 27SB pre-rRNAs and about 50 ribosome biogenesis proteins, primarily components of early pre-60S ribosomal particles. To a lesser extent, some protein factors of 90S preribosomal particles and the 35S and 27SA pre-rRNAs also copurify with TAP-tagged Spb4. Moreover, we have obtained by site-directed mutagenesis an allele that results in the R360A substitution in the conserved motif VI of the Spb4 helicase domain. This allele causes a dominant-negative phenotype when overexpressed in the wild-type strain. Cells expressing Spb4(R360A) display an accumulation of 35S and 27SB pre-rRNAs and a net 40S ribosomal subunit defect. TAP-tagged Spb4(R360A) displays a greater steady-state association with 90S preribosomal particles than TAP-tagged wild-type Spb4. Together, our data indicate that Spb4 is a component of early nucle(ol)ar pre-60S ribosomal particles containing 27SB pre-rRNA. Apparently, Spb4 binds 90S preribosomal particles and dissociates from pre-60S ribosomal particles after processing of 27SB pre-rRNA.

In all organisms, the biogenesis of ribosomes is a fundamental process mediated by *trans*-acting factors. Experimental evidence strongly suggests that the ribosome synthesis process is well conserved throughout eukaryotes (reference 69 and references therein). Most of our knowledge on this process comes from studies performed with *Saccharomyces cerevisiae* (reviewed in references 16, 25, and 68). In this yeast, more than 225 protein *trans*-acting factors and 75 small nucleolar RNAs (snoRNAs) involved in ribosome synthesis have been identified (reviewed in references 16, 25, and 48). These factors, whose precise functions are still largely unknown, likely allow the ribosome maturation process to proceed with the required speed, accuracy and directionality (reviewed in references 30, 31, and 61).

Biogenesis of eukaryotic ribosomes involves the transcription, processing, and modification of the precursors of rRNAs (pre-rRNAs), the folding and the assembly of the pre-rRNAs with the ribosomal proteins (r-proteins), and the export of the resulting preribosomal particles to the cytoplasm, where the last assembly reactions take place (16, 25, 39, 44, 63, 65). Pre-rRNA processing is a well-defined pathway and involves a complex series of sequential endo- and exonucleolytic reactions (see Fig. S1 in the supplemental material). Much less is known about the reactions that allow formation, maturation, and movement of preribosomal particles through the nucleus and their subsequent export (see Fig. S2 in the supplemental material). However, the use of many different experimental approaches, in particular the purification of preribosomal particles via the tandem affinity purification (TAP) method (for reviews, see references 8, 16, 25, and 64) and the combination of this technique with molecular genetics (see references 36 and 55 for examples) and, more recently, with electron microscopy analyses (43, 56, 66), have allowed the conclusions that (i) the *trans*-acting factors undergo cycles of association with and dissociation from the successive preribosomal particles and (ii) these particles undergo structural rearrangements during maturation.

In this work, we have studied the dynamics of the association and release of Spb4 from preribosomal particles. Spb4 is a nucleolar putative ATP-dependent RNA helicase that is required for the synthesis of 60S r-subunits (9, 52). Mutation (*spb4-1*) and depletion of Spb4 lead to a strong inhibition of processing of the 27SB pre-rRNAs (9). Our results strongly suggest that Spb4 associates with 90S preribosomal particles and dissociates from pre-60S r-particles after the endonucleolytic cleavage of 27SB pre-rRNAs at site C_2. We have also studied the consequences for ribosome biogenesis of the expression of a dominant-negative (DN) Spb4(R360A) protein. We show that Spb4(R360A) blocks pre-rRNA processing of 35S and 27SB pre-rRNAs. This leads to a strong inhibition of the overall biogenesis of both r-subunits, with the synthesis

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† Supplemental material for this article may be found at http://mcb.asm.org/.

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▽ Published ahead of print on 8 August 2011.

of the small subunit being more strongly diminished than that of the large one. TAP purification suggests that the Spb4(R360A)-TAP protein stalls on 90S preribosomal particles.

MATERIALS AND METHODS

Strains and microbiological methods. The yeast strains used in this study are listed in Table S5 in the supplemental material. Growth and handling of yeast and standard media were by established procedures (27). Yeast cells were grown at 30°C in either rich medium (1% yeast extract, 2% peptone) or synthetic minimal medium (0.15% yeast nitrogen base, 0.5% ammonium sulfate) supplemented with the appropriate amino acids and bases as nutritional requirements and containing either 2% galactose (YPGal and SGal), 2% glucose (YPD and SD), 3% glycerol and 2% lactate (SGly-Lac), or 2% raffinose (SRaf). To prepare plates, 2% agar was added to the media before sterilization. *Escherichia coli* strain DH5α was used for cloning and propagation of plasmids (53).

Plasmids. The plasmids used in this study are listed in Table S6 in the supplemental material. To generate plasmid pTAPC111-SPB4, the intermediate pJJ-SPB4 plasmid was constructed as follows. A 3.1-kb HindIII/EcoRI fragment from YCplac111-SPB4 (9) containing the complete *SPB4* gene was made blunt ended and cloned into SmaI-restricted pTAPC111 (51) (a gift from D. Kressler). A PCR was then performed using YCplac111-SPB4 as a template and oligonucleotides SPB4+2003 and SPB4+XBAI (see Table S7 in the supplemental material). The resulting PCR product (0.8 kb) contains the 3′ part of the *SPB4* coding region but lacks the termination codon. This product was cloned in the pGEM-T plasmid (Promega), and then an NdeI-XbaI fragment of 0.5 kb from this clone was used to replace the NdeI-XbaI fragment released from pJJ-SPB4.

Plasmid pAS24-NTAP/2xFLAG-SPB4 was a gift from D. Kressler. This plasmid contains a *GAL1-10* promoter, a start codon followed by a TAP tag, a double Flag tag, and the complete *SPB4* coding region plus 250 bp from its 3′ contiguous region. To generate plasmid pAS24-NTAP/2xFLAG-DNSPB4, which harbors the dominant-negative *SPB4*(R360A) allele, a two-step overlapping fusion PCR method was applied as previously described (62). Briefly, two PCRs were performed using the oligonucleotide pairs IN1-SPB4-UP/SPB4R2-P2 and POST-SPB4-LO/SPB4R2-P1 (see Table S7 in the supplemental material) and YCplac111-SPB4 as a template. The PCR products contain the desired mutation (AGA → GCA, R360A) and the 5′ or the 3′ end of the *SPB4* coding region, respectively. The two PCR fragments were purified, and a second PCR was done with an aliquot of each fragment and the IN1-SPB4-UP and POST-SPB4-LO oligonucleotides. The resulting PCR product (ca. 1.3 kb) was cloned in the pGEM-T plasmid. Some clones were checked by DNA sequencing. The XhoI-HpaI fragment (ca. 1 kb) from a positive one was then subcloned into XhoI-HpaI-restricted pAS24-NTAP/2xFLAG-SPB4.

