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Citation for published version:

Digital Object Identifier (DOI):
10.1128/EC.3.6.1619-1626.2004

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher’s PDF, also known as Version of record

Published In:
Eukaryotic Cell

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The Small-Subunit Processome Is a Ribosome Assembly Intermediate

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Received 11 August 2004/Accepted 15 September 2004

The small-subunit (SSU) processome is a large ribonucleoprotein required for the biogenesis of the 18S rRNA and likely corresponds to the terminal knobs visualized by electron microscopy on the 5′ end of nascent rRNAs. The original purification of the SSU processome of Saccharomyces cerevisiae resulted in the identification of 28 proteins. Here, we characterize 12 additional protein components, including five small-ribosomal-subunit proteins (Rps4, Rps6, Rps7, Rps9, and Rps14) that had previously been copurified. Our multiple criteria for including a component as bona fide SSU processome component included coimmunoprecipitation with Mpp10 (an SSU processome component), the U3 snoRNA, and the anticipated pre-rRNAs. Importantly, the association of specific ribosomal proteins with the SSU processome suggests that the SSU processome has roles in both pre-rRNA processing and ribosome assembly. These ribosomal proteins may be analogous to the primary or secondary RNA binding proteins first described in bacterial in vitro ribosome assembly maps. In addition to the ribosomal proteins and based on the same experimental approach, we found seven other proteins (Utp18, Noc4, Utp20, Utp21, Utp22, Emg1, and Krr1) to be bona fide SSU processome proteins.

Ribosomes are essential for the translation of mRNA into protein. Ribosome biogenesis in Saccharomyces cerevisiae begins with the transcription of the 35S pre-rRNA, which is then cleaved and processed at more than 10 different processing sites to give rise to the mature 18S, 25S, and 5.8S rRNAs (Fig. 1). Small nucleolar ribonucleoproteins (snoRNPs) are required for many of the different processing steps and modifications that occur relative to the pre-rRNA (16). There are three classes of snoRNPs (H/ACA box, C/D box, and RNase mitochondrial RNA processing) that are required for ribosome biogenesis, each of which contains a small nucleolar RNA (snoRNA). H/ACA box snoRNAs are required for site-specific pseudouridylation of rRNA, while C/D box snoRNAs are required for 2′-O-ribose methylation of specific nucleotides in rRNA.

The U3 snoRNA and its associated proteins are required for the processing of the small ribosomal subunit at cleavage sites A0, A1, and A2 (Fig. 1). Cleavages at A0 and A1 in the 5′ end of the pre-rRNA. Cleavage at A2 or A3 in internal transcribed spacer 1 separates the small-ribosomal-subunit precursor rRNA from the large-ribosomal-subunit precursors. Defects in cleavage at the A0, A1, and A2 sites lead to a reduction in the levels of the 18S rRNA. This reduction causes accumulation of the 35S and 23S pre-rRNAs and a reduction in the levels of the 27SA2 and 20S pre-rRNAs (39).

A large RNP required for the processing of the small-ribosomal-subunit rRNA, called the small-subunit (SSU) processome, has recently been purified (4). This preribosomal complex contains the U3 snoRNA and at least 28 proteins. We defined the SSU processome components as having the following properties. (i) They are nucleolar. (ii) They are able to coimmunoprecipitate with the U3 snoRNA and Mpp10 (a protein specific to the SSU processome). (iii) They are required for 18S RNA biogenesis. Subsequent large-scale tandem affinity purification studies have also purified several 80 to 90S preribosomal complexes that contain many SSU processome components (9, 33). Additional proteomic studies have also revealed similar proteins required for ribosome biogenesis and subcomplexes, including SSU processome proteins (17, 29). Collectively, these studies have purified an 80S or 90S preribosome which serves as a precursor to the 43S preribosome and is required for cleavages at A0, A1, and A2. Here, we present results that expand upon these studies and validate the role of seven nonribosomal proteins in pre-rRNA processing.

