The Putative RNA Helicase Dbp6p Functionally Interacts With Rpl3p, Nop8p and the Novel trans-acting Factor Rsa3p During Biogenesis of 60S Ribosomal Subunits in Saccharomyces cerevisiae

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ABSTRACT
Ribosome biogenesis requires at least 18 putative ATP-dependent RNA helicases in Saccharomyces cerevisiae. In this study, we have performed a synthetic lethal screen with dbp6 alleles. We have previously characterized the nonessential Rsa1p, whose null allele is synthetically lethal with dbp6 alleles. Here, we report on the characterization of the four remaining synthetic lethal mutants, which reveals that Dbp6p also functionally interacts with Rpl3p, Nop8p, and the so-far-uncharacterized Rsa3p (ribosome assembly 3). The nonessential Rsa3p is a predominantly nucleolar protein required for optimal biogenesis of 60S ribosomal subunits. Both Dbp6p and Rsa3p are associated with complexes that most likely correspond to early pre-60S ribosomal particles. Moreover, Rsa3p is co-immunoprecipitated with protA-tagged Dbp6p under low salt conditions. In addition, we have established a synthetic interaction network among factors involved in different aspects of 60S-ribosomal-subunit biogenesis. This extensive genetic analysis reveals that the rsa3 null mutant displays some specificity by being synthetically lethal with dbp6 alleles and by showing some synthetic enhancement with the npl8-101 and the rsa1 null allele.

THE synthesis of ribosomes is a major cellular activity that, in eukaryotes, takes place primarily in a specialized subnuclear compartment termed the nucleolus (Olson et al. 2000). Ribosome synthesis is evolutionarily conserved throughout eukaryotes (Eichler and Craig 1994), and so far most of our knowledge concerning this highly complex and dynamic process comes from studies with Saccharomyces cerevisiae. In yeast nucleoli, the ribosomal RNA (rRNA) genes are transcribed as precursors (pre-rRNAs), which undergo processing and covalent modification (Kressler et al. 1999b; Venema and Tollervey 1999; for a simplified pre-rRNA processing scheme, see Figure 4A). Three of the four rRNAs (18S, 5.8S, and 25S) are transcribed as a single large pre-rRNA by RNA polymerase I, whereas the fourth rRNA (5S) is transcribed by RNA polymerase III. The maturation of pre-rRNAs is intimately linked to their assembly with the 78 ribosomal proteins (r-proteins). The large 60S ribosomal subunits (r-subunits) are composed of 46 r-proteins and three rRNA species (5.8S, 25S, and 5S), while the small 40S r-subunits contain 32 r-proteins and the 18S rRNA (Planta and Mager 1998).

While the processing steps, as well as the protein trans-acting factors involved therein, that lead to the pre-rRNA intermediates and the mature rRNAs are fairly well characterized, less is known about the assembly, intranuclear transport, and export of preribosomal particles. Studies performed in the 1970s outlined a ribosome assembly pathway through identification of a 90S preribosomal particle, containing the 35S pre-rRNA, that is matured into 43S and 66S preribosomes, which contain the 20S and the 27S precursors to the mature 18S and 25S/5.8S rRNAs, respectively (Trapman et al. 1975; Kressler et al. 1999b). In addition, the application of proteomic approaches allowed a more detailed insight (Fatica and Tollervey 2002; Fromont-Racine et al. 2003). The 90S preribosomal particles contain, in addition to the 35S pre-rRNA, the U3 small nucleolar ribonucleoprotein particle, protein trans-acting factors implicated in the biogenesis of 40S r-subunits and small subunit r-proteins; however, they predominantly lack 60S biogenesis protein transacting factors and large subunit r-proteins (Dragon et al. 2002; Grandi et al. 2002). This has led to the proposal of a biphasic model in which the machinery required for 40S r-subunit synthesis almost exclusively assembles onto the 35S pre-rRNA, whereas the 60S r-subunit assembly factors are recruited only to form an early 66S preribosomal particle after the separation of the 20S and the 27S r-pre-rRNAs by

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cleavage at site A2 (Fatica and Tollervey 2002; Grandi et al. 2002). The early 60S particle is found in the nucleo-
lus and exchange of the Noc1p-Noc2p complex by the
Noc2p-Noc3p complex has been suggested to trigger
its intranuclear transport to the nucleoplasm (Milker-
eit et al. 2001). Several distinct pre-60S complexes have
been identified (Bassler et al. 2001; Harnpicharnchai
et al. 2001; Saveanu et al. 2001; Fatica et al. 2002;
Nissan et al. 2002) and they can be placed in a tentative
pre-60S assembly pathway (Fatica and Tollervey
2002). In addition to providing a refined framework
for the course of ribosome assembly, the above proteomic
analyses identified additional protein trans-acting fac-
tors, thus raising the inventory to ~150 proteins. Some
of these protein trans-acting factors can be grouped
according to their physical association with snoRNAs
or their proposed enzymatic function (Kressler et al.
1999b). In agreement with the dynamic nature of the
process, several putative GTPases (Bassler et al. 2001;
Gelperin et al. 2001; Saveanu et al. 2001; Wegierski
et al. 2001), two AAA-type ATPases (Bassler et al. 2001;
Gadal et al. 2001a), and 18 putative ATP-dependent
RNA helicases of the DEAD-box and related protein
families (Kressler et al. 1999b; Bond et al. 2001; Emery
et al. 2004) are implicated in ribosome assembly. These
proteins likely promote or monitor structural rearrange-
ments of rRNA:rRNA, rRNA:protein, or protein:protein
interactions within preribosomal particles, which finally
lead to the generation of export and translation-compe-
tent r-subunits.

Despite the success of the proteomic approach in
identifying preribosomal particles and their compo-
nents, not all protein trans-acting factors implicated in
ribosome assembly have been found in the identified
preribosomal particles. One such factor is the putative
RNA helicase Dbp6p, which is an essential nucleolar
protein that is required for 60S r-subunit assembly and
has been proposed to act at an early step during this
process (Kressler et al. 1998). As for almost all of the
putative RNA helicases involved in ribosome biogenesis,
the functional environment of Dbp6p is not well estab-
lished. To gain more insight into the cellular role of
Dbp6p, we performed a synthetic lethal (sl) screen with
conditional dbp6 alleles (Kressler et al. 1999a). We
previously reported the isolation and characterization of
Rsa1 (ribosome assembly 1), whose null allele is
synthetically lethal with dbp6 alleles (Kressler et al.
1999a). Rsa1p is a nonessential nucleoplasmic protein
that is likely involved in a nucleoplasmic assembly step
of 60S r-subunits and their subsequent nuclear export
(Kressler et al. 1999a; Gadal et al. 2001b). Here we
describe the cloning and the genetic analysis of three
additional sl genes: the large subunit r-protein gene
RPL3, the known protein trans-acting factor encoding
gene NOP8 (Zanchin and Goldfarb 1999), and the
so-far-uncharacterized gene RSA3 (ribosome assembly
3). We provide evidence that the predominantly nucleo-
lar Rsa3p is required for optimal biogenesis of 60S
r-subunits. Furthermore, we show that Rsa3p and Dbp6p
coassembly on sucrose gradients and that Rsa3p is effi-
ciently co-immunoprecipitated with Dbp6p, suggesting
that these two proteins functionally interact within the
same early pre-60S ribosomal particles.

