Dbp6p Is an Essential Putative ATP-Dependent RNA Helicase Required for 60S-Ribosomal-Subunit Assembly in *Saccharomyces cerevisiae*

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A previously uncharacterized *Saccharomyces cerevisiae* open reading frame, YNR038W, was analyzed in the context of the European Functional Analysis Network. YNR038W encodes a putative ATP-dependent RNA helicase of the DEAD-box protein family and was therefore named *Dbp6p* ( DEAD-box protein 6). Dbp6p is essential for cell viability. In vivo depletion of Dbp6p results in a deficit in 60S ribosomal subunits and the appearance of half-mer polysomes. Pulse-chase labeling of pre-rRNA and steady-state analysis of pre-rRNA and mature rRNA by Northern hybridization and primer extension show that Dbp6p depletion leads to decreased production of the 27S and 7S precursors, resulting in a depletion of the mature 25S and 5.8S rRNAs. Furthermore, hemagglutinin epitope-tagged Dbp6p is detected exclusively within the nucleolus. We propose that Dbp6p is required for the proper assembly of preribosomal particles during the biogenesis of 60S ribosomal subunits, probably by acting as an rRNA helicase.

In eukaryotes, ribosome biogenesis is a complex process where approximately 80 ribosomal proteins (r-proteins) and four rRNAs are assembled into mature ribosomal subunits. The 60S ribosomal subunit consists of three rRNAs (5S, 5.8S, and 25S or 28S) and approximately 45 r-proteins, whereas the 40S ribosomal subunit is formed by one 18S rRNA molecule and approximately 35 r-proteins (74). In all eukaryotes, rRNAs are synthesized as precursors (pre-rRNAs) that require maturation by a large number of nonribosomal trans-acting factors. Transcription of the rRNA genes, processing (cleavage and modification) of the pre-rRNAs, and assembly with r-proteins are concomitant processes that take place primarily in a specialized subnuclear compartment termed the nucleolus (35). Although ribosome biogenesis has been extensively studied for higher eukaryotes (14), biochemical strategies and genetic approaches with the yeast *Saccharomyces cerevisiae* have provided the best-characterized picture regarding the various cis elements and trans-acting factors participating in this process (59, 60, 64, 67). In yeast, three of the four rRNAs (18S, 5.8S, and 25S) are produced as a single 35S precursor by RNA polymerase I, whereas the fourth rRNA (5S) is transcribed independently by RNA polymerase III (67, 74). In the 35S pre-rRNA, the mature RNA sequences are separated by two internal transcribed spacers, ITS1 and ITS2, and flanked by two external transcribed spacers, 5′ETS and 3′ETS (Fig. 1A). During the maturation of the 35S pre-rRNA, these transcribed spacers are removed by a series of ordered endo- or exonucleolytic steps requiring small nucleolar RNAs (snRNAs) and proteins as trans-acting factors (Fig. 1B) (59, 67). Concomitantly, the pre-rRNAs are also covalently modified, mostly by 2′O methylation of ribose groups and conversion of uridine residues to pseudouridine (60). In addition to snoRNAs, enzymes for rRNA modification, and endo- and exonucleases, another class of trans-acting factors predicted to function enzymatically in ribosome biogenesis is the ATP-dependent RNA helicases.

RNA helicases originate from many organisms, ranging from bacteria to humans (51). They are involved in a variety of RNA metabolic processes, including translation initiation (42), premRNA splicing (48), ribosome biogenesis (67), and RNA degradation (25, 34). An ATP-dependent RNA unwinding activity has been observed for some of these proteins (e.g., see references 22, 30, and 46), whereas only an RNA-dependent ATPase activity could be attributed to others (e.g., see references 17, 27, and 53). However, based on sequence analysis, most are regarded as putative ATP-dependent RNA helicases. Many putative ATP-dependent RNA helicases are grouped in the DEAD-box protein family. This family is characterized by a core region of about 300 to 350 amino acids that shows strong homology to the translation initiation factor eIF4A, which is the prototype of the DEAD-box protein family (32). The core region consists of eight motifs with strong sequence conservation among all members of the family (51). To date, biochemical properties have been attributed only to four of the motifs within the mammalian eIF4A (42). Sequence divergence within the DEAD box gives rise to the DEXH subgroup, whose members are more heterogeneous with respect to both sequence and function (16). Moreover, individual members of the family have distinct amino- and carboxy-terminal regions that vary in length (51). These regions and differences within the core region may confer substrate specificity, direct the protein to its subcellular localization, include RNA binding motifs, or bind to accessory proteins, which could by themselves carry out the aforementioned functions.