During the original purification, analysis was limited to proteins whose peptide sequences were present more than once in the mass spectrometric analysis (4). Due to this stringent criterion, we hypothesized that there may be some additional SSU processome components that had been eliminated in the original purification. In addition, the initial purification included the presence of five ribosomal proteins (Rps4, Rps6, Rps7, Rps14, and Rps28), but it was not known at that time whether they were components of the SSU processome or contaminants. Therefore, we aimed to determine whether these five ribosomal proteins are SSU processome components by testing (i) their association via coimmunoprecipitation with known SSU processome components, i.e., Mpp10 and the U3 snoRNA, and (ii) their association with precursors to the 18S rRNA. Since there is no in vitro ribosome assembly system in yeast, the order of assembly of ribosomal proteins in eukaryotes is currently unknown. We have found a subset of ribosomal proteins to be associated with the SSU processome, suggesting that these proteins are able to associate with early precursors to the 18S rRNA. Therefore, the association of specific ribosomal proteins with the SSU processome suggests...
that it has roles in both pre-rRNA processing and ribosome assembly.

We have also tested whether seven other nonribosomal proteins (Utp18, Noc4, Utp20, Utp21, Utp22, Emg1, and Krr1), present only once in the purifications or subsequently found by others to coimmunoprecipitate with SSU processome components, were additional components of the SSU processome (7, 8, 32). A subset of these proteins has been partially characterized as being involved in ribosome biogenesis (3, 7, 9, 17, 25, 29, 33). For example, Utp18 was first identified (9), localized to the nucleolus (12), and shown to coimmunoprecipitate with the 5′ external transcribed spacer and the U3 snoRNA (17).

Here we show that Utp18 is required for pre-18S rRNA processing by Northern blot analysis of pre-rRNAs; we also show that Utp18 coimmunoprecipitates with the SSU processome protein, Mpp10, by Western blot analysis. Similarly, Noc4 was previously identified (9, 29) and localized (9, 26), and the pre-rRNA processing phenotype of the temperature-sensitive Noc4 mutant was analyzed (26). However, Noc4 was found not to coimmunoprecipitate with the U3 snoRNA (9). Here, we determined the resulting defects in pre-rRNA processing of cells depleted of Noc4 and report that, contrary to previously published results, Noc4 does indeed interact with both the U3 snoRNA and the SSU processome protein, Mpp10. In this study, we determined that these proteins are components of the SSU processome, which therefore places them in a specific preribosomal complex.

Collectively, we have validated that these 12 proteins are indeed bona fide SSU processome components and are essential for 18S rRNA biogenesis. Together, these results suggest that the SSU processome, in addition to its role in pre-rRNA processing, also has a role as a ribosome assembly intermediate.

MATERIALS AND METHODS

Yeast strains and media. All yeast strains were derived from YPH499 (MATa ura3-52 lys2-80 ade2-101 trp1-D61 his3-D200 leu2-D1). Yeast strains were grown in rich medium, either YEPD (1% yeast extract, 2% peptone, 2% glucose) or YPG/R (1% yeast extract, 2% peptone, 2% galactose, 2% raffinose) as specified below.

Expression of proteins from a conditional promoter. Strains which expressed N termini that were tagged with a triple hemagglutinin epitope tag (3×HA) from a galactose-inducible and glucose-repressible promoter were created as described previously (22) with plasmid pFA6a-kanMX6-PGAL1-3×HA and with primers with 50 nucleotides of complementarity to the gene of interest (the Utp1, Utp7, Utp18, Noc4, Utp20, Emg1, Brf2, Enp1, Rps9B, or Rps14A gene).

C-terminal 3×HA tagging. Yeasts expressing proteins with 3×HA tags were constructed as described previously (15) with plasmid pYMI (kanMX6 selectable marker) and 50 nucleotides with complementarity to the gene of interest (the Utp1 to Utp10, Utp12 to Utp17, Utp21, Utp22, Krr1, Enp1, Enp2, Rpf2, Imp4, Rps4A, Rps6B, Rps7B, Rps27A, Rps28A, Rpl33A, Nop7, or Sof1 genes).
Analysis of pre-rRNA processing by Northern blotting. Strains expressing an N-terminal 3×HA tag were grown in YPG/R and then washed and resuspended in YEPD. RNA was extracted from 10 ml of cells grown to an optical density at 600 nm of 0.4 to 0.5 in YPG/R and of cells grown in YEPD (24 h). RNA extraction and Northern blotting were carried out as previously described (20).