MATERIALS AND METHODS

Strains, media, and genetic methods: Most S. cerevisiae strains
used in this study (Table 1) are derivatives of the diploid
strain W303 (MATa/MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-
3,112/leu2-3,112 tryr1-1/tryr1-1 ura3-1/ura3-1). Preparation of
standard media and genetic manipulations were according to
established procedures (Ausubel et al. 1994; Kainer et al.
1994). Yeast cells were transformed by a lithium acetate
method (Gietz et al. 1992). Deletion disruptions with the
heterologous kanMX4 or HIS3MX6 marker modules were
carried out as previously described (Kressler et al. 1999a).
For tetrad dissection, a Singer MSM micromanipulator was
used.

Plasmids: All recombinant DNA techniques were done ac-
gording to established procedures using Escherichia coli DH10B
and DH5α for cloning and plasmid propagation (Sambrook et al.
1989). Relevant plasmids used in this study are listed in
Table 2. More information on the plasmids is available on
request.

Cloning of RSA3 and rsa3-1: The original sl-mutant strain
sl263210 was outcrossed once with a haploid W303-derived
wild-type strain to yield strain sl263210-6B, which was trans-
formed with both a pSEY18 (gift of M. N. Hall) and a pFL44L
(gift of F. Lacroute)-based yeast genomic library and screened
for clones complementing the slow-growth (sg) phenotype
at 37°C. Candidate plasmids were isolated from yeast, amplified
in E. coli, and retransformed into sl263210-6B. Sequence analy-
sis of three clones revealed that all had the YLR221C (RSA3)
open reading frame (ORF) in common.

To determine whether sl263210 indeed had the RSA3 gene
mutated, this gene was amplified by PCR from genomic DNA
prepared from a wild-type control strain and sl263210. The
wild-type PCR product was digested and cloned into pHAC33
and pHAC111 (gift of M. N. Hall). Screening of the rsa3-1
PCR product as well as of the cloned RSA3 revealed that
the rsa3-1 allele contains two mutations: first, a C-to-T transi-
tion at nucleotide position +329, which changes S110(TCT) to
F(TtT), and second, an insertion of one A at nucleotide posi-
tion +331, which causes a frameshift followed by a premature
stop codon leading to a truncated protein of 144 amino acids
(aa).

Cloning of NOP8 and nop8-101: The original sl-mutant strain
sl264409 was outcrossed once with a haploid W303-derived
wild-type strain to yield strain sl264409-42D, which was trans-
formed with both a YCplac111- and a YEplac181-based yeast
genomic library and screened for clones complementing the
slow-growth (sg) phenotype at 37°C. Candidate plasmids were
isolated from yeast, amplified in E. coli, and retransformed into
sl264409-42D. Sequence analysis of six clones revealed that all
clones had the NOP8 ORF in common. To confirm that the
NOP8 ORF indeed harbored the complementing activity, YC-
plac111-NOP8, which contains only the NOP8 gene, was con-
structed. This construct complements the sg phenotype and
the polyosomal defect of sl264409-42D to the wild-type extent.

To determine whether sl264409-42D indeed had the NOP8
gene mutated, this gene was amplified by PCR from genomic
DNA prepared from a wild-type control strain and sl264409-
42D. The PCR products were digested and cloned into
\begin{table}
\caption{Yeast strains used in this study}
\begin{tabular}{lll}
\hline
Strain$^a$ & Relevant genotype & Source \\
\hline
s263210 & MATa dbp6::kanMX4 ade3::kanMX4 ras3::1 [pRS414-db6p-2] & This study \\
 & [pHT4467-HA-DBP6] & \\
s263210-6B & MATa dbp6::kanMX4 ras3::1 [pRS415-HA-DBP6] & This study \\
s264409 & MATa dbp6::kanMX4 ade3::kanMX4 nop8::101 [pRS414-db6p-3] & This study \\
 & [pHT4467-HA-DBP6] & \\
s264409-42D & MATa nop8::101 & This study \\
s263509 & MATa dbp6::kanMX4 ade3::kanMX4 rpl3::101 [pRS414-db6p-2] & This study \\
 & [pHT4467-HA-DBP6] & \\
s264205 & MATa dbp6::kanMX4 ade3::kanMX4 rpl3::102 [pRS414-db6p-3] & This study \\
 & [pHT4467-HA-DBP6] & \\
YMD3-1A & MATa rsa3::HIS3MX6 & This study \\
YMD3-2A & MATa & This study \\
YMD3-2D & MATa rsa3::HIS3MX6 & This study \\
YDK8-1A & MATa dbp6::kanMX4 [pRS416-DBP6] & KRESSLER et al. (1998) \\
J DY139 & MATa dbp6::kanMX4 ras3::HIS3MX6 [pRS415-protA-DBP6-T7] & This study \\
 & [pHTAC33-RSA3] & \\
MCD1-3B & MATa dbp7::HIS3MX6 [YCplac33-DBP7] & DAUGERON and LINDER (1998) \\
MCD1-7C & MATa dbp7::HIS3MX6 [YCplac33-DBP7] & DAUGERON and LINDER (1998) \\
MCD9H-2D & MATa dbp9::HIS3MX6 [YCplac33-DBP9] & DAUGERON et al. (2001) \\
MCD9H-4C & MATa dbp9::HIS3MX6 [YCplac33-DBP9] & DAUGERON et al. (2001) \\
MMY2-3B & MATa qvl-1 & EISINGER et al. (1997) \\
J DY318 & MATa rpl3::HIS3MX6 [YCplac33-RPL3] & This study \\
J DY319 & MATa rpl3::HIS3MX6 [YCplac33-RPL3] & This study \\
YDK4-1B & MATa rsa1::kanMX4 & KRESSLER et al. (1999a) \\
YDK4-1C & MATa rsa1::kanMX4 & KRESSLER et al. (1999a) \\
YDK3-7A & MATa spb4::kanMX4 [YCplac111-spb4+1] & KRESSLER et al. (1999a) \\
rbb1-TAP & MATa ade2 arg4 leu2-3,112 trp1-289 ura3-52 RRB1-TAP::TRP1 & SCHAPER et al. (2001) \\
\hline
\end{tabular}
\end{table}

$^a$ All strains used in this study, except the rbb1-TAP strain, are derivatives of the diploid strain W305 (see materials and methods for its genotype).

pGEM (Promega, Madison, WI). Sequencing revealed that the nop8-101 mutation is an insertion of one A at nucleotide position +765, which causes a frameshift followed by a premature stop codon leading to a truncated protein of 257 aa.