Plasmid pAS24-SPB4 has been previously described (9). To generate pAS24-DNSPB4, the dominant-negative *SPB4*(R360A) allele was subcloned as an SphI fragment (ca. 1.5 kb) from pAS24-NTAP/2xFLAG-DNSPB4 into pAS24-SPB4.

Induction of the dominant-negative *SPB4*(R360A) allele. The wild-type W303-1A strain was transformed with the pAS24-NTAP/2xFLAG-SPB4 or the pAS24-NTAP/2xFLAG-DNSPB4 plasmid, which encode the *GAL::SPB4*-TAP and *GAL::SPB4*(R360A)-TAP genes, respectively. These strains were grown during the day in SD-Leu medium at 30°C. To remove any traces of intracellular glucose, the cultures were diluted and grown overnight in SGly/Lac-Leu. The following day, filter-sterilized galactose or glucose was added to the medium to a final concentration of 2%. Cell growth was monitored over time, with the cultures being regularly diluted into fresh SGal-Leu or SD-Leu, respectively, to maintain an optical density at 600 nm (OD_{600}) of between 0.5 and 0.8. Samples for Western blotting, polysome analyses, affinity purification, and RNA extraction were taken at different times.

Western blot analyses. Western blotting was performed according to a standard procedure (53). The following primary antibodies were used: mouse monoclonal antihemagglutinin (anti-HA) antibodies (Covance), mouse monoclonal anti-green fluorescent protein (anti-GFP) (Roche), rabbit polyclonal anti-Has1 (13), rabbit polyclonal anti-L1 (a gift from F. Lacroute) (47), and rabbit polyclonal anti-Mrt4 (50). Goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies (Bio-Rad) were used as secondary antibodies. Peroxidase-antiperoxidase (PAP) soluble complex (Sigma) was used to detect the wild-type and the dominant-negative forms of the Spb4-TAP protein. Proteins were visualized using an enhanced chemiluminescence detection kit (Super-Signal West Pico; Pierce).

Sucrose gradient centrifugation. Polysome and r-subunit preparations and analyses were performed as previously described (29). Ten A_{260} units of cell extracts were loaded on sucrose gradient. Profiles were obtained by continuously monitoring A_{254} with an ISCO UA-6 system.

RNA analyses. RNA extraction, Northern hybridization, and primer extension analyses were carried out according to standard procedures (29, 67). In all experiments, RNA was extracted from samples corresponding to 10 OD_{600} units of exponentially grown cells. Equal amounts of total RNA were loaded on gels or used in primer extension reactions. The sequences of oligonucleotides used for Northern hybridization are listed in Table S7 in the supplemental material. Phosphorimager analysis was performed in a FLA-5100 imaging system (Fujifilm) at the Biology Service of the University of Seville (CITIUS).

Fluorescence microscopy. To test preribosomal particle export in the *GAL::SPB4-TAP* and *GAL:SPB4(R360A)-TAP* strains, these were transformed with the pRS314-RFP-NOP1-RPL25-eGFP or the pRS314-RFP-NOP1-RPS3-eGFP plasmid (gifts from J. Bassler) (66), which harbor the reporters Nop1-mRFP and L25-eGFP or Nop1-mRFP and S3-eGFP, respectively (see Table S6 in the supplemental material). Transformants were grown in SD-Leu-Trp medium or shifted as described above to SGal-Leu-Trp for 24 h to induce overexpression of the wild-type or the DN form of the Spb4-TAP protein. Cells were examined with a Leica DMR fluorescence microscope equipped with a DC350F digital camera and its software. Images were processed with Adobe Photoshop CS2 (Adobe Systems Incorporated).

Tandem affinity purification. Complexes associated with the wild-type or the DN form of the Spb4-TAP protein were purified by a standard two-step TAP procedure (49) exactly as previously described (18, 34). After two successive affinity purifications using an immunoglobulin G (IgG) Sepharose column (GE Healthcare) followed by a calmodulin affinity resin (Stratagene), the proteins from the purified complexes were separated in Novex Tris-glycine–SDS 4 to 20% gradient polyacrylamide gels (Invitrogen) and visualized by Coomassie blue staining.

Compositional analysis of TAP-purified complexes. To identify the proteins associated with the wild-type or the dominant-negative form of Spb4-TAP, eluted proteins from the calmodulin resin were precipitated with trichloroacetic acid, separated in Novex Tris-glycine–SDS 4 to 20% gradient polyacrylamide gels, and identified by mass spectrometry exactly as described in previously (18, 34).

To identify the RNAs associated with the wild-type or the DN form of Spb4-TAP, a one-step purification was performed with IgG-Sepharose beads as described previously (18, 34). RNA was recovered from total cell extracts and the beads with phenol-chloroform exactly as described previously (18, 51). RNA was analyzed by Northern blotting as described above.

Affinity purification of GFP-tagged proteins. GFP-tagged factors representative of 90S, pre-60S, and pre-40S r-particles were purified following a one-step GFP-Trap_A procedure (Chromotek) as described in reference 1 with slight modifications. Briefly, 200 ml of GFP-tagged or untagged negative-control cells was transformed with YCplac111-HA-SPB4 and grown in SD-Leu medium to an OD_{600} of 0.8. Cells were washed with cold water, harvested, and concentrated in 500 μl of ice-cold lysis buffer (20 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 150 mM CH_3COOK, 1 mM dithiothreitol [DTT], 0.2% Triton X-100) containing a protease inhibitor cocktail (Complete; Roche). Cells were disrupted by vigorous shaking with glass beads in a Fastprep-24 (MP Biomedicals) at 4°C, and total extracts were clarified by centrifugation in a microcentrifuge at the maximum speed (ca. 16,000 × *g*) for 10 min at 4°C. To each of the resulting supernatants, 50 μl of GFP-Trap_A beads, equilibrated with the same buffer, was added, and the mixture was incubated for 1.5 h at 4°C with end-over-end tube rotation. After incubation, the beads were extensively washed six times with 1 ml of the same buffer at 4°C and resuspended in 100 μl of Laemmli buffer. Proteins were extracted by boiling for 10 min, separated in NuPAGE-SDS 4 to 12% gradient polyacrylamide gels (Invitrogen), and visualized by silver staining or Western blotting. To normalize the amount of purified complex to be loaded in comparative studies, a sample was previously resolved under the same conditions and dyed with a silver stain according to the manufacturer's instructions (Bio-Rad).