Equal amounts of RNA (5 μg) were loaded in each lane.

Immunoprecipitations. Immunoprecipitations were carried out with N- and C-terminally 3×HA-tagged strains. Protein-protein coimmunoprecipitations were carried out with 200 μl each of anti-HA (12CAS hybridoma culture supernatant) with glass bead protein extracts and blotted with anti-Mpp10 antibodies (20). Immunoprecipitations for protein, RNA, and pre-rRNA were carried out with extracts from strains that had been N-terminally tagged with GAL-3×HA and C-terminally tagged with 3×HA as previously described.

Protein-snoRNA coimmunoprecipitations were carried out with 200 μl of anti-HA (12CAS) on tagged protein extracts made by glass bead disruption, and RNA was extracted and analyzed as previously described (20). Protein-pre-rRNA coimmunoprecipitations were carried out as previously described (40), except that Rps4A and Rps5B pre-rRNA coimmunoprecipitates were probed with oligonucleotides e and b.

Immunofluorescence. Yeast strains expressing 3×HA-tagged proteins were used in indirect immunofluorescence assays to determine subcellular localization as described previously (1, 5). Mouse anti-HA (12CAS hybridoma culture supernatant; dilution, 1:1,000) and rabbit anti-Mpp10 polyclonal antibodies (dilution, 1:2,000) (6) were detected with tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse immunoglobulin G (dilution, 1:1,000) and fluorescein isothiocyanate-conjugated donkey anti-rabbit immunoglobulin G (dilution, 1:300) secondary antibodies (Jackson ImmunoResearch). The localization of tagged Utp4, Utp18, Noc4, Utp20, Bfr2, and Emg1 was carried out with C-terminally 3×HA-tagged strains. Protein-snoRNA coimmunoprecipitations were carried out with extracts from strains that had been N-terminally tagged with GAL-3×HA and C-terminally tagged with 3×HA as previously described.

Four of the five SSU processome-copurifying ribosomal proteins (Rps4, Rps6, Rps7, Rps9, Rps14, Rps27, Rps28, and Rpl33) were immunoprecipitated with beads conjugated with HA antibodies. 3×HA-tagged Utp9 (a bona fide SSU processome component) and YPH499 (the untagged parental strain) were used as positive and negative controls, respectively. Ribosomal proteins were tested with Western blotting for their abilities to coimmunoprecipitate Mpp10. Ribosomal proteins were also tested with Northern blotting for their abilities to coimmunoprecipitate the U3 snoRNA. Results for totals (lanes T), representing 5% (Western blot) and 10% (Northern blot) of the proteins extracted, and immunoprecipitates (lanes IP) are shown.

RESULTS

Previous work identified 28 protein components and the U3 snoRNA as components of the SSU processome (4). Here, we identify a number of additional proteins which, based on a combination of factors, are SSU processome candidates.

During the original SSU processome purification, five copurifying ribosomal proteins, Rps4, Rps6, Rps7, Rps14, and Rps28, were identified (4). In order to assess whether these and other ribosomal proteins might be components of the SSU processome, 3×HA-tagged ribosomal proteins Rps4, Rps6, Rps7, Rps9, and Rps28, were identified (4). In order to assess whether these and other ribosomal proteins might be components of the SSU processome, 3×HA-tagged ribosomal proteins Rps4, Rps6, Rps7, Rps9, Rps14, Rps27, Rps28, and Rpl33 were tested for their ability to coimmunoprecipitate with Mpp10 and the U3 snoRNA, two components of the SSU processome (Fig. 2). Four of the five SSU processome-copurifying ribosomal proteins (Rps4, Rps6, Rps7, and Rps14) were able to coimmunoprecipitate with Mpp10 and the U3 snoRNA, though to different degrees. Rps9 was also able to coimmunoprecipitate with Mpp10 and the U3 snoRNA. Relative to the other ribosomal proteins, Rps7 was able to coimmunoprecipitate only with small amounts of Mpp10 and the U3 snoRNA. In contrast, ribosomal proteins Rps27, Rps28, and Rpl33 did not coimmunoprecipitate with Mpp10 or the U3 snoRNA (Fig. 2).