Cloning of RPL3, rpl3-101, and rpl3-102: The sl strains sl263309 and sl264205 were transformed with a YCplac111-based yeast genomic library and screened for clones complementing the ug and sl phenotypes. Candidate plasmids were isolated from yeast, amplified in based yeast genomic library and screened for clones comple-
thetic enhancement phenotypes, the crosses described below


Synthetic interaction crosses: To determine if different mu-
tants affecting assembly of 60S r-subunits were showing syn-
thetic enhancement phenotypes, the crosses described below were performed.

dbp6 ras3::HIS3MX6: YDK8-1A pRS416-DBP6 was crossed to

YMD3-2D pRS413, the resulting diploid was sporulated, and

tetrads were dissected. Spore clones from two complete tet-
ratype tetrads were transformed with pRS414, pRS414-HA-

DBP6, pRS414-db6p-2, and pRS414-db6p-4. Transformants were

restreaked on SD-Trp plates and subjected to plasmid shuffling

on 5-fluoroorotic (5-FOA)-containing plates. No viable dbp6-2

ras3::HIS3MX6 and dbp6-4 ras3::HIS3MX6 double mutants could be recovered.

dbp6 nop8-101: YDK8-2A pRS416-DBP6 was crossed to

sl264409-42D pRS413, the resulting diploid was sporulated, and
tetrads were dissected. Spore clones from two complete tet-
ratype tetrads were transformed with pRS414, pRS414-HA-

DBP6, pRS414-db6p-2, and pRS414-db6p-4. Transformants were

restreaked on SD-Trp plates and subjected to plasmid shuffling

on 5-FOA-containing plates. No viable dbp6-2 nop8-101 and
dbp6-4 nop8-101 double mutants could be recovered.

dbp6 rpl3: YDK8-1A pRS416-DBP6 was crossed to JDY319

YCplac111-RPL3, the resulting diploid was sporulated, and
tetrads were dissected. Two complete tetratype tetrads were
selected, and two G418<sup>+</sup>, His<sup>+</sup>, Ura<sup>+</sup>, and Leu<sup>+</sup> spore clones were transformed with the plasmids pRS414-HA-DBP6, pRS414-protA-DBP6-T7, pRS414-Dbp6-2, and pRS414-Dbp6-4. Transformants were restreaked on SD-Trp plates, subjected to plasmid shuffling on 5-FOA-containing plates, and then transformed with YCplac33-RPL3. Transformants were restreaked on SD-Ura, subjected to segregation of YCplac111-RPL3 and then they were transformed with YCplac111, YCplac111-RPL3, YCplac111-rpl3-101, and YCplac111-rpl3-102. Transformants were restreaked on SD-Leu plates and subjected to plasmid shuffling on 5-FOA-containing plates. No viable dbp6-2 rpl3-101, dbp6-2 rpl3-102, dbp6-4 rpl3-101, and dbp6-4 rpl3-102 double mutants could be recovered.

Information on the crosses for the analysis of the synthetic enhancement phenotypes of other double-mutant combinations can be found on the Linder laboratory website (http://www.medecine.unige.ch/linder/S1_delacruz_2003.pdf).

**Sucrose gradient analyses:** Polysome and r-subunit preparations and their analyses were as previously described (Kressler et al. 1997). Gradient analysis was performed with an ISCO UA-6 gradient UV detector and fraction collection system with continuous monitoring at A<sub>254</sub>.

For fractionation analyses, extract preparations and gradient centrifugation conditions were identical to those used for r-subunit analysis, except that 10 A<sub>260</sub> units of cell extract was layered onto the gradients. Fractions of ~500 μl were collected. The gradient position of the 40S and 60S r-subunits was determined from the UV profile. To analyze the gradient position of protA-Dbp6p, Rsa3-HAp, and Rpl3p, fractions (200 μl) were processed exactly as described (Kressler et al. 1999a) and subjected to Western blot analysis. Polyclonal rabbit antiprotein A (at 5 ng/μl, Sigma, St. Louis), monoclonal mouse 16B12 (1:10,000 dilution, BabCo), and monoclonal mouse anti-Rpl3p antibodies (1:10,000 dilution, gift of J. R. Warner) were used as primary antibodies. Blots were decorated with goat anti-mouse or anti-rabbit IgG horseradish-peroxidase-conjugated secondary antibodies (1:15,000 dilution, Bio-Rad), and then they were incubated with 50 μl IgG Sepharose 6 Fast Flow beads (Amerham, Buckinghamshire, UK) for 1.5 hr at 4°C. Aliquots of cleared lysates (0.35 ml) were diluted with 0.7 ml lysis buffer and incubated with 50 μl IgG Sepharose 6 Fast Flow beads (Amerham, Buckinghamshire, UK) for 1.5 hr at 4°C with end-over-end tube rotation. The beads were first washed five times with 2 ml lysis buffer, three times with 2 ml lysis buffer containing.

### TABLE 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Plasmid</th>
<th>Features</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBP6</td>
<td>pRS416-DBP6</td>
<td>CEN, URA3</td>
<td>Kressler et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>pRS415-protA-DBP6-T7</td>
<td>CEN, LEU2</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>pRS414-HA-DBP6</td>
<td>CEN, TRP1</td>
<td>Kressler et al. (1999a)</td>
</tr>
<tr>
<td></td>
<td>pRS414-Dbp6-2</td>
<td>CEN, TRP1, dbp6-2</td>
<td>Kressler et al. (1999a)</td>
</tr>
<tr>
<td></td>
<td>pRS414-Dbp6-4</td>
<td>CEN, TRP1, dbp6-4</td>
<td>Kressler et al. (1999a)</td>
</tr>
<tr>
<td>DBP7</td>
<td>YCplac35-DBP7</td>
<td>CEN, URA3</td>
<td>Daugeron and Linder (1998)</td>
</tr>
<tr>
<td>DBP9</td>
<td>YCplac35-DBP9</td>
<td>CEN, URA3</td>
<td>Daugeron et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>YCplac111-DBP9</td>
<td>CEN, LEU2</td>
<td>Daugeron et al. (2001)</td>
</tr>
<tr>
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<td>CEN, LEU2, dbp9-1</td>
<td>Daugeron et al. (2001)</td>
</tr>
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<td></td>
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<td>Daugeron et al. (2001)</td>
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<td>YCplac111-HA-dbpl9-5</td>
<td>CEN, LEU2, dbp9-5</td>
<td>Daugeron et al. (2001)</td>
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<td>pUN100-GFP-NOP1</td>
<td>CEN, LEU2</td>
<td>Teixeira et al. (2002)</td>
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<td>NOP8</td>
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<td>This study</td>
</tr>
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<td>RPL3</td>
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<td>pHAC111-RSA3</td>
<td>CEN, LEU2, RSA3HA</td>
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<td>SPB4</td>
<td>YCplac111-spb4-1</td>
<td>CEN, LEU2, spb4-1</td>
<td>Kressler et al. (1999a)</td>
</tr>
</tbody>
</table>