RESULTS AND DISCUSSION

Spb4 is a component of pre-60S ribosomal complexes. We have previously found Spb4 in high-molecular-mass complexes of a size overlapping 60S/80S peaks in cell extracts by sucrose gradient fractionation (9). Due to the fact that Spb4 is required for 60S r-subunit biogenesis, these complexes are most likely pre-60S r-particles. Indeed, Spb4 has been identified as a component of several purified pre-60S r-particles by the TAP

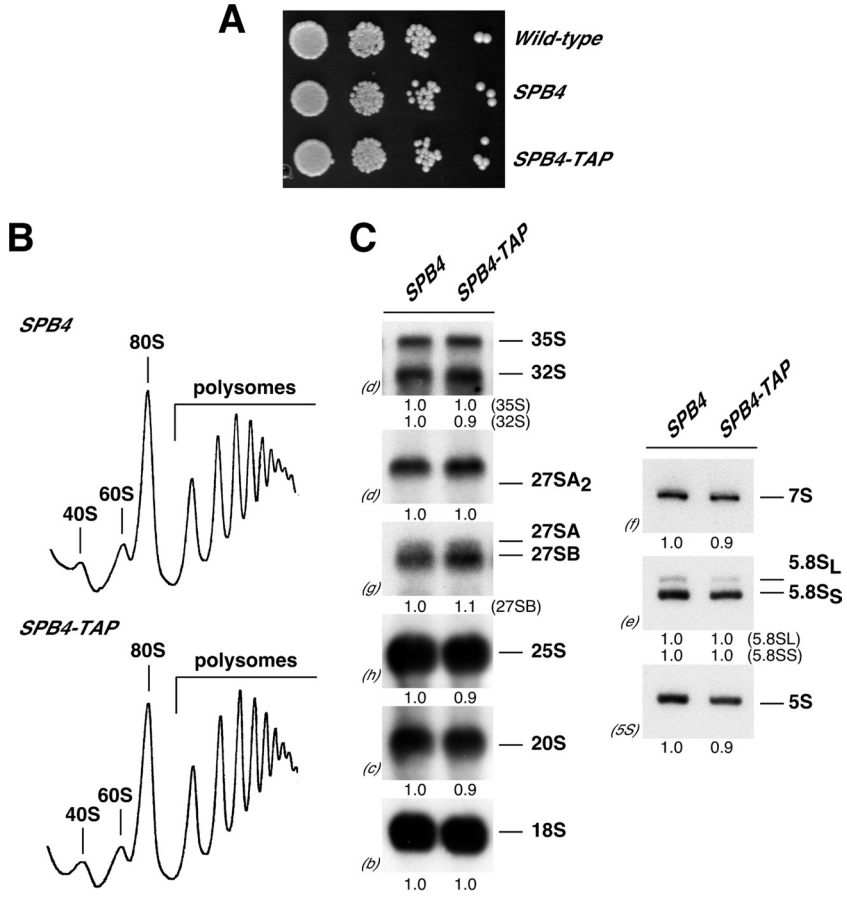

FIG. 1. The Spb4-TAP fusion protein is fully functional. (A) The W303-1A strain harboring the empty YCplac111 vector (wild type) and the YDK37-1B strain containing the YCplac111-SPB4 (*SPB4*) or pTAPC111-SPB4 (*SPB4-TAP*) plasmid were grown in liquid SD-Leu medium. Ten-fold serial dilutions were spotted on SD-Leu plates and incubated at 30°C for 2 days. (B) The *SPB4* and *SPB4-TAP* strains were grown in SD-Leu medium at 30°C and harvested at an OD_{600} of 0.8. Cell extracts were prepared, and 10 A_{260} units of each extract was centrifuged in 7% to 50% sucrose gradients. The A_{254} was read continuously. Sedimentation is from left to right. The peaks of free 40S and 60S r-subunits, 80S free couples/monosomes, and polysomes are indicated. (C) Total RNA was isolated from cell extracts of the strains described above and analyzed by Northern blotting. Probes (in parentheses) are described in Fig. S1A and Table S7 in the supplemental material. Signal intensities were measured by phosphorimager scanning. Values, indicated below each lane, were normalized to those obtained for the wild-type control, which were arbitrarily set at 1.0.

method (5, 16, 19, 32, 33, 42, 55, 60). To confirm its association with pre-60S r-particles, we tagged Spb4 with a C-terminal TAP cassette (see Materials and Methods). Growth of the strain expressing Spb4-TAP as a sole source of Spb4 was indistinguishable from that of the wild-type isogenic counterpart (Fig. 1A). Moreover, the Spb4-TAP-expressing strain led to polysome and pre-rRNA processing profiles virtually identical to those of the wild-type strain (Fig. 1B and C). Thus, the Spb4-TAP fusion protein is fully functional. We then performed a two-step TAP purification of TAP-tagged Spb4 (see Materials and Methods). The strain W303-1A, which expresses a nontagged Spb4 protein, was used as a negative control. Coprecipitated proteins were separated by SDS-PAGE and stained with Coomassie blue (Fig. 2). Mass spectrometry identification of the different polypeptides revealed many proteins, including about 50 ribosome biogenesis factors (Fig. 2; see Tables S1 to S3 in the supplemental material). The majority of these factors have been described as components of pre-60S r-particles and implicated in the production of 60S r-subunits. The remaining factors are components of 90S preribosomal particles (see Table S1). Some proteins not involved in ribosome biogenesis were also found associated with Spb4-TAP (see Tables S2 and S3). Some of these proteins, such as translation factors, chaperones, or histones, are found frequently in TAP complexes (see Table S2), and therefore the specificity of their interaction with Spb4 is uncertain. Likewise, TAP complexes can be contaminated by r-proteins (see Table S2); thus, it is also unclear whether those we identified are the result of a contamination or are truly components of the purified preribosomal complexes.