As expected, no coimmunoprecipitation was observed with the untagged parental strain, YPH499, while Utp9 (a known SSU processome component) did coimmunoprecipitate with both Mpp10 and the U3 snoRNA (4). We verified that each ribosomal protein (both positive and negative) was enriched by immunoprecipitation by stripping the blot and reprobing it for the HA-tagged protein (data not shown).

We examined whether proteins represented as single peptides by mass spectrometry were SSU processome components (YJL069c and Enp1). In addition, we tested whether several proteins identified in tandem affinity purification-tagged SSU processome protein coimmunoprecipitates (8, 9) were also members of the SSU processome (YJL069c, YBL004w, YLR409c, YGR090w, Noc4, Emg1, Krr1, Bfr2, Enp1, and Enp2) (Tables 1 and 2). Proteins would be considered components of the SSU processome (i) if they were nucleolar, (ii) if they could coimmunoprecipitate with Mpp10 and the U3 snoRNA, both known SSU processome components, and (iii) if their depletion led to a decrease in the levels of the 18S rRNA.

Since ribosome biogenesis occurs within the nucleolus, we reasoned that proteins that were part of the SSU processome would also be nucleolar and colocalize with another nucleolar protein, Mpp10. Therefore, we first determined the subcellular localization of the candidate SSU processome proteins. We localized HA-tagged YJL069c, YBL004w, YLR409c, YGR090w, Noc4, Emg1, Krr1, Bfr2, Enp1, and Enp2 (Fig. 3). An untagged parental strain, YPH499, and a known SSU processome component, Utp4-3×HA, were used as negative and positive controls, respectively. All of these proteins colocalized with Mpp10 and are therefore localized primarily to the nucleolus. Enp1, Krr1, and Noc4 have previously been localized to the nucleolus (3, 9, 25, 32). While the present work was in progress, YJL069c, YGR090w, and Bfr2 were localized to the nucleolus, whereas YBL004w, YLR409c, and Noc4 were localized to other cellular compartments (12). When overexpressed, Enp2 appears to accumulate predominantly in the nucleoplasm; however, we found that it localizes primarily to the
nucleolus when expressed from its own promoter (19). Further analysis (see below) indicates that only seven of these proteins are part of the SSU processome. Bfr2 and Enp2 were localized to the nucleolus but are not SSU processome components, since they did not coimmunoprecipitate with Mpp10 and the U3 snoRNA (Fig. 3B and data not shown). Therefore, four of these proteins have subsequently been named to reflect their function in the biogenesis of the small ribosomal subunit (Utp18 for YJL069c, Utp20 for YBL004w, Utp21 for YLR409c, and Utp22 for YGR090w) (Fig. 3A).

Collectively, our results suggest that these proteins are involved in ribosome biogenesis. To substantiate this finding, we determined whether the new nonribosomal SSU processome components have functions in ribosome biogenesis. Since all of the proteins tested are encoded by essential genes, we fused a galactose-inducible promoter and a 3HA tag to each gene of interest in the chromosome. When these strains are grown in medium with galactose and raffinose, the gene is transcribed and the protein is expressed. However, when the yeast strains are grown in medium containing dextrose, the promoter is unable to induce gene expression, and its protein levels are depleted over time. RNA was extracted from strains grown to early log phase in medium containing galactose and raffinose (undepleted) and from strains grown for 24 h in dextrose (depleted). Upon RNA analysis by Northern blotting, Utp1, Utp7, Utp18, Noc4, Utp20, and Emg1 revealed similar prerRNA processing defects (Fig. 5). In all of these strains, 35S and 23S prerRNAs accumulated, and 27SA2, 20S, and 18S rRNAs were no longer present. Accumulation of these precursors suggests defects in prerRNA processing at cleavage sites A0, A1, and A2 (Fig. 5). Different processing defects were also observed in cells depleted of Bfr2 and Enp1. Strikingly, when the Bfr2 protein was depleted, the levels of all the prerRNAs were reduced; however, 18S rRNA levels were more affected.