The yeast strain used for the sucrose gradient fractionation and co-immunoprecipitation analyses was obtained by crossing YDR8-1A pRS415-protA-DBP6-T7 (this plasmid expresses N-terminally protA- and C-terminally T7-tagged Dhp6p from its cognate promoter) to YMD3-2D pHAC33-RSA3. The resulting diploid was sporulated and tetrads were dissected to yield the meiotic segregant JDY139 pRS415-protA-DBP6-T7 pHAC33-RSA3.

**Co-immunoprecipitation:** For immunoprecipitation experiments, strains JDY139 pRS415-protA-DBP6-T7 pHAC33-RSA3 and YMD3-1A pHAC33-RSA3 were grown to an OD<sub>600</sub> of ~1 in 100 ml SD-Ura medium at 30°C. Cells were first washed with 50 ml ice-cold water and then with 50 ml ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM 2-mercaptoethanol, 0.1% NP-40) and cell pellets were finally resuspended in 0.7 ml of ice-cold lysis buffer containing 1 mM phenylmethylsulfonyl fluoride. Cells were lysed by vortexing eight times for 30 sec in the presence of an equal volume of glass beads (425–600 μm, Sigma) at 4°C. Aliquots of cleared lysates (0.35 ml) were diluted with 0.7 ml lysis buffer and incubated with 50 μl IgG Sepharose 6 Fast Flow beads (Amerham, Buckinghamshire, UK) for 1.5 hr at 4°C with end-over-end tube rotation. The beads were first washed five times with 2 ml lysis buffer, three times with 2 ml lysis buffer containing
RESULTS

Synthetic lethality with \( \text{dbp6} \) alleles identifies \( \text{RPL3}, \ \text{NOP8}, \ \text{and the novel} \ \text{RSA3} \): To dissect the functional environment of Dbp6p, we carried out an \( \text{sl} \) screen with \( \text{dbp6} \) alleles that led to the isolation of six \( \text{sl} \) strains (Kressler et al. 1999a). We previously described the cloning and functional characterization of \( \text{RSA1} \), which complemented two of the six \( \text{sl} \) strains (Kressler et al. 1999a). Rsa1p is a nonessential nucleoplasmic protein involved in the assembly and nuclear export of 60S r-subunits (Kressler et al. 1999a; Gadal et al. 2001b). To extend the above study, we subjected the remaining four \( \text{sl} \) strains to polysome profile analysis, which revealed that, compared to a wild-type strain, they all had a defect in free 60S r-subunits and had accumulated half-mer polysomes (Figure 1, A–C; data not shown and see also Figure 3A). To clone the genes that complemented the four \( \text{sl} \) strains, we transformed the original \( \text{sl} \) strains or outcrossed mutant strains with yeast genomic materials and methods (see MATERIALS AND METHODS); we therefore refer to the mutations as \( \text{rsa3-1} \) (sl263210), \( \text{rpl3-101} \) (sl263309), \( \text{rpl3-102} \) (sl264205), and \( \text{nop8-101} \) (sl264409). Second, we determined whether the \( \text{rsa3} \) null, \( \text{rpl3-101}, \ \text{rpl3-102}, \ \text{or nop8-101} \) mutant alleles were synthetically lethal with the mildly affected \( \text{dbp6-2} \) mutant or the more strongly affected \( \text{dbp6-4} \) mutant (see MATERIALS AND METHODS). In agreement with their isolation in the \( \text{sl} \) screen with \( \text{dbp6} \) alleles, no viable \( \text{dbp6} \ \Delta \text{rsa3}, \ \text{dbp6} \ \text{rpl3}, \ \text{and dBp6} \ \text{nop8-101} \) double mutants could be recovered (for \( \text{dbp6-2} \), see Figure 2, B–D; data not shown for \( \text{dbp6-4} \)).

**Figure 1.**—Synthetic-lethal mutant strains with \( \text{dbp6} \) alleles show a deficit in free 60S r-subunits and an accumulation of half-mer polysomes. (A) sl263210 (\( \text{rsa3-1} \)), (B) sl264205 (\( \text{rpl3-102} \)), and (C) sl264409-42D (\( \text{nop8-101} \)) were grown in YPD at \( 30^\circ \) up to an \( \text{OD}_{600} \) of \( \sim 0.8 \). Cell extracts were resolved in 7–50% sucrose gradients. Gradients were analyzed by continuous monitoring at \( A_{260} \). Sedimentation is from left to right. The peaks of free 40S and 60S subunits, 80S ribosomes (free couples and monosomes), and polysomes are indicated. Half-mers are indicated by arrows.

different salt concentrations (50–500 mM NaCl), and finally another three times with 2 ml lysis buffer. Bound proteins were eluted with 200 \( \mu \)l 0.5 M acetic acid and they were concentrated by lyophilization. Total, nonbound, and bound proteins were subjected to Western blot analysis using polyclonal rabbit anti-protein A (at 5 ng/ml) and monoclonal mouse 16B12 (1:10,000 dilution) antibodies.