To determine which pre-rRNA species are associated with Spb4-TAP, we performed precipitation experiments using IgG-conjugated Sepharose with total cell extracts of the Spb4-TAP strain and the nontagged control strain (see Materials and Methods). Coprecipitated RNAs were analyzed by Northern blotting. Consistent with the previous proteomic results, two of the most efficiently coprecipitated pre-rRNAs with Spb4-TAP are the $27SA_2$ and 27SB pre-rRNAs, which are

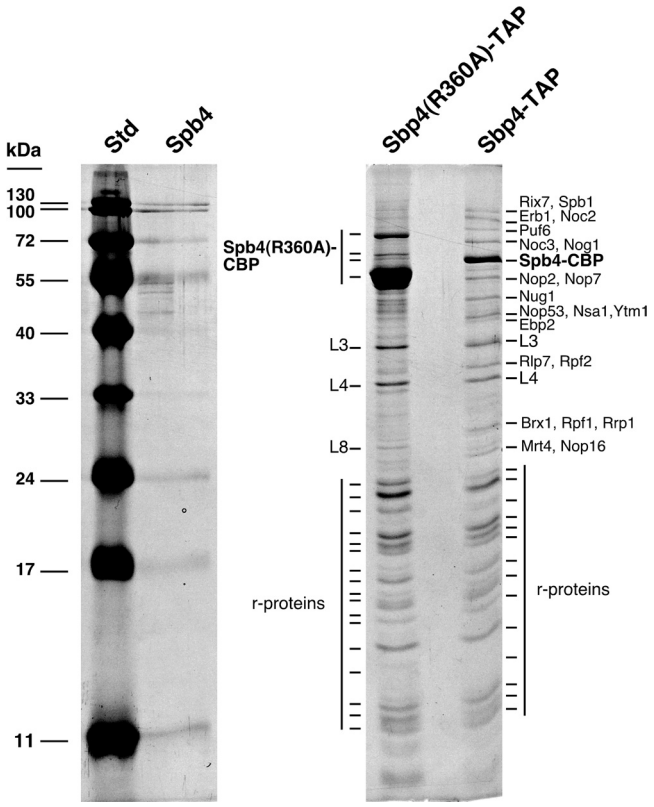

FIG. 2. Protein composition of the Spb4-TAP and Spb4(R360A)-TAP complexes. Strain YDK37-1B/ pTAPC111-SPB4, which expresses a TAP-tagged Spb4 fusion protein as a sole source of Spb4, was grown in YPD at 30°C to an OD_{600} of around 0.8. Strain W3031-A/pAS24-NTAP/2xFLAG-DNSPB4, which expresses the dominant-negative Spb4(R360A)-TAP protein under the control of a *GAL* promoter, was grown at 30°C in SD-Leu, transferred to SGly/Lac-Leu, and then shifted to SGal-Leu for 24 h. Spb4-TAP and Spb4(R360A)-TAP were affinity purified by the TAP procedure, and the final EGTA eluate was separated on a 4 to 12% SDS-polyacrylamide gradient gel and subjected to Coomassie blue staining. The wild-type W303-1A strain (Spb4) served as a negative control. The indicated proteins and those described in Tables S1 to S4 in the supplemental material were identified by mass spectrometry. Std, molecular mass protein standards. Despite the fact that the figure is divided into two pieces, all samples were run in the same gel. Note that TAP-tagged Spb4(R360A) carries two consecutive N-terminal Flag epitopes that are not present in the TAP-tagged wild-type Spb4 construct.

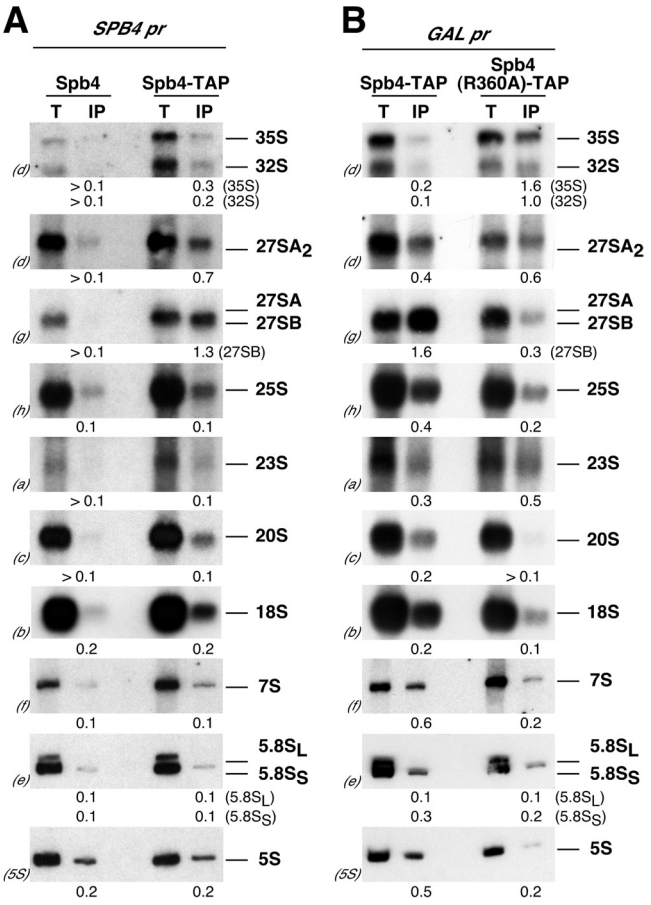

FIG. 3. Wild-type Spb4 is associated mainly with early pre-60S ribosomal particles, but dominant-negative Spb4(R360A) also coenriches 90S preribosomal particles. (A) Immunoprecipitation experiments were carried out using IgG-Sepharose and whole-cell extracts from the YDK37-1B strain harboring the pTAPC111-SPB4 plasmid, which expressed Spb4-TAP (Spb4-TAP) from its cognate promoter (*SPB4 pr*). RNA was extracted from the pellets obtained after purification (lanes IP) or from an amount of total extract corresponding to 1/30 of that used for the purification (lanes T). Pre-rRNAs and mature rRNAs were analyzed by Northern blotting. Strain YDK37-1B harboring the YCplac111-SPB4 plasmid, which expressed nontagged Spb4 (Spb4) from its cognate promoter, was used as a negative control. (B) Immunoprecipitation experiments were carried out as described above on whole-cell extracts from the wild-type W303-1A strain harboring either the pAS24-NTAP/2xFLAG-SPB4 (Spb4-TAP) or the pAS24-NTAP/2xFLAG-DNSPB4 [Spb4(R360A)-TAP] plasmid, which expressed the wild-type or the DN form of Spb4-TAP, respectively, from the *GAL* promoter (*GAL pr*). Cells were grown in SGal-Leu for 24 h. RNA was extracted from whole-cell extracts (lanes T) and affinity-purified samples (IP) and subjected to Northern analysis as described above. Probes (in parentheses) are described in Fig. S1A and Table S7 in the supplemental material. For panels A and B, signal intensities were measured by phosphorimager scanning to derive the percentage of input RNA precipitated together with tagged Spb4 proteins (values are indicated below each IP lane).