To date, nine putative RNA helicases have been shown to be implicated in ribosome biogenesis in yeast. Dhp4p, Fal1p, Rok1p, and Rrp3p are required for 18S rRNA synthesis (28, 31, 41, 65). Dbp3p, Dbp7p, Drs1p, and Spb4p are involved in 25S rRNA maturation (10, 44, 49, 72). Dob1p is required for correct 3′-end processing of the 5.8S rRNA (12). Considering their putative RNA unwinding activity, RNA helicases could play different roles during ribosomal biogenesis reactions.
FIG. 1. Scheme of pre-rRNA processing in *S. cerevisiae*. (A) Structure and processing sites of the 35S precursor. This precursor contains the sequences for the mature 18S, 5.8S, and 25S rRNAs, which are separated by two internal transcribed spacers, ITS1 and ITS2. Two external transcribed spacers, 5' ETS and 3' ETS, are present at either end. The locations of the various probes (numbered from 1 to 9) used in this study are also indicated. Thick lines represent mature rRNA species, and thin lines represent transcribed spacers. (B) Pre-rRNA processing pathway. The 35S pre-rRNA is cleaved at site A₀ by endonuclease Rnt1p (1), generating the 33S pre-rRNA. This molecule is subsequently processed at sites A₁ and A₂ to give rise to the 20S and 27SA₂ precursors, resulting in the separation of the pre-rRNAs destined for the small and large ribosomal subunits. It is thought that the early pre-rRNA cleavages from A₀ to A₂ are carried out by a large small nucleolar RNP complex (67), which is likely to be assisted by the putative ATP-dependent RNA helicases Dbp4p (31), Fal1p (28), Rok1p (65), and Rrp3p (41). The final maturation of the 20S precursor takes place in the cytoplasm, where endonucleolytic cleavage at site D yields the mature 18S rRNA. The 27SA₂ precursor is processed by two alternative pathways that both lead to the formation of mature 5.8S and 25S rRNAs. In the major pathway, the 27SA₂ precursor is cleaved at site A₃ by RNase MRP (67). The putative ATP-dependent RNA helicase Dbp3p (72) assists in this processing step. The resulting 27SA₃ precursor is 5'⁰⁰⁰⁰-to-3'⁰⁰⁰⁰ exonucleolytically digested up to site B₁S to yield the 27SB₃ precursor, a reaction requiring exonuclease Xrn1p and Rat1p (21). The minor pathway processes the 27SA₂ molecule at site B₁L, producing the 27SB₄ pre-rRNA. While processing at site B₁ is completed, the 3'⁰⁰⁰⁰ end of mature 25S rRNA is generated by processing at site B₂. The subsequent ITS2 processing of both 27SB₃ species appears to be identical. Cleavage at sites C₁ and C₂ releases the mature 25S rRNA and the 7S pre-rRNA. The latter undergoes exosome-dependent 3'⁰⁰⁰⁰-to-5'⁰⁰⁰⁰ exonuclease digestion to the 3'⁰⁰⁰⁰ end of the mature 5.8S rRNA (36, 37); this reaction also requires the putative ATP-dependent RNA helicase Do61p (12). The data presented in this study suggest that Dbp6p is required for the assembly of 60S ribosomal subunits, a process that may also involve three other putative ATP-dependent RNA helicases: Dbp7p (10), Drs1p (44), and Spb4p (49). See references 59 and 67 for reviews on pre-rRNA processing and trans-acting factors.
They might be required to provide an accessible sequence in pre-rRNA substrates for proper endonucleolytic processing to take place, as has been suggested for Dbp3p or Fallp (28, 72), or they could disrupt secondary structures that might otherwise stall or block the activity of an exonuclease, as has been suggested for Doblp (12). In addition, many snoRNAs, including U3, U14, and the methylation and pseudouridylation guide snoRNAs, form extensive base pair interactions with pre-rRNAs (60). Thus, RNA helicases may play roles in the association or dissociation reactions of these snoRNAs with the pre-rRNA, as has been suggested for Dbp3p and Roklp (31, 65). Finally, extensive structural rearrangements between pre-rRNAs and r-proteins probably occur during the assembly reactions, and these may require RNA helicases. It is suggested that Dbp7p, Dps1p, and Spl3p are involved in such processes (10, 44, 49).

In this report, we describe the functional analysis of the previously uncharacterized putative ATP-dependent RNA helicase Dbp6p (DEAD-box protein 6). We show that Dbp6p is essential for cell viability and that it localizes to the nucleolus. In vivo depletion of Dbp6p results in a defect in 60S ribosomal subunits and the appearance of lower-molecular mass polypeptides. This defect is accompanied by decreased production of the 27S and 7S precursors, which leads to a decrease in the levels of the mature 2S and 5.8S rRNAs. These phenomena lead us to conclude that Dbp6p depletion results in improper assembly of preribosomal particles during the biogenesis of 60S ribosomal subunits.

**MATERIALS AND METHODS**

Strains, media, and methods. The *S. cerevisiae* strains used in this study are derivatives of diploid strain W303 (MATa/MATa ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3 A159-115 trp1-1/ trp1-1 can1-100 HIN3 len2a/LEU2 trp1 SP63/TRPI) (73). Separate PCRs were set up to obtain the 5’ long flanking homology region (including the start codon and the pA6a poly(A) tail sequence at its 3’ end) and the 3’ long flanking homology region (including the DBP6 ORF sequence, starting at +1741, and the pA6a poly(A) tail sequence at its 5’ end). These LFH1-PCR products were then used as primers to amplify the kanMX4 heterologous marker module from the EcoRV-linearized plasmid pFA6a-kanMX4 (71). The LFH2-PCR product was used to directly transform yeast. Selection for transformants was done on yeast extract-peptone-dextrose (YPD) plates containing 200 mg of G418 (Gibco BRL) per liter. Integration at the correct genomic locus was verified by Southern blotting. The oligonucleotides used with LFH1-PCR was P5 (‘ATT TCA GTC CCA CGA ACT GAT’) (starting 418 bp upstream of the DBP6 start codon), P5’ long (‘GGG GAT CAT CGC TCG ACC TGC AGC G’ (5’)) (starting 418 bp upstream of the DBP6 start codon), Sph I-prepared vector pRS416 (55), resulting in the plasmid pRS416-DBP6. Subcloning of a 2.8 kb SalI fragment into pRS415 (55), YCplac111 (19), and YEplac111-DBP6, and YEplac111-DBP6, respectively. The four plasmids constructed complemented the dbp6 null allele to the wild-type extent.