**Pulse-chase labeling of pre-rRNA:** Strains YMD3-2A (\( \text{RSA3} \)) and YMD3-2D (\( \Delta \text{rsa3} \)) were first grown in SD-Met medium at \( 30^\circ \) and then for 14 hr at \( 37^\circ \) up to an \( \text{OD}_{600} \) of \( \sim 0.8 \). A total of 30 \( \text{OD}_{600} \) units of cells were harvested by centrifugation and the pre-rRNA was labeled with \( [\text{methyl}^3\text{H}] \) methionine and analyzed as previously described (Kressler et al. 1999a). Two of the six \( \text{sl} \) strains or outcrossed mutant strains with yeast genomic materials and methods (see MATERIALS AND METHODS) were analyzed as previously described (Kressler et al. 1999a). We previously described the cloning and functional characterization of \( \text{RSA1} \), which complemented two of the six \( \text{sl} \) strains (Kressler et al. 1999a). Rsa1p is a nonessential nucleoplasmic protein involved in the assembly and nuclear export of 60S r-subunits (Kressler et al. 1999a; Gadal et al. 2001b). To extend the above study, we subjected the remaining four \( \text{sl} \) strains to polysome profile analysis, which revealed that, compared to a wild-type strain, they all had a deficit in free 60S r-subunits and had accumulated half-mer polysomes (Figure 1, A–C; data not shown and see also Figure 3A). To clone the genes that complemented the four \( \text{sl} \) strains, we transformed the original \( \text{sl} \) strains or outcrossed mutant strains with yeast genomic materials and methods (see MATERIALS AND METHODS); we therefore refer to the mutations as \( \text{rsa3-1} \) (sl263210), \( \text{rpl3-101} \) (sl263309), \( \text{rpl3-102} \) (sl264205), and \( \text{nop8-101} \) (sl264409). Second, we determined whether the \( \text{rsa3} \) null, \( \text{rpl3-101}, \ \text{rpl3-102}, \ \text{or nop8-101} \) mutant alleles were synthetically lethal with the mildly affected \( \text{dbp6-2} \) mutant or the more strongly affected \( \text{dbp6-4} \) mutant (see MATERIALS AND METHODS). In agreement with their isolation in the \( \text{sl} \) screen with \( \text{dbp6} \) alleles, no viable \( \text{dbp6} \ \Delta \text{rsa3}, \ \text{dbp6} \ \text{rpl3}, \ \text{and dBp6} \ \text{nop8-101} \) double mutants could be recovered (for \( \text{dbp6-2} \), see Figure 2, B–D; data not shown for \( \text{dbp6-4} \)).

\( \text{RSA3} \) encodes a relatively short protein of 220 aa with a calculated molecular mass of 24.7 kDa, which, on the basis of a codon adaptation index of 0.17, is predicted to be of low cellular abundance. Furthermore, \( \text{Rsa3p} \) is predicted to be a rather acidic protein (pI 4.83) and to
localize to the nucleus (PSORTII program) due to the presence of four overlapping “4 residue pattern” nuclear localization signals located between aa 18 and aa 24. Rsa3p is likely a yeast-specific protein since no homologous proteins could be identified by sequence comparison searches in Drosophila, Caenorhabditis, or mammalian databases. However, there is a significant homology (38% identity and 50% similarity) between Rsa3p and the Candida albicans ORF IPF3878. A more detailed sequence analysis revealed a region (aa 29–45) within the N-terminal part of Rsa3p that contains almost exclusively serine, aspartic acid, and glutamic acid residues (S/D/E-rich region); similar S/D/E-rich regions are found in other nucleolar proteins and in the case of Nsr1p it is much more extended (Yan and Mélese 1993; Kressler et al. 1998). Moreover, aa 129–150 of Rsa3p may adopt a coiled-coil conformation (COILS program; Lupas et al. 1991), which could serve as a homo- or heterodimerization surface (see discussion). This coiled-coil region is likely to be important for the function of Rsa3p since the rsa3-1 mutation, which leads to a frameshift after aa 110, confers the same phenotypes as the rsa3 null mutation does.

Absence of Rsa3p leads to a deficit in free 60S r-subunits, an accumulation of half-mer polysomes, and a slightly impaired production of 25S rRNA: To analyze the function of Rsa3p, we first constructed a rsa3-null mutant strain by replacing one copy of the RSA3 ORF in the diploid strain W303 with the HIS3MX6 marker module. Tetrad dissection and restreaking of spore clones on YPD plates at 18°, 30°, and 37° showed that the rsa3-null mutant strain had a mild slow-growth phenotype at 37° (Figure 2A). Doubling times of 1.5 hr for the wild-type and of 1.9 hr for the rsa3-null mutant strain were obtained in liquid YPD medium at 37°. As a first step to the functional analysis, we performed polysome profile analysis in a rsa3-null mutant strain (YMD3-1A YCplac33) and a wild-type control strain (YMD3-1A phAC33-RSA3) that were grown in SD-Ura at 30° and then shifted for 4 hr to 37°. The rsa3-null mutant strain displayed a reduction in the amount of free 60S r-subunits as well as an accumulation of half-mer polysomes (Figure 3B). In contrast, the Δrsa3 strain transformed with phAC33-RSA3 had normal “wild-type” polysome profiles (Figure 3A). Quantification of total r-subunits in cell extracts from strains grown at 37° in YPD by sucrose gradient centrifugation under low-Mg2+ conditions confirmed the imbalance between 40S and 60S r-subunits. An A254 60S to 40S subunit ratio of 2.1 was observed for the wild-type control strain (YMD3-2A) whereas the ratio decreased to 1.7 for the Δrsa3-null mutant strain (YMD3-2D). We conclude that the absence of Rsa3p leads to a slight underaccumulation of 60S r-subunits, which likely accounts for the mild growth defect associated with the rsa3 null mutation.

Next, we attempted to study the role of Rsa3p during 60S r-subunit metabolism in more detail. To this end, we analyzed the effects of the rsa3 null mutation on the synthesis and processing of pre-rRNA by [methyl-3H]methionine pulse-chase labeling experiments. The
wild-type control strain YMD3-2A (RSA3) and the rsa3-null-mutant strain YMD3-2D (Δrsa3) were first grown in SD-Met medium at 30°C and then for 14 hr at 37°C up to an OD_{600} of ~0.8. Cells were harvested by centrifugation, pulse-labeled for 1 min with [methyl-^3H]methionine, and chased for 2, 5, and 15 min with an excess of cold methionine. Total RNA was extracted, resolved on an agarose-formaldehyde gel, transferred to a nylon membrane, and finally subjected to fluorography. Compared to the wild-type strain, the Δrsa3 mutant displayed a mild decrease in the synthesis of the mature 25S rRNA while the formation of 18S rRNA was not affected (Figure 4B). We could not observe a reduced synthesis of the 27S precursors to the mature 25S rRNA (Figure 4B). lanes 1 and 5); however, this might be due to the fact that there is only a mild growth defect associated with the rsa3-null-mutant strain. Also, the loading of equal counts (20,000 cpm) in all lanes might mask some of the effects of the absence of Rsa3p on the production of the 27S pre-rRNAs.