components of early pre-60S r-particles (Fig. 3A; see Fig. S2 in the supplemental material). A modest enrichment of 35S and 32S pre-rRNAs, which are the components of 90S preribosomal particles, was also found (Fig. 3A; see Fig. S2). In contrast, 7S pre-rRNAs, which are components of intermediate nuclear pre-60S r-particles, and mature 25S rRNAs, which define late pre-60S r-particles and mature 60S r-subunits, were not significantly coprecipitated over the background levels obtained using untagged strain extracts (Fig. 3A; see Fig. S2). On the 40S r-subunit maturation pathway, only background levels of 20S pre-rRNA and 18S rRNA were found (Fig. 3A).

To further study the stage in the 60S r-subunit maturation pathway at which Spb4 associates with preribosomal particles, we screened for the presence of HA-tagged Spb4 following purification of GFP-tagged proteins specific for 90S (Pwp2/Utp1 and Nop58/Nop5) (10, 11, 21), early, intermediate, late, and cytoplasmic pre-60S r-particles (Ssf1, Nop7/Yph1, Rix1/Ipi2, Arx1, and Kre35/Lsg1) (14, 24, 28, 33, 42), and mature 60S r-subunits (P0) (54). Purified particles were analyzed by SDS-PAGE, and HA-Spb4 was detected by Western blotting using anti-HA antibodies. Antibodies against other selected

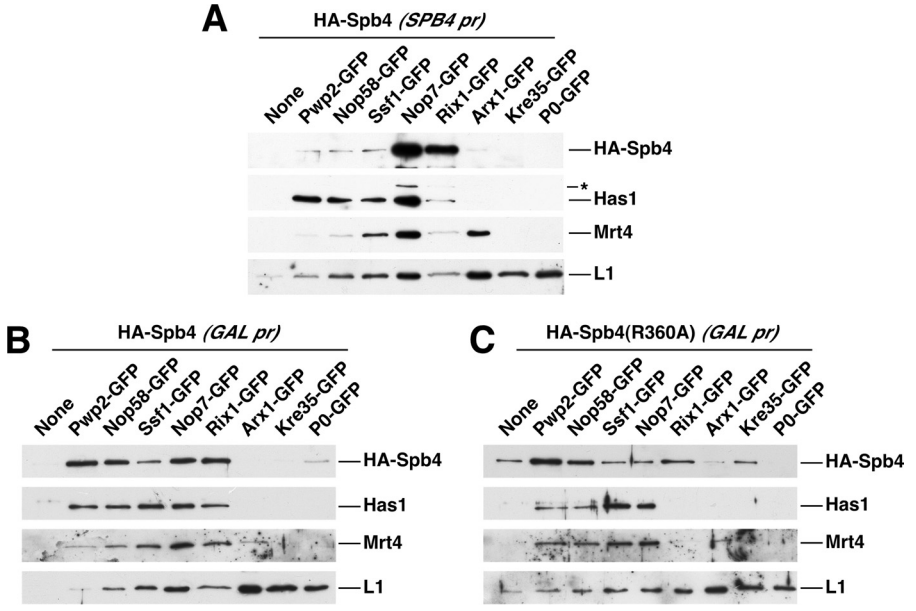

FIG. 4. Wild-type Spb4 associates with pre-60S ribosomal particles, and the dominant-negative Spb4(R360A) protein stalls in 90S preribosomal particles. (A) The indicated GFP-tagged bait proteins were affinity purified by using the GFP-Trap_A procedure (see Materials and Methods) from cells expressing HA-Spb4 under control of its cognate promoter. (B and C) In addition, we also used cells expressing under control of the *GAL* promoter the wild-type HA-Spb4 (B) or the dominant-negative HA-Spb4(R360A) (C) protein. Cells were grown for 12 h in galactose-containing medium. Equivalent amounts of the corresponding purified complexes were separated on 4 to 12% gradient SDS-polyacrylamide gels and subjected to Western blot analysis using specific antibodies against the HA epitope and the Has1, Mrt4, and L1 proteins. The cross-reacting band (marked with an asterisk) in the panel for Has1 most likely corresponds to nonstripped HA-Spb4 signal.

preribosomal factors (Has1 and Mrt4) and 60S r-subunits (L1) were also used to define the pre-60S particles at the different stages of their maturation. As shown in Fig. 4A, HA-Spb4 was strongly enriched in the Nop7-GFP- and Rix1-GFP-containing particles, present to some extent in 90S (Pwp2-GFP and Nop58-GFP), and late pre-60S r-particles, and practically absent from cytoplasmic (Arx1-GFP and Lsg1-GFP) and mature (P0-GFP) 60S r-subunits. As previously described, Nop7 is associated with a larger number of different pre-60S particles, although enriched within early E_2 and intermediate nucleolar particles (see Fig. S2 in the supplemental material) (24, 42). Rix1, which is part of a protein complex together with Ipi1, Ipi3, and Rea1 (17, 33), stably associates with intermediate nucleolar/nucleoplasmic pre-60S particles (see Fig. S2) (33, 42). Moreover, we found that HA-Spb4 is absent from pre-40S r-particles (Tsr1 and Nob1) (15, 20, 57) (data not shown).

Altogether, these results indicate that Spb4 is predominantly associated with early nucle(ol)ar pre-60S r-particles containing 27SB pre-rRNAs. A fraction of Spb4 is also present in earlier 66S r-particles containing $27SA_2$ and in 90S preribosomal particles containing either 35S or 32S pre-rRNA. Thus, Spb4 could bind 90S preribosomal particles and remain associated with pre-60S r-particles following cleavages of the 35S pre-rRNA at sites A_0 to A_2. Spb4 apparently efficiently dissociates from pre-60S particles after 27SB pre-rRNA processing.