**DBP6 HA epitope tagging and cloning under the control of a galactose-inducible promoter.** DBP6 was cloned using PCR (polymerase) with oligonucleotides introducing the restriction sites SalI (‘5’GCA CGC GAT CTC GAT TTG GCA TCG AGA TTA GAT CAG’ (the SalI site is underlined, and the DBP6 ORF homology region, starting with the second codon, is in bold type)) and SphiI (‘GGG AGG CAT AAC CTG GAA CAA CTG CGT GAT ATG CCG TCA TTG GCT AAG ATT GCT GAT’ (the SphiI site is underlined, and the DBP6 3’ downstream homology region, starting 176 bp after the stop codon, is in bold type)). The SalI/SphiI-restricted PCR product was cloned into the SalI/SphiI-cut YCplac111-based plasmid pAS24 (52). The resulting plasmid, pAS24-DBP6, containing a GAL1 promoter-fragmented DBP6 ORF followed by a double hemagglutinin (HA) tag, and the DBP6 ORF and its 3’-flanking homology region. This construct was transformed into strain YDK8-1A(pRS416-DBP6), and segregation of the plasmid harboring URA3 DBP6 (fluoroorotic acid selection) resulted in strain YDK8-1A(pAS24-DBP6). We also refer to this strain as the GAL::DBP6 strain or, if grown in YPD medium, as the Dbp6p-depleted strain.

In order to express an N-terminally HA-tagged Dbp6p fusion protein from its cognate promoter at approximately wild-type levels, a fusion PCR was performed (25). Briefly, two fragments with sequence overlap were generated in a first PCR series with SacI-restricted pRS416-DBP6 as a template and the oligonucleotides 5’GGG AGG CAT AAC CTG GAA CAA CTG CGT GAT ATG CCG TCA TTG GCT AAG ATT GCT GAT’ (the SphiI site is underlined) and 5’GTA AAA CGA CGG CCA GT3’ (universal primer), 5’TGA TTG ACC TCC CTA TGA CGT CCG GCC GTA CAG AGC TCT GTG AAT ATG AAG TGG TAA ATG TGG AAC TAT GCA ATT CAT CGA ATT TCG TAT TGG ATG ATC TAA AAG ATC TAC T (the DBP6 ORF homology region is in bold type, and the overlapping part of the double HA tag is underlined), 5’GTA AAA CGA CGG CCA GT3’ (universal primer), and 5’t5’ (‘5’CTT TTA CCA TGA TAC T3’) (reverse complement sequence within the DBP6 ORF, starting at +318) as primers. The PCR products, together with the universal primer and the SphiI primer, were used for the fusion PCR. The final product was cloned as a XhoI/Nhel fragment into the XhoI/Nhel-restricted plasmid pRS415-DBP6 to yield plasmid pRS415-HA-DBP6. The HA fusion and DBP6 ORF sequences originating from the fusion PCR were verified by sequencing. This construct complemented the dbp6 null allele to the wild-type extent, and HA-tagged Dbp6p was detected by Western blotting as a band that migrated at the expected molecular mass of approximately 73 kDa.

**Polysome analysis and total ribosomal subunit quantification.** Polyribosome preparation, polysome analysis, and ribosomal subunit preparation were done according to Fioani et al. (15) as previously described (26). Gradient analysis was performed with an ISCO UV-6 gradient collector with continuous monitoring at λ260.

**Indirect immunofluorescence.** Strains YDK8-1A(pRS415-HA-DBP6) and YDK8-1A(pRS415-DBP6) were grown to an optical density of 600 nm (OD600) of about 0.5 in YPD medium, and 5 ml of cells was harvested by centrifugation. Preparation of yeast cells for immunofluorescence was done according to standard procedures (43). DAPI (4’,6-diamidino-2-phenylindole dihydrochloride; Fluka) was used to stain DNA. Primary monoclonal mouse anti-HA antibody 12B12 (BAbCO; at a dilution of 1/1000, and 5000) was used to detect HA-DBP6. Polyclonal rabbit anti-Nop1p antibodies (obtained from E. C. Hurt, University of Heidelberg), at a dilution of 1/500, and secondary goat anti-rabbit fluorescein-conjugated antibodies (Pierce), at a dilution of 1/200, were used to detect the endogenous protein. Immunofluorescence microscopy was done in a Zeiss Axioskop fluorescence microscope with the Plan-NEOFUOR 100X.1.3 objective. Photographs were taken with Kodak Tmax p3200 and transferred to Kodak PhotoCD. Figures were arranged with Adobe Photoshop and printed on a Kodak Digital Science 6560 PS color printer.

**Pulse-chase labeling of pre-rRNA.** Cells of strains YDK8-1A(pAS24-DBP6) and YDK8-1A(pRS415-DBP6) were grown in yeast extract peptone-galactose (YPGAL) medium, shifted to YPD medium, grown in 40 ml of synthetic dextrose (SD) medium lacking methionine (SD-Met) to an OD600 of about 1, and concentrated in 1 ml of SD-Met. Cells were then pulse-labeled for 1 min with 250 μCi of [5,6-3H]uracil (Amersham; 45 to 50 Ci/mmol). The chase was initiated by diluting 200-μl aliquots of the pulse-labeled cells in 4 ml of 3H medium containing 1 mg of cold uracil per ml. Cells were harvested after 0, 5, 15, 30, and 60 min of chase and washed twice in ice-cold water, and frozen in liquid nitrogen. Total RNA was extracted by the acid-phenol method (4).