We conclude that the absence of Rsa3p causes a mild deficit in 60S r-subunit levels and therefore leads to a slight reduction in the levels of mature 25S rRNA.

**Rsa3p localizes predominantly to the nucleolus:** To distinguish between a cytoplasmic, nuclear, or nucleolar role for Rsa3p in maintenance of 60S r-subunit levels, we analyzed the subcellular localization of a C-terminally HA-tagged version of Rsa3p that was expressed from the cognate RSA3 promotor (plasmid pHAC33-RSA3). The Rsa3-HAP fusion protein was fully functional since it complemented the deficit in 60S r-subunits of the Δrsa3 mutant (see Figure 3A) and the synthetic lethality of a dhp6-2 Δrsa3 double mutant (data not shown). Immunofluorescence was performed on cells of the rsa3 null strain YMD3-1A transformed with pHAC33-RSA3 and pUN100-GFP-NOP1 (Teixeira et al. 2002). The HA-tagged Rsa3p was detected by anti-HA antibodies, followed by decoration with goat anti-mouse Cy3-conjugated antibodies (Figure 5B). For precise subnuclear localization, the nucleoplasm was visualized by staining the DNA with 4′,6-diamidino-2-phenylindole (DAPI; Figure 5C) and the nucleolus was revealed by the green fluorescent protein (GFP)-tagged Nop1p (Figure 5D).

**Synthetic lethality network with dhp6 alleles:** To assess the specificity of the sl interactions observed between dhp6 alleles and the nop8, rpl3, and rsa3 mutants, we tested the synthetic interaction relationship among mutants of genes encoding 60S r-subunit proteins or protein trans-acting factors that have been shown to genetically interact with Dhp6p (see Figure 6). In addition to Dhp6p, Nop8p, Rpl3p, and Rsa3p, these factors included two DEAD-box proteins (Dbp7p and Dbp9p), Rsa1p, and the large r-subunit protein Rpl10p/Qsr1p. Like Dhp6p, Dbp7p and Dbp9p are likely involved in early 60S r-subunit assembly steps since their inactivation leads to reduced steady-state levels and decreased synthesis of the 27S precursors to mature 25S and 5.8S rRNAs (Daugeron and Linder 1998; Daugeron et al. 2001). In addition, the combination of certain dhp6 and dhp9 alleles leads to synthetic lethality and elevated dosage of Dbp9p has been shown to suppress the growth defect of certain dhp6 mutants (Daugeron et al. 2001). Rsa1p, also identified in the sl screen with conditional dhp6 alleles, is important for efficient export of 60S r-subunits and has been suggested to assist the assembly of the large subunit r-protein Rpl10p/Qsr1p (Eisinger et al. 1997) onto 60S r-subunits in the nucleoplasm (Kressler et al. 1999a; Gadal et al. 2001b). Rpl10p/Qsr1p is the docking site for the nuclear export adaptor Nmd3p, which contains a nuclear export sequence and mediates export of 60S r-subunits (Ho et al. 2000; Gadal et al. 2001b). As a specificity control, we selected the

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**Figure 3.—** The Δrsa3 mutant has a deficit in free 60S r-subunits and accumulates half-mer polysomes. (A) YMD3-1A pHAC33-RSA3 (wild type) and (B) YMD3-1A YCplac33 (Δrsa3) were grown in SD-Ura at 30°C and then shifted for 4 hr to 37°C (OD_{600} ~0.8). Cell extracts were resolved in 7–50% sucrose gradients. Gradients were analyzed by continuous monitoring at A_{254}. Sedimentation is from left to right. The peaks of free 40S and 60S subunits, 80S ribosomes (free couples and monosomes), and polysomes are indicated. Halfmers are indicated by arrows.
Figure 4.—Absence of Rsa3p leads to a mild reduction in the synthesis of the mature 25S rRNA. (A) Simplified pre-rRNA processing scheme. The early cleavage reactions A₀, A₁, and A₂ process the 35S pre-rRNA in which the mature rRNA sequences are separated by two internal transcribed spacer sequences, ITS1 and ITS2, and are flanked by two external transcribed spacer sequences, 5'ETS and 3'ETS, into the 20S and 27SA₂ pre-rRNAs. Endonucleolytic cleavage of the 20S pre-rRNA at site D in the cytoplasm yields the mature 18S rRNA. The 27SA₂ precursor is processed by two alternative pathways: in the major pathway, the 27SA₂ pre-rRNA is first cleaved at site A₃ and is then 5'–3' exonucleolytically digested up to site B₁S to yield the 27SB precursor; a minor pathway processes the 27SA₂ pre-rRNA at site B₁L, thus producing the 27SBL precursor. The subsequent ITS2 processing of both 27SB species appears to be identical and leads to the formation of the mature 5.8S and 25S rRNAs. For details see Venema and Tollervey (1999) and Kressler et al. (1999b). (B) Strains YMD3-2A (RSA3) and YMD3-2D ( rsa3) were first grown in SD-Met at 30°C and then for 14 hr at 37°C up to an OD₆₀₀ of 0.8. 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 cold 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. The positions of the different pre-rRNAs and mature rRNAs are indicated.

DEAD-box protein Spb4p since we had previously shown strongly suggests that these two proteins functionally interact during assembly of 60S r-subunits.

**Dbp6p and Rsa3p are associated with preribosomal particles:** To address whether the genetic interaction between Dbp6p and Rsa3p may represent a functional interaction within pre-60S ribosomal particles, we first investigated the migration of Dbp6p and Rsa3p in sucrose gradients under polysome runoff and low Mg²⁺ concentration conditions that are normally used for the analysis of r-subunits. To this end, strain JDY139, which coexpresses protA-Dbp6p and Rsa3-HAp from centromeric plasmids, was constructed (see Table 1). Both the Rsa3-HAp (see above) and the protA-Dbp6p fusion protein were fully functional, and the latter complemented the lethality of the dbp6 null mutant to the wild-type extent (data not shown). Under the abovementioned experimental conditions, the majority of protein-A-tagged Dbp6p and HA-tagged Rsa3p were present in large complexes. These complexes had a sedimentation behavior similar to that of 60S r-subunits (Figure 7), as evidenced by the absorption at A₂₅₄ (data not shown). Importantly, the Δrsa3 null mutant, which displays only a minor growth phenotype, was already synthetically lethal with very mild dbp6 alleles (see Figure 2B) and by showing some mild synthetic enhancement with the nop8-101 and the rsa1 null mutant (data not shown). The specific genetic interaction between Dbp6p and Rsa3p...
and Rsa3p are mainly nucleolar proteins, these data are consistent with the direct association of both Dpb6p and Rsa3p with nucleolar preribosomal particles to mature 60S r-subunits.