The dominant-negative Spb4(R360A)-TAP protein stalls in 90S preribosomal particles. To further address the properties of binding of Spb4 to preribosomal particles, we converted to alanine the conserved second arginine residue within motif VI (356-HRCGRTGR-363; the second arginine is underlined) of Spb4-TAP. It has been previously shown for different RNA helicases that mutation of this arginine drastically reduced the ATPase activities of the mutated enzymes *in vitro* (for examples, see references 45 and 58; reviewed in reference 7). *In vivo*, this mutation is lethal and results in a DN phenotype when overexpressed in a wild-type strain (for examples, see references 26, 34, and 40; reviewed in reference 7). Since RNA helicases seem to interact transiently with their substrates, it has been hypothesized that DN versions of these enzymes would sequester these substrates and would not be released from the large ribonucleoprotein complexes to which they bind (2, 22, 59). This scenario has been suggested for DN mutants of Prp2 (12), Prp16 (58, 59), Prp22 (58), and Prp43 (34, 40).

To test whether the Spb4(R360A)-TAP protein could confer a DN phenotype, we placed its corresponding allele under the control of the inducible *GAL* promoter and expressed it in the wild-type strain. This allele could not restore the viability of an *spb4*-null strain (data not shown). As shown in Fig. 5A, the overexpression of Spb4(R360A)-TAP in galactose-containing medium led to a strong inhibition of growth of the wild-type strain, while that of Spb4-TAP caused no change in the growth phenotype. Western blot analysis confirmed that both Spb4(R360A)-TAP and Spb4-TAP were comparably expressed in galactose-containing medium (Fig. 5B). Next, we tested the ability of the mutant Spb4(R360A)-TAP to affect the ribosome maturation pathway. The galactose-inducible TAP-tagged wild-type and DN versions of Spb4 were expressed in the wild-type strain, and polysome profile analyses were performed. The profile of the wild-type strain harboring the *GAL::SPB4*(R360A)-*TAP* construct was completely normal when grown in SD-Leu (Fig. 5C). However, despite Spb4 hav-

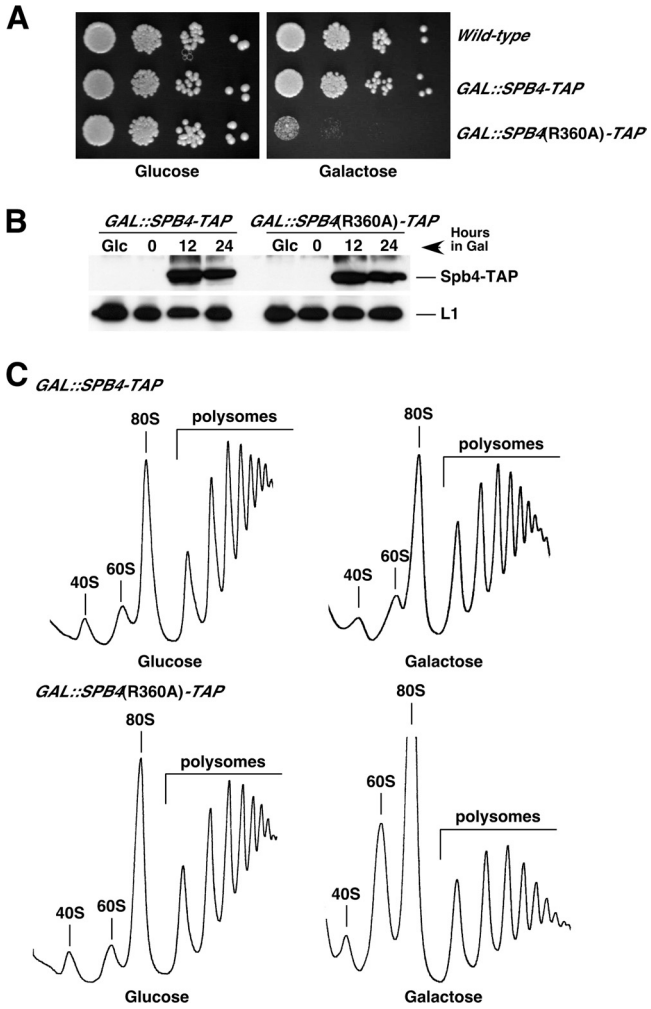

FIG. 5. Expression of the Spb4(R360A)-TAP protein leads to a dominant-negative growth phenotype and a deficit in 40S ribosomal subunits. (A) The W303-1A strain was transformed with plasmid YCplac111 (wild type), pAS24-NTAP/2xFLAG-SPB4 (*GAL::SPB4-TAP*), or pAS24-NTAP/2xFLAG-DNSPB4 [*GAL::SPB4*(R360A)-*TAP*]. Transformants were grown in liquid SD-Leu and spotted in 10-fold serial dilutions on SD-Leu (glucose) or SGal-Leu (galactose). SD-Leu plates were incubated for 2 days at 30°C and SGal-Leu plates for 3 days at 30°C. (B) The *GAL::SPB4-TAP* and *GAL::SPB4*(R360A)-*TAP* strains were grown at 30°C in liquid SD-Leu medium (Glc) or transferred to SGly/Lac-Leu and then shifted to SGal-Leu (Gal) for 12 or 24 h. Total protein extracts were prepared from each sample. Equivalent amounts of extracts were subjected to SDS-PAGE and Western blot analysis with PAP and anti-L1 antibodies. (C) Polysome profiles from the *GAL::SPB4-TAP* and *GAL::SPB4*(R360A)-*TAP* strains. Cells were grown at 30°C in SD-Leu medium (glucose) or shifted to SGal-Leu (galactose) for 12 h. Cell cultures were maintained at an OD_{600} of around 0.5 to 0.8. Cells were harvested, and extracts were prepared. Ten A_{260} units of each extract was centrifuged in 7% to 50% sucrose gradients. The A_{254} was continuously measured. Sedimentation is from left to right. The peaks of free 40S and 60S ribosomal subunits, 80S free couples/monosomes, and polysomes are indicated.