They were measured by scintillation counting, and 30,000 cpm per RNA extract was loaded and resolved on 1.2% agarose–formaldehyde gels as previously described (26, 49).

For [5-3H]uracil pulse-chase labeling, cells of strains YDK8-1A(pAS24-DBP6)(pRS416) and YDK8-1A(pRS416-DBP6) were grown in synthetic galactose (SGal)-Ura and then in 40 ml of SD-Ura to an OD600 of about 1. The cells were concentrated in 1 ml of SD-Ura and pulse-labeled for 2 min with 100 μCi of [5-3H]uracil (Amersham; 45 to 50 Ci/mmol). The chase was initiated by diluting 250-μl aliquots of the pulse-labeled cells in 4 ml of 3H medium containing 1 mg of cold uracil per ml. Cells were harvested after 0, 5, 15, 30, and 60 min of chase and washed twice in ice-cold water, and frozen in liquid nitrogen. Total RNA was extracted by the acid-phenol method (4).

Uracil incorporation was measured by scintillation counting, and 30,000 cpm per RNA extract was loaded and resolved on 1.2% agarose–formaldehyde and 7% polyacrylamide–8 M urea gels. RNA was transferred to Hybond N membrane, dried, and autoradiographed as described previously (28, 66), and the filters were baked for 2 h at 80°C, sprayed.
with En'Hance (Du Pont), dried, and exposed to X-ray films for 4 days at −80°C with an intensifying screen.

Northern and primer extension analyses. Steady-state levels of pre-rRNA and mature rRNA were assessed by Northern and primer extension analyses. Oligonucleotides (numbered from 1 to 9 according to the scheme in Fig. 1A) 5′-A0, 18S, D/A0, A0/3′A, A0/B0, 5.8S, E/C0, C0/C, and 25S (and the oligonucleotide 5′-5′GATCACCCAATCATACCTGG3′) were end labeled with 30 μCi of [γ-32P]ATP (Amersham; 5,000 Ci/mmol) by use of T4 polynucleotide kinase (Appligene). Total RNA was extracted as described above, and 5 μg was loaded and resolved on 1.2% agarose-formaldehyde gels. For analysis of low-molecular-weight rRNA species, RNA samples (ca. 2.5 μg) corresponding to equal amounts of OD260 units of cells were separated on 7% polyacrylamide–8 M urea gels. RNA bands were transferred to and immobilized on nylon membranes as described above. Prehybridization and hybridization were done with Church buffer (9). Washes were done with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate, and 0.1× SSC–0.5% sodium dodecyl sulfate, and the membranes were exposed to X-ray films at −80°C with an intensifying screen.

Primer extension was done with the same RNA samples as those used for Northern analysis according to Venema and Tolleri (68). Oligonucleotides 6′-A0, 5′-A0, 5′-A0, 5′-A0, 5′-A0, and 5′-A0 were used. To identify the positions of the primer extension stops, plasmid-borne rRNA genes were sequenced with the oligonucleotides listed above. Avian myeloblastosis virus reverse transcriptase and RNaseA were purchased from Pharmacia.

Miscellaneous. Total yeast protein extracts were prepared and analyzed by Western blotting according to standard procedures (4, 50). Monoclonal antibody 16B12 and goat anti-mouse alkaline phosphatase-conjugated antibody (Bio-Rad) were used as primary and secondary antibodies, respectively. DNA manipulations were done according to Sambrook et al. (50) with Escherichia coli DH10B for subcloning and amplification of plasmid DNA. Forideoxy sequencing, a T7 sequencing kit (Pharmacia) was used. Sequence comparisons were performed at the Saccharomyces Genome Database (Stanford University) and NCBI facilities.

RESULTS

Dbp6p is a putative ATP-dependent RNA helicase that is essential for cell viability. Dpb6p (YNR038W) is an ORF of S. cerevisiae chromosome XIV. The Dpb6p ORF encodes a protein of 629 amino acids with a predicted molecular mass of 70.4 kDa. Dpb6p is also predicted to be acidic (pI, 5.88) and to localize within the cytoplasm (SwissProt Psort program [3]). Calculation of the codon adaptation index (54) suggests that Dpb6p, with a codon adaptation index of 0.19, is of moderate to low intracellular abundance.

Sequence analysis revealed that Dpb6p belongs to the DEAD-box protein family of putative ATP-dependent RNA helicases (Fig. 2). Seven of the eight conserved motifs characteristic of DEAD-box proteins are found in Dpb6p (bold type and underlined). The helicase core region extends from amino acid 234 (A motif) to amino acid 596 (HRVGR motif). A portion of the N-terminal domain, starting at amino acid 39 and ending at amino acid 124, is highly enriched in serine and the negatively charged amino acids aspartic acid and glutamic acid (underlined).

FIG. 2. Dpb6p encodes a putative ATP-dependent RNA helicase of the DEAD-box protein family. Seven of the eight conserved motifs characteristic of DEAD-box proteins are found in Dpb6p (bold type and underlined). The helicase core region extends from amino acid 234 (A motif) to amino acid 596 (HRVGR motif). A portion of the N-terminal domain, starting at amino acid 39 and ending at amino acid 124, is highly enriched in serine and the negatively charged amino acids aspartic acid and glutamic acid (underlined).

As a first approach to the functional analysis of Dpb6p, we constructed a dbp6 null allele. We replaced most of one Dpb6p ORF copy in the diploid strain W303 with the kanMX4 marker module (see Materials and Methods). Correct integration at the DBP6 ORF complemented its null allele after transformation of the DBP6-dbp6::kanMX4 heterozygote (YDK8) with pRS416-DBP6 and subsequent sporulation and tetrad analyses (data not shown). Furthermore, Dpb6p was required for vegetative growth, as judged by the lack of growth of YDK8-1A(pRS416-DBP6) on 5-fluoroorotic acid-containing plates (data not shown). These results showed that Dpb6p is essential for cell viability.