### Table 1. Additional nonribosomal components of the SSU processome

<table>
<thead>
<tr>
<th>Protein</th>
<th>Alias</th>
<th>Gene product</th>
<th>Mol wt (10^3)</th>
<th>Essential</th>
<th>Homolog (GenBank no. and/or % homology)</th>
<th>Motif and/or comment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Utp18</td>
<td>YJL069c</td>
<td>66.3</td>
<td>Yes</td>
<td>CGI-48 (25)</td>
<td>WD repeats, interacts with Utp21; one peptide in purification</td>
<td>4, 12</td>
<td></td>
</tr>
<tr>
<td>Noc4</td>
<td>YPR144c</td>
<td>63.5</td>
<td>Yes</td>
<td>MGC3162 (31)</td>
<td>Noc domain and ribosome biogenesis</td>
<td>8, 9, 12, 24, 29</td>
<td></td>
</tr>
<tr>
<td>Utp20</td>
<td>YBL004w</td>
<td>287</td>
<td>Yes</td>
<td>DRIM^a (NP05531 8.1; 23)</td>
<td>HEAT repeats and homolog of DRIM</td>
<td>8, 9, 12, 33, 34</td>
<td></td>
</tr>
<tr>
<td>Utp21</td>
<td>YLR409c</td>
<td>105</td>
<td>Yes</td>
<td>TA-WDRP (NP644810.1; 32) NO6 (NRAP; 24)</td>
<td>WD repeats, coiled-coil domains, interacts with Utp18 Ribosome biogenesis and homolog of human Nol6</td>
<td>8, 9, 12, 13, 33</td>
<td></td>
</tr>
<tr>
<td>Utp22</td>
<td>YGR090w</td>
<td>141</td>
<td>Yes</td>
<td>C2F^b (53)</td>
<td>Required for 40S biogenesis and interacts with Nop14; one peptide in purification</td>
<td>4, 9, 21</td>
<td></td>
</tr>
<tr>
<td>Emg1</td>
<td>Nep1</td>
<td>YLR186w</td>
<td>27.3</td>
<td>Yes</td>
<td>HRB2 (NP008974.3; 61)</td>
<td>Required for 18S, KH domain, KRR-R motif</td>
<td>8, 9, 13, 14, 32, 37</td>
</tr>
<tr>
<td>Krr1</td>
<td>YCL059c</td>
<td>37.2</td>
<td>Yes</td>
<td>HRB2 (NP008974.3; 61)</td>
<td>WD repeats, interacts with Utp21; one peptide in purification</td>
<td>4, 9, 21</td>
<td></td>
</tr>
</tbody>
</table>

^a DRIM, down-regulated in metastasis.
^b HEAT, Huntington-elongation-A subunit-TOR; KH, lysine homology; KRR-R, lysine arginine arginine arginine.
^c C2F is a possible homolog.