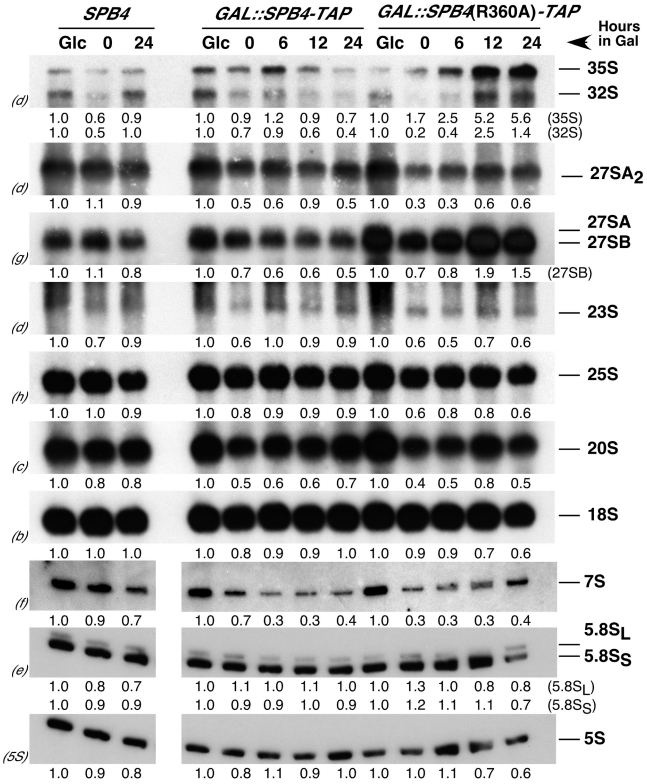

FIG. 6. Expression of the dominant-negative Spb4(R360A)-TAP protein inhibits pre-rRNA processing. YDK37-1A cells harboring YCplac111-SPB4 (*SPB4*) were grown at 30°C in SD-Leu medium or transferred to SGly/Lac-Leu and then shifted to SGal-Leu (Gal) for 24 h. W303-1A cells harboring either the pAS24-NTAP/2xFLAG-SPB4 (*GAL::SPB4-TAP*) or the pAS24-NTAP/2xFLAG-DNSPB4 [*GAL::SPB4*(R360A)-*TAP*] plasmid were also grown at 30°C in SD-Leu (Glc) or transferred to SGly/Lac-Leu and then shifted to SGal-Leu (Gal) for 24 h. Cells were harvested at the indicated times. Total RNA was isolated and analyzed by Northern blotting. Probes (in parentheses) are described in Fig. S1A and Table S7 in the supplemental material. Signal intensities were measured by phosphorimager scanning. Values, indicated below each lane, were normalized to those obtained for each strain in SD-Leu (Glc), which were arbitrarily set at 1.0.

ing a role in 60S r-subunit biogenesis (9, 52), induction of *GAL::SPB4*(R360A)-*TAP* for 12 h in SGal-Leu led to a mild 40S r-subunit deficit as revealed by an obvious increase in free 60S versus 40S r-subunits and a decrease in polysome levels (Fig. 5C). An induction for a longer time period of 24 h resulted in a further enhancement of this deficit (data not shown).

To assess whether this detected 40S r-subunit shortage was due to a biogenesis defect, we first analyzed pre-rRNA processing by Northern hybridization at various time points after induction by galactose of either the Spb4-TAP or the Spb4(R360A)-TAP protein in the wild-type strain. As shown in Fig. 6, induction of DN Spb4(R360A)-TAP but not that of wild-type Spb4-TAP resulted in a clear time-dependent accumulation of 35S, 32S, and 27SB pre-rRNAs. Interestingly, the levels of the aberrant 23S RNA species did not increase. The levels of the other pre-rRNAs and those of mature rRNAs are not significantly altered upon induction of DN Spb4(R360A)-TAP compared to the induction of wild-type Spb4-TAP (Fig. 6). We also assessed the levels of 25.5S and the different 27S pre-rRNAs by primer extension analysis. As shown in Fig. S3 in the supplemental material, the steady-state levels of 25.5S, $27SA_2$, and $27SA_3$ slightly increased upon overexpression of Spb4(R360A)-TAP; consistent with the results obtained by Northern hybridization, the steady-state levels of both $27SB_L$

and $27SB_S$ pre-rRNAs significantly increased upon overexpression of Spb4(R360A)-TAP.

Finally, to determine whether or not the overexpression of Spb4(R360A)-TAP impairs nuclear export of preribosomal particles, we analyzed the localization of the L25-eGFP and S3-eGFP r-subunit protein reporters upon induction by galactose of either the Spb4-TAP or the Spb4(R360A)-TAP protein in the wild-type strain. Our results indicate that the localization of neither reporter was affected upon induction of either wild-type or DN Spb4 proteins (see Fig. S4A in the supplemental material). As a positive control for nuclear retention of r-protein reporters, we depleted the cells of the Spb4 and Fal1 proteins (see Fig. S4B). In all cases, the tested r-protein reporters did incorporate into preribosomal particles (see Fig. S5) (data not shown).

Altogether, these analyses suggest that DN Spb4(R360A)-TAP would be trapped into 90S preribosomal particles, delaying their further maturation. Alternatively, the ribosome maturation defects observed would indirectly arise as the consequence of the inefficient recycling of *trans*-acting factors of 90S preribosomal particles that could not dissociate from the defective pre-60S r-particles that accumulate in the DN mutant. To distinguish between these two possibilities, we investigated the protein and RNA compositions of preribosomal particles purified with TAP-tagged DN Spb4(R360A) after overexpression from a *GAL* promoter (Fig. 2 and 3). As shown in Table S1 in the supplemental material, we identified only a few components of pre-60S r-particles, which were purified only using Spb4(R360A)-TAP as bait (e.g., Cgr1, Dbp10, Drs1, Mak5, Mak11, Nop4, Rsa4, and Ssf2). However, most coenriched pre-60S factors were also found in the affinity purification of wild-type Spb4 (see Table S1). Interestingly, Spb4(R360A)-TAP also copurified several components of RNA polymerase I and about 20 components of 90S preribosomal particles (see Table S1). Thus, we decided to determine which pre-rRNA species associate with Spb4(R360A)-TAP. To do this, RNA was extracted from TAP-purified complexes of both galactose-induced Spb4(R360A)-TAP and Spb4-TAP and analyzed by Northern blotting. As shown in Fig. 3, the profile of RNAs precipitated with overexpressed Spb4-TAP is overall similar to that of RNAs precipitated with Spb4-TAP expressed from its cognate promoter (compare Fig. 3A and B). Interestingly, 35S and 32S pre-rRNA-containing preribosomal particles were precipitated substantially more efficiently with Spb4(R360A)-TAP than with the TAP-tagged wild-type Spb4 fusion protein; in contrast, 27SB pre-rRNA was precipitated much less efficiently with Spb4(R360A)-TAP than with the TAP-tagged wild-type Spb4 fusion protein (Fig. 3B). In agreement with these results, DN HA-Spb4(R360A) protein is present in two peaks of high molecular mass as determined by sucrose gradient fractionation, one of them corresponding to 66S and 90S preribosomal particles (see Fig. S6B, lanes 13 to 16, in the supplemental material). Moreover, the DN HA-Spb4(R360A) protein was enriched within 90S preribosomal particles purified using Pwp2-GFP and Nop58-GFP as baits (Fig. 4C). Affinity purifications from *GAL:HA-SPB4* cells yielded similar coenrichment of the wild-type HA-Spb4 protein in those 90S preribosomal complexes containing Pwp2-GFP or Nop58-GFP (Fig. 4B). However, while substantial wild-type HA-Spb4 protein is still detected in the pre-60S r-particles purified using Nop7-GFP or Rix1-GFP (Fig. 4B), practically background levels of DN HA-Spb4(R360A) protein are detected following Nop7-GFP or Rix1-GFP purifications (Fig. 4C, compare lanes Nop7-GFP and Rix1-GFP with lane None). Altogether, these results suggest that the DN mutant Spb4(R360A)-TAP is readily incorporated into 90S preribosomal particles but cannot properly dissociate from them. This likely impedes the correct processing of 35S pre-rRNA, thus producing a negative effect on the 40S r-subunit biogenesis pathway. Concomitantly, processing of 27SB pre-rRNA is also inhibited.