Construction of a GAL::DBP6 strain. To determine the essential Dpb6p function, a conditional system for phenotypic analysis was established. The Dpb6p ORF and its 3′ downstream sequence were cloned under the control of a galactose-inducible promoter, which allows gene expression in medium containing galactose (YPGal) and represses gene expression in glucose-based medium (YPD). The resulting plasmid, pAS24-DBP6, expressed an N-terminally HA-tagged Dpb6p that complemented the dbp6 null mutant (YDK8-1A) on YPGal plates to the wild-type extent and resulted in a strong slow-growth phenotype on YPD plates at 30°C (data not shown). After YDK8-1A(pAS24-DBP6) was shifted from YPGal medium to YPD medium, the growth rate remained similar to that of the wild-type control strain YDK8-1A (YPGal 111-DBP6) for the first 12 h but then progressively decreased to a doubling time of more than 8 h after 36 h in YPD medium (Fig. 3A). Comitant with the decrease in the growth rate, the cells were depleted of HA-Dpb6p, as detected by Western blot analysis (Fig. 3B).

In vivo depletion of Dpb6p leads to a deficiency in 60S ribosomal subunits. As some DEAD-box proteins are involved in either translation initiation (6, 8, 11) or ribosome biogenesis (28, 44, 72), we first investigated by polysome profile analysis whether Dpb6p was involved in one of these two processes. For this purpose, YDK8-1A(pAS24-DBP6) was grown at 30°C in YPGal medium and shifted to YPD medium, and polysomes were extracted at different times. Wild-type polysome profiles were obtained after 6 h (Fig. 4A). Comitant with the decrease in the growth rate and in HA-Dpb6p levels after 24 h, the Dpb6p-depleted strain showed a deficit of free 60S versus 40S ribosomal subunits, an overall decrease in 80S ribosomes (free couples and monosomes) and polysomes, and an accumulation of half-mers polysomes (Fig. 4B). The 60S-
Dbp6p is involved in 60S-ribosomal-subunit biogenesis

To distinguish between a cytoplasmic role and a nucleolar role of Dbp6p, the subcellular localization of Dbp6p was analyzed by indirect immunofluorescence. For this purpose, DBP6 was HA tagged at its 5′ end by fusion PCR and cloned into pRS415 to express the N-terminally epitope-tagged Dbp6p from its cognate promoter at approximately wild-type levels (see Materials and Methods). The resulting plasmid (pRS415-HA-DBP6) or a control plasmid harboring the untagged DBP6 gene (pRS415-DBP6) was transformed into strain YDK8-1A(pRS415-DBP6) (data not shown). The predominant localization of HA-Dbp6p was detected by anti-HA antibodies, followed by decoration with goat anti-mouse rhodamine-conjugated antibodies (Fig. 5A). For precise localization, the nucleus was visualized by staining of DNA with DAPI (Fig. 5C) and the nucleolus was stained with anti-Nop1p antibodies (Fig. 5B). The fluorescence photographs demonstrated that HA-Dbp6p is restricted to the nucleolus (Fig. 5A), where it colocalizes with Nop1p (Fig. 5B). In most cells, Dbp6p and Nop1p showed the typical crescentic or cap-like staining pattern of nucleolar proteins. No signal was obtained with the combination of anti-HA and goat anti-mouse rhodamine-conjugated antibodies when cells of strain YDK8-1A(pRS415-DBP6) were analyzed by indirect immunofluorescence (data not shown). The predominant localization of HA-Dbp6p in the nucleolus, the specialized compartment for ribosome biosynthesis (35), indicated that Dbp6p is implicated in the biogenesis of 60S ribosomal subunits.

Formation of the mature 25S and 5.8S rRNAs is impaired upon Dbp6p depletion. To study the role of Dbp6p in 60S-ribosomal-subunit biogenesis, we performed polysome runoff and low-Mg²⁺ cell extracts. The 60S-to-40S ratio was determined from the OD₆₀₀ of about 0.5, and cells were processed for immunofluorescence. The HA-tagged Dbp6p was detected by anti-HA antibodies, followed by decoration with goat anti-mouse rhodamine-conjugated antibodies (Fig. 5A). For precise localization, the nucleus was visualized by staining of DNA with DAPI (Fig. 5C) and the nucleolus was stained with anti-Nop1p antibodies (Fig. 5B). The fluorescence photographs demonstrated that HA-Dbp6p is restricted to the nucleolus (Fig. 5A), where it colocalizes with Nop1p (Fig. 5B). In most cells, Dbp6p and Nop1p showed the typical crescentic or cap-like staining pattern of nucleolar proteins. No signal was obtained with the combination of anti-HA and goat anti-mouse rhodamine-conjugated antibodies when cells of strain YDK8-1A(pRS415-DBP6) were analyzed by indirect immunofluorescence (data not shown). The predominant localization of HA-Dbp6p in the nucleolus, the specialized compartment for ribosome biosynthesis (35), indicated that Dbp6p is implicated in the biogenesis of 60S ribosomal subunits.