To assess whether Dpb6p and Rsa3p are present within the same cellular complexes, we also subjected extracts obtained from cells of strain JDY139 to immunoprecipitation with IgG Sepharose beads at the ionic strength of 50 mM NaCl. Then, equivalent aliquots of total cell extracts, unbound fractions, and immunoprecipitates were subjected to Western blotting. As shown in Figure 8, these analyses revealed that Rsa3-HAp was efficiently co-immunoprecipitated with protA-Dpb6p. Rsa3-HAp was not detected in immunoprecipitates of extracts from control cells (strain YMD3-1A pHAC33-RSA3) that coexpress Rsa3-HAp and untagged wild-type Dpb6p. These results indicate that Rsa3p interacts in vivo with Dpb6p-containing complexes. However, Rsa3-HAp could not be co-immunoprecipitated with protA-Dpb6p when we increased the salt concentration in the wash buffer from 50 to 500 mM NaCl (data not shown); therefore, the association of Rsa3p with Dpb6p-containing complexes or the Dpb6p-containing complexes themselves seem not to be very stable. Indeed, when we explored further the immunoprecipitates of the protA-Dpb6p strain, we were unable to specifically coprecipitate Rpl3p or any pre-rRNA, despite efficient protA-Dpb6p precipitation (data not shown).

Altogether, these results suggest that a substantial amount of Dpb6p and Rsa3p is associated with the same cellular complexes, which most likely correspond to nucleolar preribosomal particles to mature 60S r-subunits. However, Dpb6p might be loosely bound to these preribosomal particles and therefore dissociated from them following purification by immunoprecipitation.

**DISCUSSION**

In this study, we have further dissected the functional environment of the putative ATP-dependent RNA helicase Dpb6p by cloning the complementing genes of four previously isolated mutants that were synthetically lethal with *dbp6* alleles (*Kressler et al. 1999a*). These genes encode the large subunit r-protein Rpl3p (*Planta and Mager 1998*), the protein *trans*-acting factor Nop8p, which is involved in 60S r-subunit biogenesis (*Zanchin and Goldfarb 1999*), and the so-far-uncharacterized YLR221Cp protein, which we named Rsa3p. Several
Figure 7.—Dbp6p and Rsa3p are associated with pre-60S ribosomal particles. Strain JDY139 pRS415-protA-DBP6p-T7 pHAC33-RSA3 was grown at 30° up to an OD600 of ~0.8. Cell extracts were prepared in a buffer containing 50 mM NaCl, 50 mM Tris-HCl pH 7.4, and 1 mM dithiothreitol. Then, 10 A260 units of cell extract were resolved in 7–50% sucrose gradients containing a low concentration of Mg2+ to dissociate ribosomes into subunits and fractions of 500 μl were collected.

Sedimentation is from left to right and numbers (1–16) indicate the fractions. Proteins were extracted from each fraction (200 μl) and equal volumes were resolved on a 12% SDS-PAGE and subjected to Western blotting using polyclonal rabbit anti-Rpl3p antibodies to detect Rpl3p (bottom), monoclonal mouse 16B12 antibodies to detect Rsa3-HAp (middle), and monoclonal mouse anti-Rpl3p antibodies to detect Rpl3p (bottom). The gradient position of the 40S and 60S ribosomal subunits was determined from the A260 profile by continuous monitoring. To analyze the gradient position of pre-rRNAs, fractions (250 μl) were processed and subjected to Northern blot analysis with probes revealing the 20S, 27SA/B, and 35S pre-rRNAs. As a control, a total extract (T) was also subjected to Western and Northern blot analyses.

lines of evidence indicate that the nonessential Rsa3p is required for efficient biogenesis of 60S r-subunits:

i. The absence of Rsa3p leads to a slight deficit in 60S r-subunits (Figure 3).

ii. The Δrsa3 null mutant interacts synthetically with selected factors involved in the biogenesis of 60S r-subunits (Dbp6p, Nop8p, and Rsa1p).

iii. Rsa3-HAp localizes predominantly to the nucleolus (Figure 5).

iv. Rsa3-HAp is likely associated with preribosomal complexes since a majority of the cellular Rsa3-HAp sediments below the cytoplasmic 60S r-subunits in sucrose gradients (Figure 7). Consistent with these data, Rsa3p has been found in a Noc1p-containing complex (Gavin et al. 2002; Milkereit et al. 2003), which represents either a late 90S or an early 60S preribosomal particle.

v. Finally, RSA3 shows an expression profile similar to many ribosome synthesis genes at the mRNA level (Gasch et al. 2000).

Our initial phenotypic analysis of Dbp6p suggested that the predominantly nuclear Dbp6p is required for early assembly steps within pre-60S ribosomal particles (Kressler et al. 1998). Here we show that a substantial amount of protA-tagged Dbp6p sediments faster than cytoplasmic 60S r-subunits (Figure 7), which likely represents an association with early pre-60S ribosomal particles. The observed synthetic lethality between dbp6 alleles and dbp9, nop8, and rpl3 mutants also supports a function of Dbp6p during early pre-60S r-subunit assembly reactions. Dbp9p is found in the so-called early E1 66S preribosomal particle and its genetic depletion leads to reduced formation and steady-state levels of all 27S species, especially the 275B pre-rRNAs (Daugeron et al. 2001; Fatica and Tollervey 2002; Gavin et al. 2002). Nop8p is a mainly nuclear protein whose genetic depletion leads to a reduced synthesis and/or stability of 27S pre-rRNAs (Zanchin and Goldfarb 1999), and Rpl3p belongs to a group of large subunit r-proteins that associate early with preribosomal particles (Kruijver et al. 1978). The fact that there is an intimate sl network among dbp6, dbp9, nop8, and rpl3 alleles (note that all combinations of double mutants are sl; see Figures 2 and 6) opens up the possibility that all the corresponding proteins operate in the same environment within early pre-60S ribosomal particles. In agreement with this model, we have previously revealed a physical interaction between Dbp6p and Dpb9p by using the yeast two-hybrid system (Daugeron et al. 2001).