### Table 2. Nucleolar proteins that are not components of the SSU processome

<table>
<thead>
<tr>
<th>Protein</th>
<th>Alias</th>
<th>Gene product</th>
<th>Mol wt (10^3)</th>
<th>Essential</th>
<th>Homolog (GenBank no. and/or % homology)</th>
<th>Motif and/or comment</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Enp1</td>
<td>Meg1</td>
<td>YBR247c</td>
<td>55</td>
<td>Yes</td>
<td>BYSL^a (42) NLS, coiled-coil domains, interacts with Nop1; one peptide in purification</td>
<td>3, 4, 9, 31–33</td>
<td></td>
</tr>
<tr>
<td>Enp2</td>
<td>YGR145w</td>
<td>81.7</td>
<td>Yes</td>
<td>FLJ14075 (38)</td>
<td>WD repeats, interacts with Mpp10 and Bfr2, and has homology to Spb1 Interacts with Lcp5, Crm1, and Enp2</td>
<td>8, 13</td>
<td></td>
</tr>
<tr>
<td>Bfr2</td>
<td>YDR299w</td>
<td>61.2</td>
<td>Yes</td>
<td>AATF (NP036270.1; 25)</td>
<td>WD repeats, interacts with Mpp10 and Bfr2, and has homology to Spb1 Interacts with Lcp5, Crm1, and Enp2</td>
<td>8, 12–14, 37</td>
<td></td>
</tr>
</tbody>
</table>

^a BYSL is a possible homolog.
^b NLS, nuclear localization sequence; WD, tryptophan-aspartate repeat.
than those of 25S rRNA. In Enp1 depletion, accumulation of the processing intermediates 35S, 23S, and 21S pre-rRNA (A1 to A3) was observed, and levels of the 27SB and 18S rRNAs were reduced. The 21S pre-rRNA has previously been noted to be a precursor that is normally present in the strains we studied (20). The observed increase in the accumulation of the 21S pre-rRNA precursor suggests a defect in cleavage at A2. While this work was in progress, pre-rRNA processing defects were reported in Noc4, Utp20, Utp21, Utp22, Emg1, Krr1, and Enp1. All proteins were 3×HA tagged and colocalized using anti-HA(red) or nucleolar protein Mpp10 (green) with anti-Mpp10 by indirect immunofluorescence. DAPI (4',6'-diamidino-2-phenylindole) (blue) was used to stain the nuclear DNA. Merge, merge of tagged protein, Mpp10, and DAPI. (A) Nucleolar localization of Utp18, Noc4, Utp20, Utp21, Utp22, and Emg1. The parental strain YPH499 (untagged) and Utp4 (a bona fide SSU processome component) were used as negative and positive controls, respectively. (B) Nucleolar localization of Bfr2 and Enp2.

Since the SSU processome proteins affect the processing of RNA precursors to the small-ribosomal-subunit rRNA, we determined with which specific precursor rRNAs they were associated. 3×HA-tagged SSU processome proteins were immunoprecipitated, RNA was extracted, and Northern blots were probed with oligonucleotides specific for pre-rRNAs. Utp1, Utp4, Utp9, Utp16, Utp17, Utp20, Utp21, Rps4, and Rps6 were all tested for their ability to coimmunoprecipitate with pre-rRNA species. Utp1, Utp4, Utp9, Utp16, Utp17, Utp20, Utp21, Rps4, and Rps6 were all tested for their ability to coimmunoprecipitate with pre-rRNA species. Utp1, Utp4, Utp9, Utp16, and Utp17 were all able to coimmunoprecipitate with the 35S, 33/32S, and 23S rRNA precursors (Fig. 6). Utp20 and Utp21 were able to efficiently coimmunoprecipitate with the 23S pre-rRNA (Fig. 6). Pre-rRNA coimmunoprecipitation experiments demonstrate that SSU processome proteins efficiently associate with the 23S pre-rRNA. None of these proteins were able to coimmunoprecipitate with the 20S pre-rRNA. Rps4 and Rps6 also coimmunoprecipitated with the 23S pre-rRNA (Fig. 6). This suggests that a subset of small-ribosomal-subunit proteins is found on pre-rRNA precursors with the SSU processome. As expected, Rps4 and Rps6 also coimmunoprecipitated with the 20S pre-rRNA, the RNA that is exported from the nucleolus to the cytoplasm, where the small ribosomal subunit undergoes a final cleavage step. No coimmunoprecipitation of pre-rRNA was observed in the untagged strain, YPH499. In contrast, an SSU processome component, Imp4, was able to coimmunoprecipitate with the 27SB pre-rRNA specie, but not
with precursors to the 18S rRNA, as previously described (40). Together, these data suggest that SSU processome components are associated with the 35S, 33/32S, and 23S pre-rRNA species. In addition, Rps4 and Rps6 are also associated with the 23S and 20S pre-rRNA, the latter of which represents the fully assembled 40S preribosome.