Depletion of Dbp6p resulted in a deficiency in 60S ribosomal subunits. YDK8-1A(pAG24-DBP6) was grown in YPgal medium and shifted to YPD medium for up to 24 h. Polysome analysis was done after 6 h (A) and 24 h (B). Cell extracts were resolved in 7 to 50% sucrose gradients. The peaks of free 40S and 60S ribosomal subunits, 80S ribosomes (free couples and monosomes), and polysomes are indicated. Half-mr polysomes are indicated by arrows.
Dbp6p is required for normal pre-rRNA processing. To define the pre-rRNA processing steps that are affected upon Dbp6p depletion, steady-state levels of pre-rRNA and mature rRNA were determined by Northern blot and primer extension analyses. Different oligonucleotides hybridizing to defined regions of the 35S pre-rRNA transcript (Fig. 1A) were used to monitor specific processing intermediates in a wild-type control strain and during a time course of Dbp6p depletion. Depletion of Dbp6p resulted in a slight decrease in 18S rRNA and a more drastic decrease in 25S rRNA steady-state levels (Fig. 7A). Probing with oligonucleotide 1 (Fig. 7B), which hybridizes 5' to site A3, revealed that the GAL::DBP6 strain accumulated, with ongoing depletion of Dbp6p, an aberrant processing product that had already been detected by the pulse-chase labeling experiments. This aberrant species could also be detected with oligonucleotides 3 (Fig. 7C) and 4 (Fig. 7D) but not with oligonucleotide 5 (Fig. 7E), indicating that this rRNA molecule might extend from the 5' end of the 5'S ETS to the A3 site and thus correspond to the previously described aberrant 23S processing product (67). Concomitant with Dbp6p depletion, the amounts of the 32S pre-rRNA (Fig. 7D) and the 20S pre-rRNA (Fig. 7C) diminished slightly. More importantly, the 27SA and 27SB pre-rRNAs were strongly depleted (Fig. 7D to F; data not shown for oligonucleotide 8).

To assess the steady-state levels of low-molecular-weight ribosomal-subunit biogenesis, we first analyzed the effects of Dbp6p depletion on the synthesis and processing of pre-rRNA by [methyl-3H]methionine pulse-chase labeling experiments. For this purpose, YDK8-1A(pAS24-DBP6) and the wild-type control strain YDK8-1A(pRS415-DBP6) were grown first as logarithmic cultures in YPGal medium, then for 12 h in YPD medium, and finally for another 10 h to an OD₆₀₀ of about 1 in SD-Met. At this time, the GAL::DBP6 strain was doubling every 5.5 h, compared to 2.5 h for the wild-type strain. The cells were pulse-labeled for 1 min and then chased for 2, 5, and 15 min with an excess of cold methionine. We observed a net decrease in the ratio of labeled 25S to 18S mature rRNAs in the Dbp6p-depleted strain relative to the wild-type strain (Fig. 6A, lanes 3, 4, 7, and 8). Furthermore, processing of the 35S precursor was slightly delayed, and practically no 32S precursor was formed. Interestingly, different bands of high molecular weight appeared at the 1-min pulse time point. These bands presumably correspond to 35S pre-rRNA-derived intermediates or degradation products that became metastable in the absence of Dbp6p. Moreover, less of the 27S species formed, with the 27SA and 27SB precursors not persisting until the 2-min chase time point (Fig. 6A, lanes 5 and 6). The formation of the 18S rRNA was also weakly impaired, as revealed by the lower levels of its 20S precursor and the mature species itself and by the accumulation of an aberrant 23S species (Fig. 6A, lanes 5 and 6). However, the kinetics of processing of the 20S precursor to the mature 18S rRNA seemed not to be affected.

To exclude a defect in rRNA methylation and to monitor the processing and formation of low-molecular-weight RNAs, cells were also pulse-labeled with [5,6-3H]uracil. Strains YDK8-1A(pAS24-DBP6)(pRS416) and YDK8-1A(pRS416-DBP6) were grown first as logarithmic cultures in SGal-Ura and then for 22 h in SD-Ura to an OD₆₀₀ of about 1. At this time, the GAL::DBP6 strain was doubling every 4.5 h, compared to 2.75 h for the wild-type strain. The cells were pulse-labeled for 2 min and then chased for 5, 15, 30, and 60 min with an excess of cold uracil. Results comparable to those shown in Fig. 6A were obtained after total RNA samples were analyzed by agarose gel electrophoresis (data not shown), thus excluding an rRNA methylation defect following Dbp6p depletion. Analysis of low-molecular-weight RNAs by polyacrylamide gel electrophoresis showed that the synthesis of mature 5.8S rRNA was substantially reduced upon Dbp6p depletion. The synthesis of 5S rRNA and the labeling of tRNAs, however, were comparable in both kinetics and levels in wild-type and Dbp6p-depleted cells (Fig. 6B). Altogether, these results indicated that the deficit in 60S ribosomal subunits following Dbp6p depletion, steady-state levels of pre-rRNA and mature rRNAs, and tRNAs are indicated.