Importantly, the rsa3 null mutant displays some specificity by being synthetically lethal only with dbp6 alleles (Figures 2 and 6). In addition to this specific genetic interaction, the similar sedimentation pattern of Dbp6p and Rsa3p in sucrose gradients (Figure 7), as well as the relatively efficient co-immunoprecipitation of Rsa3-HAp with protA-Dbp6p (Figure 8), strongly suggest that the two proteins functionally interact within the same 66S preribosomal particles. Unfortunately, attempts to explore these preribosomal particles by co-immunoprecipitation with protA-Dbp6p failed, suggesting that Dbp6p might only loosely and transiently interact with preribosomal particles and dissociate during immunoprecipitation. We also found that HA-Dbp6p is not mislocalized in rsa3-null-mutant cells and overexpression of Dbp6p from a multicopy plasmid neither restores the underaccumulation of 60S r-subunits associated with the rsa3 null mutation nor exacerbates the slow-growth phenotype of the rsa3 null mutant (data not shown). Moreover, overexpression of Rsa3p does not suppress the growth defect of the dbp6-2 or the dbp6-4 allele (data not shown). A classical model to explain the synthetic lethality between dbp6 alleles and the Δrsa3 null mutant would be the synergistic destabilization of the Dbp6p- and Rsa3p-containing preribosomal particle or subcomplex when both factors are mutated. However, we prefer to propose a model in which Rsa3p acts as a specific cofactor of Dbp6p that either stimulates the enzymatic activity of Dbp6p or facilitates its substrate recognition.
Even though we have so far not gathered any evidence for a direct physical interaction between Dpb6p and Rsa3p, it is intriguing that regions in both Dpb6p [aa 288–309; COILS program (Lupas et al. 1991)] and Rsa3p (aa 129–150; see results) may adopt coiled-coil conformations, which could serve as heterodimerization surfaces. Moreover, the potential coiled-coil region of Rsa3p is likely to be important for its function since the rsa3-1 mutation, which leads to a frameshift after aa 110, confers the same phenotypes as the rsa3 null mutation confers. The rsa3 null mutant also mildly exacerbates the slow-growth phenotype of the nop8-101 and the rsa1 null allele (Figure 6 and data not shown). The fact that Rsa3p functionally interacts with Dpb6p, together with the above-mentioned possibility that both Dpb6p and Nop8p might work in the same precise environment within early 60S preribosomal particles, could fully explain the genetic interaction between the rsa3 null and the nop8-101 allele. Previous results have shown that the rsa1 null allele is cis with dbp6 alleles (Kressler et al. 1999a). At present, we cannot provide a simple explanation for the cis interaction between dbp6 alleles and the rsa1 null mutant; we can speculate only that the absence of nucleoplasmic Rsa1p leads to a synergistic destabilization of late pre-60S r-subunits that are qualitatively altered in dbp6 mutant strains (Kressler et al. 1999a). The fact that the rsa3 null mutant also genetically interacts with the rsa1 null allele reinforces the model in which Rsa3p and Dpb6p work together in the same complex. In this sense, it is worthwhile to mention that Rsa3p seems not to be required for export of pre-60S r-subunits since, in contrast to the rsa1 null mutant, there is no nuclear accumulation of the Rpl25p-eGFP large subunit reporter (Gadal et al. 2001b) in rsa3-null-mutant cells at either 30° or 37° (data not shown).

Intriguingly, there are no readily discernible homologs of Dpb6p and not even potential homologs of Nop8p, Rsa1p, and Rsa3p in higher eukaryotes. At least and unlike Rsa1p, Dpb6p (CaDBP6; 38% identity and 58% similarity) and Rsa3p (IPF3878; 33% identity and 50% similarity) have likely homologs in C. albicans whereas Nop8p has weakly conserved homologs in Schizosaccharomyces pombe (accession NP_595780; 20% identity and 40% similarity) and C. albicans (IPF16935; 24% identity and 39% similarity). Therefore, it seems that certain aspects of eukaryotic ribosome biogenesis are yeast specific or even restricted to S. cerevisiae. An appealing model that may explain why Dpb6p and its functionally interacting partners Rsa1p, Rsa3p, and Nop8p are not evolutionarily conserved is based on the finding that increased dosage of Dpb9p, which has clear homologs in higher eukaryotes (Zirnes et al. 2000), suppresses the sg phenotype of dbp6 alleles (Daugeron et al. 2001). Accordingly, the Dpb9p homologs may have taken over the yeast-specific function of Dpb6p in higher eukaryotes. The sg screen with dbp6 alleles also identified the evolutionarily conserved large subunit r-protein Rpl3p. Given that both the rpl3-101 and the rpl3-102 mutation conferred synthetic lethality to dbp6, dbp9, and nop8 alleles but not to the slow-growing dbp7 null allele (see Figure 6), we conclude that there is some specificity associated with the functional interaction between the r-protein Rpl3p and the protein trans-acting factors Dpb6p, Dpb9p, and Nop8p. Both the rpl3-101 and rpl3-102 mutants strongly underaccumulate cytoplasmic 60S r-subunits (Figure 1B and data not shown), indicating that these mutations affect proper assembly of 60S r-subunits. Importantly, this assembly defect is not due to instability of the mutant Rpl3 proteins, since both Rpl3-101p (Q371H) and Rpl3-102p (K30E) are expressed at the same level as wild-type Rpl3p (D. Kressler, unpublished results). Simple interpretations of the sg interaction between the rpl3-101 and rpl3-102 mutants and the dbp6, dbp9, and nop8 alleles are either that these proteins trans-acting factors help the efficient recruitment of Rpl3p or that proper assembly of Rpl3p is a prerequisite for Dpb6p, Dpb9p, and Nop8p to function. It has been proposed that the protein trans-acting factor Rb1p functions as the assembler of Rpl3p onto early 66S preribosomal particles (Iouk et al. 2001; Schaper et al. 2001). In agreement with the above-mentioned interpretations, the rb1-TAP allele, which displays a sg and temperature-sensitive phenotype (Schaper et al. 2001), is synthetically lethal with the rpl3-102 and dbp6-4 alleles and synthetically enhances the growth defect of the dbp6-2 and the rsa3 null strain (data not shown).

We have started to explore the functional environment of putative RNA helicases of the DEAD-box protein family involved in ribosome synthesis by genetic means. Since not all protein trans-acting factors, as, for example, Dpb6p and Nop8p, that have been implicated in ribosome assembly have been found in the so-far...
identified preribosomal particles (Gavin et al. 2002; Nissan et al. 2002; Frommont-Racine et al. 2003; Milker- eit et al. 2003), sl screens, among other different genetic tools, represent a necessary complementary strategy not only to identify all protein trans-acting factors involved in ribosome biogenesis, but also to reveal functional interaction networks between these factors. For instance, this study clearly supports the idea that at least Dpb6p, Dpb9p, Nop8p, Rpl3p, and Rsa3p all functionally interact. Future studies that combine proteomic and genetic approaches are required to address the exciting issues of identifying the precise substrates and the timing of action of the energy-consuming enzymes, such as putative RNA helicas, involved in ribosome biogenesis.

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Rsa3p and Dbp6p Functionally Interact


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