Conclusions and perspectives. In this work, we have studied the physical environment of Spb4 by an affinity purification procedure. As expected from our previous functional analysis (9) and different global interaction data sets (60), Sbp4 associates mainly with pre-60S complexes. These complexes seem to be a heterogeneous mixture of distinct early nucle(ol)ar pre-60S r-particles (i.e., Nop7-purified complex) containing predominantly 27SB pre-rRNAs but also 27SA pre-rRNAs. Little association with pre-60S r-particles containing 7S pre-rRNAs was found, suggesting that the release of Spb4 from pre-60S r-particles might occur concomitantly with cleavage of 27SB pre-rRNAs. A small fraction of 35S pre-rRNA also coprecipitates with Spb4-TAP; therefore, Spb4 could bind at the level of 90S preribosomal particles. The association with the latter particles was more obvious when the DN Spb4(R360A) protein was analyzed, strongly suggesting that the mutant Spb4 binds to the 90S preribosomal particles but is inefficiently released from them. The copurification of several RNA polymerase I components with Spb4-TAP but more clearly with DN Spb4(R360A)-TAP protein strongly suggests that the association of Spb4 with 90S preribosomal particles may be cotranscriptional. Alternatively, it is also plausible that Spb4 associates mainly with pre-60S ribosomal particles, and the weak association between Spb4 and 90S preribosomal particles that we detected might reflect its premature and unproductive binding. Under wild-type conditions, the Spb4 protein could easily dissociate from these 90S preribosomal particles, most likely following ATP hydrolysis; however, the DN Spb4 protein, which is expected to have no ATPase activity, would have a reduced ability to dissociate from the 90S preribosomal particles in which it is engaged, leading to a jammed maturation pathway.

At first glance, the pre-rRNA processing defects due to the overexpression of the Spb4(R360A) protein mirrored those previously observed upon Spb4 mutation (*spb4-1*) or depletion (9). However, polysome profile analysis suggested that the inhibition of 35S pre-rRNA processing in the DN mutant is stronger than that upon mutation or depletion of Spb4, since a 40S r-subunit shortage was detected in the first case while a reduction in the levels of 60S r-subunits was detected in the second one (9). Strikingly, this inhibition was not accompanied by the accumulation of aberrant 23S pre-rRNA. It has been clearly demonstrated that the RNA helicases Prp43 and Has1 are required for the synthesis of both 40S and 60S r-subunits (6, 13, 34, 37, 38, 41). Prp43 is present within 90S, pre-40S, and pre-60S r-particles (4, 6, 34, 37). Has1 associates with 90S and pre-60S r-particles (13, 19, 21). Whether or not the particular pre-rRNA processing defects observed for the DN Spb4(R360A) protein reflect a dual function of Spb4 in

both 40S and 60S r-subunit biogenesis needs further investigation. Thus, it must be addressed whether or not Spb4 could act twice during ribosome biogenesis, first as part of the 90S preribosomal particles, which could be a more transient interaction, and then again as part of the pre-60S particles, which would be a more stable interaction.

RNA helicases may require cofactor proteins for optimal function *in vivo*. These cofactors could stimulate or inhibit the ATPase and helicase activities and confer substrate specificity and/or modify the affinity of the RNA helicase for its substrate (discussed in references 3 and 7). To date, cofactors have been reported for only two out of the 19 RNA helicases involved in yeast ribosome biogenesis, Dpb8 and Prp43 (23, 35, 46). These cofactors, named Esf2 for Dpb8 and Pfa1 for Prp43 (23, 35), stimulate the RNA-dependent ATPase activities of their respective RNA helicase partners *in vitro*. Pfa1 also stimulates the RNA helicase activity of Prp43 *in vitro* (35). Strikingly, only Pfa1 has been demonstrated to interact directly with Prp43 (35). This interaction is stoichiometric, RNA independent, and salt resistant (35, 46). Unfortunately, no polypeptide was copurified in stoichiometric amounts with TAP-tagged wild-type and DN versions of Spb4. Further studies are needed to determine whether Spb4 has a direct cofactor(s). We firmly believe that the combination of biochemical and genetic approaches will allow the identification of such a partner(s).

ACKNOWLEDGMENTS

We are indebted to all colleagues mentioned in the text for their gifts of material used in this study. We thank M. Dosil for advice on the affinity purification of GFP-tagged proteins and D. Kressler for critical reading of the manuscript.

This work was supported by grants from the Spanish Ministry of Science and Innovation (MICINN), FEDER (BFU2007-60151, BFU2010-15690, and HF2006-0153), and the Andalusian Government (CVI-271, P07-CVI-02623, and P08-CVI-03508) to J.D.L.C., from the CNRS, Université Paul Sabatier, the Agence Nationale de la Recherche, and the EGIDE Picasso Programme to Y.H., and from the Fondation pour la Recherche Médicale (Programme Grands Equipements) and the Génopole Toulose Midi-Pyrénées to B.M. J.J.G.-G. is a recipient of an FPI fellowship from MICINN.

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