FIG. 6. Dbp6p depletion leads to reduced synthesis of the mature 25S and 5.8S rRNAs. (A) Wild-type control strain YDK8-1A(YCplac111-DBP6) (DBP6) and strain YDK8-1A(pAS24-DBP6) (GAL::DBP6) were grown at 30°C in YPGal medium, shifted for 12 h to YPD medium, and then grown for 10 h in SD-Met. Cells were pulse-labeled (p) for 1 min with [methyl-3H]methionine and then chased (c) for 2, 5, and 15 min with an excess of unlabeled methionine. Total RNA was extracted, and 20,000 cpm was loaded and separated on a 1.2% agarose–formaldehyde gel, transferred to a nylon membrane, and visualized by fluorography. (B) Strains YDK8-1A(pRS416-DBP6) (DBP6) and YDK8-1A(pAS24-DBP6)(pRS416) (GAL::DBP6) were grown at 30°C in SGal-Ura and then shifted to SD-Ura for 22 h. Cells were pulse-labeled (p) for 2 min with [5,6-3H]uracil and then chased (c) for 5, 15, 30, and 60 min with an excess of unlabeled uracil. Total RNA was extracted, and 30,000 cpm was loaded and separated on a 7% polyacrylamide–8 M urea gel, transferred to a nylon membrane, and visualized by fluorography. The positions of the different pre-rRNAs, mature rRNAs, and tRNAs are indicated.
rRNAs, samples were separated on polyacrylamide gels and subjected to Northern blot analysis with 7S-, 5.8S-, and 5S-specific oligonucleotides. Hybridizations indicated that the 7S pre-rRNA was strongly depleted (Fig. 8, top panel) and that the levels of the mature 5.8S rRNA were also decreased (Fig. 8, middle panel). However, the steady-state levels of the 5S rRNA were only very slightly affected by Dbp6p depletion (Fig. 8, bottom panel).

Because Northern hybridization poorly detects mature 5.8S, rRNA and because it does not distinguish between the 27SA1 and 27SA2 precursors and between the 27SB1 and 27SB2 precursors, we assessed the levels of these species by primer extension. This analysis confirmed that Dbp6p depletion led to a net decrease in all the 27S precursors, with the 27SB species being most drastically affected (Fig. 9A), and to diminished levels of both mature 5.8S1 and mature 5.8S2 rRNAs (Fig. 9B). Furthermore, primer extension showed that processing at all sites tested was correct at the nucleotide level during the time course of Dbp6p depletion.

Altogether, our results demonstrate that Dbp6p depletion leads to decreased formation and decreased steady-state levels of the 27S and 7S precursors and, as a consequence, of the mature 25S and 5.8S rRNAs. These phenotypes may be due to or may cause an improper assembly of preribosomal particles during the biogenesis of 60S ribosomal subunits. The nucleolar localization of HA-Dbp6p and the 60S-ribosomal-subunit deficiency upon Dbp6p depletion are in agreement with a proposed role of Dbp6p in 60S-ribosomal-subunit biogenesis.

DISCUSSION

In this paper, we describe the functional analysis of Dbp6p, a putative ATP-dependent RNA helicase of the DEAD-box protein family. Disruption analysis showed that Dbp6p is essential for cell viability. In vivo depletion of Dbp6p resulted in a deficit in 60S ribosomal subunits, which led to the appearance of half-mer polysomes. Similar polysome profiles have been described for mutants defective in r-proteins of the 60S ribosomal subunit (13, 39, 69) and for mutants defective in components involved in pre-rRNA processing and 60S-ribosomal-subunit assembly (24, 44, 56, 72, 75). Since we detected N-terminally HA-tagged Dbp6p only in the nucleolus, we concluded that Dbp6p is not a structural component of 60S ribosomal subunits but rather plays a role in their biogenesis. Interestingly, Dbp6p is predicted to be cytoplasmic and lacks a consensus nuclear localization signal. This finding may indicate
that Dbp6p carries an as-yet-unknown signal sequence for nuclear targeting or that it is imported into the nucleus by binding to a nucleus-targeted protein in the cytoplasm.

To define the role of Dbp6p in the biogenesis of 60S ribosomal subunits, we investigated the formation and steady-state levels of pre-rRNA and mature rRNA by pulse-chase labeling, Northern blotting, and primer extension analyses. Pulse-chase labeling of pre-rRNA showed that the synthesis of the mature 25S and 5.8S rRNAs was quantitatively diminished and kinetically delayed compared to the formation of the mature 18S and 5S rRNAs. Processing of the 35S precursor was slightly delayed, and practically no 32S precursor was detected. Most notably, different high-molecular-weight species, most likely corresponding to 35S pre-rRNA-derived degradation products, could be detected during the pulse. We propose that the processed 35S pre-rRNA might not have been stable in the absence of Dbp6p and, as a consequence, that less of the 27S species was formed. Furthermore, the stability of the 27S precursors was probably also affected because they did not persist until the first chase time point. Moreover, the formation of the 18S rRNA was also weakly impaired, as revealed by the appearance of the aberrant 23S processing product and by the lower levels of the 20S pre-rRNA and the mature 18S rRNA species.