**DISCUSSION**

During the original SSU processome purification, 28 components that are required for 18S rRNA biogenesis were identified (4). Upon further analysis, we have identified 12 additional components of the SSU processome. These components include seven nonribosomal proteins (Utp18, Noc4, Utp20, Utp18, Noc4, Utp20, Emg1, Bfr2, and Enp1) from galactose-inducible and glucose-repressible promoters were grown in early log phase (time point 0) in galactose- and raffinose-supplemented media (undepleted) and then shifted into glucose (depleted) for 24 h. YPH499, the untagged parental strain, was used as a control. RNA from undepleted or depleted yeast strains was analyzed for the presence of pre-rRNAs by Northern blotting. Equal amounts of RNA were separated on formaldehyde–1.2% agarose gels, transferred to membranes, and hybridized with the specific oligonucleotide probes shown in Fig. 1.

(A) Northern blot using oligonucleotide c, which hybridizes to 35S, 32S, 27SA2, 23S, and 21S pre-rRNAs; (B) Northern blot using oligonucleotides b and c, which hybridize to the 35S, 32S, 27SA, 27SB, 23S, 20S pre-rRNAs; (C) Northern blot using oligonucleotides a and y, which hybridize to the 18S and 25S rRNAs, respectively.

![FIG. 5. Depletion of new SSU processome proteins leads to defects in pre-18S rRNA processing. Strains expressing Utp1, Utp7, Utp18, Noc4, Utp20, Emg1, Bfr2, and Enp1 from galactose-inducible and glucose-repressible promoters were grown in early log phase (time point 0) in galactose- and raffinose-supplemented media (undepleted) and then shifted into glucose (depleted) for 24 h. YPH499, the untagged parental strain, was used as a control. RNA from undepleted or depleted yeast strains was analyzed for the presence of pre-rRNAs by Northern blotting. Equal amounts of RNA were separated on formaldehyde–1.2% agarose gels, transferred to membranes, and hybridized with the specific oligonucleotide probes shown in Fig. 1.](image)

The order of assembly of ribosomal proteins with rRNA was first described for *Escherichia coli* during the early 1970s (27, 35). The first steps in analysis of the ribosomal pattern of assembly came in 1966, when Staehelin and Meselson observed that 30 to 40% of the small-ribosomal-subunit proteins partially disassembled during density gradient centrifugation in 5 M cesium chloride (35). This discovery enabled the establishment of a system for reconstituting ribosomes, which facilitated the elucidation of a detailed pathway for in vitro ribosome assembly, termed the “30S assembly map” (11, 27, 28, 36). Ribosomal proteins were grouped according to their abilities to bind to rRNA and to each other. Primary binders (i.e., S4, S7, S8, S15, S17, and S20) are ribosomal proteins that bind to rRNA directly, whereas secondary and tertiary binders are ribosomal proteins that require the presence of one or more ribosomal proteins (28). Many of the bacterial primary binding proteins (for example, S7, S8, S15, and S20) do not have yeast homologues, i.e., the primary and tertiary binding proteins S4 and S11, respectively (23, 30). Since *S. cerevisiae* rRNAs and bacterial rRNAs are different, and since not all ribosomal proteins are conserved in both
organisms, *S. cerevisiae* may have a set of primary binding proteins that is distinct from that in bacteria.