The results of the pulse-chase labeling experiments were confirmed by Northern blotting and primer extension analyses, which showed that the steady-state levels of the 27SA and, more drastically, of the 27SB precursors were decreased. As a consequence, the 7S pre-rRNA was strongly depleted, and reduced steady-state levels of the mature 25S and 5.8S rRNAs were detected. In agreement with the finding that newly synthesized 5S rRNA is more stable than 5.8S rRNA upon depletion of the 60S-subunit protein L16 (38), the steady-state levels of the 5S rRNA were only very slightly affected by Dbp6p depletion. We conclude that Dbp6p is required for the normal formation of 25S and 5.8S rRNAs. The amounts of both the 32S and the 20S pre-rRNAs also were diminished slightly, and the aberrant 23S species, which is the product of direct cleavage of the 35S pre-rRNA at site A₂ when processing at sites A₀, A₁, and A₂ is delayed or inhibited, accumulated. These findings could implicate Dbp6p in 18S rRNA synthesis; however, this role would only be a minor one, since the steady-state levels of both the 40S ribosomal subunit and the 18S rRNA were less affected than the steady-state levels of the 60S ribosomal subunit and the mature 25S or 5.8S rRNA. In agreement with these findings, reduced processing of the 35S precursor, accumulation of the aberrant 23S species, and some depletion of the 20S pre-rRNA and the 18S rRNA have been reported for different mutants affecting 60S-ribosomal-subunit biogenesis (5, 12, 24, 72, 75). Thus, delayed processing at sites A₀, A₁, and A₂ may be a general feature of mutations that interfere with the synthesis of mature 25S and 5.8S rRNAs. The mechanism is unclear; however, it is likely that all of the processing machinery interacts to some extent or that there is some form of feedback inhibition due to defective 25S and 5.8S rRNA synthesis. Indeed, the biogenesis of mature 40S ribosomal units
and the biogenesis of mature 60S ribosomal subunits are not independent events. Instead, processing of the 35S pre-rRNA requires the assembly of this primary transcript into a ribonucleoprotein particle, 90S RNP, which contains many r-proteins of the mature 40S and 60S ribosomal subunits as well as non-ribosomal proteins that are most likely trans-acting factors involved in pre-rRNA processing or ribosome assembly reactions (63). Furthermore, functional interactions between the processing machinery responsible for cleavages at sites A₉, A₁, and A₃ (small nuclear RNP complex) and that responsible for cleavage at site A₂ (RNAse MRP complex) have been reported, and they probably occur via the bridging factor Rrp5p (2, 68).

In addition to Dbp6p, other proteins have been shown to be required for 60S-ribosomal-subunit biogenesis. All of these proteins can be arbitrarily grouped into two classes. The first class consists of trans-acting factors that are directly involved in pre-rRNA processing reactions and includes the endonucleolytic RNAse MRP complex, the exonucleases Xrn1p and Rat1p, the exosome, and the putative ATP-dependent RNA helicases Dbp3p and Dobl1p (see Fig. 1B and its legend for further information and references). In contrast, the precise functional roles of proteins belonging to the second class are not clear. This class includes nucleolar and r-proteins that are required for proper ribosome assembly. Mutations in or in vivo depletion of Nip7p, Nop2p, Nop3p, and the r-proteins L1 and L32 lead to an accumulation of one of the 27S precursors (13, 24, 47, 69, 75), while in vivo depletion of Nop4p/Nop77p results in some depletion of 27SA and, more drastically, of 27SB pre-rRNAs (5, 56). The phenotype observed after the depletion of Dbp6p most closely resembles that described for Nop4p/Nop77p-depleted strains (5, 56). In both cases, early cleavage at sites A₉ to A₂ is kinetically delayed, but the most striking effect is the large decrease in 27SB pre-rRNA levels. In contrast, the depletion of Nop4p/Nop77p but not of Dbp6p impairs rRNA methylation. We conclude that Dbp6p, like Nop4p/Nop77p, plays a primary role in the early ribosome assembly steps leading to the formation of mature 60S ribosomal subunits. Considering its presumed RNA helicase activity, Dbp6p could function in the unwinding of the pre-rRNA to promote specific intramolecular rRNA or rRNA-protein interactions. An abortive assembly of the pre-RNP in the absence of Dbp6p would then either be the cause or the consequence of the instability and rapid turnover of the 27S pre-rRNAs. The increased stability of these 27S precursors observed during the depletion of Nip7p, Nop2p, and Nop3p strongly suggests that these trans-acting factors are involved in ribosome assembly steps downstream of the ones assisted by Nop4p/Nop77p and Dbp6p. This involvement may occur at a point where the assembly of 60S ribosomal subunits is close to completion and assembly would more likely be arrested rather than aborted (24).

In agreement with a 60S-ribosomal-subunit assembly function for Dbp6p, we obtained predominantly cold-sensitive dbp6 mutants (at 18°C) by random PCR mutagenesis. When examined by polysome profile analysis, all of these mutants displayed a 60S-ribosomal-subunit deficiency phenotype similar to that observed upon Dbp6p depletion (29). It has been argued on the basis of thermodynamic grounds that mutations affecting assembly reactions may be intensified at low temperatures (7). Indeed, many cold-sensitive alleles blocking ribosome assembly have been found in bacteria (20, 57). Furthermore, cold-sensitive DEAD-box protein mutations that affect bacterial and yeast ribosome assembly have been identified (40, 44, 49, 62, 72).

The functional analysis of Dbp6p reported here is, to our knowledge, the first report that clearly implicates a putative ATP-dependent RNA helicase in the assembly of 60S ribosomal subunits in S. cerevisiae. However, earlier publications suggested, based on polysome profile analyses and pulse-chase labeling experiments, that mutations in two other putative ATP-dependent RNA helicases, Drs1p and Spb4p, also affect 60S-ribosomal-subunit assembly (44, 49). Unfortunately, the destiny of the 27S pre-rRNAs in drs1Δ and sbp4Δ mutants has not been assessed so far. As Dbp6p, Drs1p, and Spb4p are all essential for cell viability, it is unlikely that they carry out redundant functions. More recently, it was also shown that Dbp7p, a nonessential putative ATP-dependent RNA helicase, may also assist in early 60S-ribosomal-subunit assembly reactions (10). However, experimental evidence indicates that Dbp6p and Dbp7p are not genetically redundant (10, 29). We conclude that there are at least four putative ATP-dependent RNA helicases that play nonredundant roles in the assembly of 60S ribosomal subunits. It will be interesting to determine what their precise functions are and why so many putative RNA helicases are needed in this process. Thus, it will be crucial to determine whether these proteins exhibit RNA unwinding or RNA-dependent ATPase activities and what their specific RNA substrates are.

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