We propose that the ribosomal proteins associated with the SSU processome may be analogous to the primary or secondary binding proteins described for bacteria, since cleavages by the SSU processome represent early pre-rRNA maturation steps for the small-ribosomal-subunit rRNA. We found that the yeast ribosomal proteins Rps4, Rps6, Rps7, Rps9, and Rps14 were bona fide components of the SSU processome and may therefore represent a distinct set of yeast ribosomal proteins involved in the early stages of ribosome assembly. Because there is no in vitro ribosomal assembly system for eukaryotic ribosomes, we can only hypothesize which ribosomal proteins bind first on the basis of their association with pre-rRNAs. For example, Rps4 and Rps6 were both able to coimmunoprecipitate the 23S pre-rRNA, suggesting that they may be involved in ribosome assembly prior to cleavage at sites A0, A1, and A2 (Fig. 6). These results are consistent with those of Kruiswijk et al., who hypothesized that a specific set of ribosomal proteins (Rps23, Rps18, Rps2, Rps30, Rps5, Rps11, Rps19, Rps4, Rps21, Rps9, Rps22, and Rps3 [in new nomenclature]) were involved in the early stages of ribosomal assembly (18). In agreement with this hypothesis, we found that Rps4 and Rps9 may be required for the early steps of ribosome assembly. However, we were unable to confirm the current ribosomal protein counterpart for 10 ribosomal proteins (S19, S12, S22, S20, S21, S11, S6, S17, S5, and S29) that were found by Kruiswijk et al. to be associated with an early step of assembly of the small ribosomal subunit (18, 23, 30). In addition, two ribosomal proteins that we have found to be SSU processome components, Rps7 and Rps14, were not analyzed by Kruiswijk et al.

Our results are consistent with those of Grandi et al. and Schäfer et al., who reported the identification of a 90S RNP that contains 35 nonribosomal proteins and the U3 snoRNA (9, 33). They identified 27 SSU processome proteins that we had identified previously (Nop1, Noc4, Nop56, Mpp10, Imp3, Imp4, Sof1, Rrp5, Rrp9, Utp1, Utp2, Utp4, Utp5, Utp6, Utp7, Utp8, Utp9, Utp10, Utp11, Utp12, Utp13, Utp15, Utp16, Utp17, Utp18, Utp21, Utp22, and Emgl1) but did not identify 2 other SSU processome components (Utp3 and Utp14). Due to the large overlap of protein components, it seems highly probable that we have independently characterized the same complex. Our results are also consistent with those of Krogan et al. and Peng et al., who used genomics and proteomic experiments to identify additional proteins required for ribosome biogenesis (17, 29).

One important difference between the interpretation of our results and those previously published is whether Enp1 is a component of the SSU processome-90S preribosome (3, 9, 33). We have shown here that tagged Enp1 does not detectably coimmunoprecipitate with other SSU processome components or the U3 snoRNA, compared to positive and negative controls, because Enp1 coimmunoprecipitated with the same amounts of Mpp10 and the U3 snoRNA as did the negative controls (Rpf2 and the untagged parental strain, YPH499). In addition, genetic depletion of Enp1 led to the accumulation of the 21S pre-rRNA, a phenotype different from that caused by the depletion of any other SSU processome component. The appearance of the 21S pre-rRNA upon Enp1 depletion suggests that it may participate in a later complex, as has been suggested by Milkereit et al. (25, 33). However, sedimentation of Enp1 on sucrose density gradients shows that Enp1 sediments at both 40S and 90S (33). These results therefore suggest that Enp1 may have multiple roles in ribosome biogenesis, and we cannot rule out the possibility that it may also be present in substoichiometric amounts in the SSU processome (3).

In addition to the SSU processome’s role in pre-rRNA processing, it also likely has a role in RNA folding, as has been suggested previously (4, 39). Furthermore, the specific association of a subset of ribosomal proteins with the SSU processome suggests that the SSU processome is also an assembly intermediate for ribosome biogenesis. Therefore, folding of
the pre-rRNA for processing is intertwined with ribosome assembly.

ACKNOWLEDGMENTS

K.A.B. and J.E.G.G. were supported by predoctoral fellowships from the National Institutes of Health (GM67564 and GM20905). K.A.B. was previously supported by a Research Service Award (GM07499) from the National Institute of General Medical Sciences (NIGMS). S.G. was supported by a Leslie H. Warner fellowship in cancer research. This work was supported by NIH grant GM52581 to S.J.B.

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