Functional Analysis of Rrp7p, an Essential Yeast Protein Involved in Pre-rRNA Processing and Ribosome Assembly

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During the functional analysis of open reading frames (ORFs) identified during the sequencing of chromosome III of Saccharomyces cerevisiae, the previously uncharacterized ORF YCL031C (now designated RRP7) was deleted. RRP7 is essential for cell viability, and a conditional null allele was therefore constructed, by placing its expression under the control of a regulated GAL promoter. Genetic depletion of Rrp7p inhibited the pre-rRNA processing steps that lead to the production of the 20S pre-rRNA, resulting in reduced synthesis of the 18S rRNA and a reduced ratio of 40S to 60S ribosomal subunits. A screen for multicopy suppressors of the lethality of the GAL::rrp7 allele isolated the two genes encoding a previously unidentified ribosomal protein (r-protein) that is highly homologous to the rat r-protein S27. When present in multiple copies, either gene can suppress the lethality of an RRP7 deletion mutation and can partially restore the ribosomal subunit ratio in Rrp7p-depleted cells. Deletion of both r-protein genes is lethal; deletion of either single gene has an effect on pre-rRNA processing similar to that of Rrp7p depletion. We believe that Rrp7p is required for correct assembly of rpS27 into the preribosomal particle, with the inhibition of pre-rRNA processing appearing as a consequence of this defect.

The biosynthesis of eukaryotic ribosome is a complex process that occurs in a subnuclear organelle: the nucleolus (for reviews, see references 15 and 32). In the yeast Saccharomyces cerevisiae, between 100 and 200 copies of the rRNA gene (rDNA) are present on chromosome XII. Each repeated transcription unit is transcribed by RNA polymerase I into a 7-kb, 35S primary transcript that contains the 18S, 5.8S, and 25S rRNA sequences separated by internal transcribed spacers (ITS) and flanked by external transcribed spacers (ETS). The 35S transcript undergoes a complex series of cleavages and trimming reactions that remove the spacer regions, releasing the three mature rRNA species. The early steps in pre-rRNA processing, cleavage at sites A0, A1, and A2, separate the 20S pre-rRNA from the 27SA pre-rRNA. 20S pre-rRNA is the precursor to the 18S rRNA which is incorporated into the 40S ribosomal subunit, while 27SA contains the 5.8S and 25S rRNAs which are destined to form the 60S subunit. In many cases, mutations affect only the processing reactions on either the pathway of 18S synthesis or the pathway of 5.8S-25S synthesis (38). During the pre-rRNA processing events, many of the approximately 80 ribosomal proteins assemble onto the rRNA, but little detailed knowledge is available on the assembly pathway in eukaryotes. Mutations in r-protein genes lead to the inhibition of synthesis of the subunit of which they are components, presumably as a consequence of defects in subunit assembly (40). Ribosomal proteins L1 and rp59 are necessary for production of the 60S and the 40S subunits, respectively (11, 23), as well as the UBI1 to UBI3 genes that encode ribosomal proteins (12), while a mutation in the ribosomal protein L16 prevents the normal processing of the 27S pre-rRNA to 25S rRNA (24). A number of other yeast mutations have been identified which inhibit pre-rRNA processing but which are not predicted to encode enzymatic components of the processing machinery (for a review, see reference 38). These include several predicted ATP-dependent RNA helicases (26, 30, 31) and Nsr1p, the yeast homolog of vertebrate nucleolin (17, 18, 20). It seems likely that many of these also lead to defects in ribosome assembly, but in no case is the specific function of an assembly cofactor known.

In this paper, we describe an approach that led to the identification of a new component of the ribosome synthesis machinery. The initial project was the systematic functional analysis of open reading frames (ORF) located on chromosome III of S. cerevisiae. A small ORF, YCL031C, was found to be essential for cell viability, and the lethality of the deletion was suppressed by overexpression of a ribosomal protein. Starting from this finding, we have demonstrated that the protein encoded by YCL031C is required for pre-rRNA processing, and we therefore called this locus RRP7 (for ribosomal RNA processing).

MATERIALS AND METHODS

Strains, media, and microbiological methods. S. cerevisiae W303-1B (MATα ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15 can1-100) was used as the wild-type parent. The strain BMA59-1B is isogenic except that it was transformed with the UR43-1pGAL10::rrp7 construct, which is integrated at the RRP7 locus. For the purpose of selecting multicopy suppressors, BMA59-1B was UV irradiated (0.256 nm) for 40 s (80% killing rate), grown on complete galactose medium for 24 h, and harvested. A total of 5 × 10⁶ cells were plated on galactose medium containing 5-fluoro-orotic acid in order to select ura⁻ colonies. Fifteen colonies were selected. Among those 15, one colony that did not revert was named BMA65-1B and used for Rrp7p depletion and selection of multicopy suppressors.

Strains BMA55-1B (MATα ura3-1 ade2-1 leu2-3,112 his3-11,15 can1-100 trp1-1 pFL38::RRP7), BMA67-1B (MATα ura3-1 ade2-1 leu2-3,112 his3-11,15 can1-100 trp1-1 UBI1::TRP1), and BMA66-1B (MATα ura3-1 ade2-1 leu2-3,112 his3-11,15 can1-100 UBI3::TRP1) were obtained as described below (see Results).

Growth and handling of S. cerevisiae involved standard techniques. Strains were grown in complete medium (YPGA [1% yeast extract, 1% Bacto Peptone, 2% glucose, 2% Bacto yeast nitrogen base without amino acids]) at 30°C.
Extraction of RNA, hybridization of pre-rRNA, primer extension, and pulse-chase labeling. Total yeast RNA was isolated from exponentially growing cells essentially as described by Sherman et al. (33). The extraction buffer was 50 mM Tris-HCl (pH 7.5)–10 mM EDTA–150 mM NaCl–0.5% sodium dodecyl sulfate (SDS).

For RPS27 mRNA analysis, RNAs were fractionated on 1.5% agarose-formaldehyde gels and transferred to nitrocellulose membranes. Ten picomoles of oligonucleotide 8238, GATGTTGAAACACCGTGTCG, or 1854 (29) was 5′ end labeled with 250 Ci of [γ-32P]ATP by using T4 polynucleotide kinase. The hybridization was performed at 37°C for 12 h. The membrane was washed three times for 5 min at 25°C and for 15 min at 42°C. The membrane was subjected to several autoradiographies with the Molecular Dynamics PhosphorImage to measure the hybridization signal intensities.

For Northern analysis of pre-rRNA, 10 μg of total RNA was loaded on 1.2% agarose-formaldehyde gels and transferred to nitrocellulose membranes. Hybridization was performed as described above. Primer extension was performed as described previously (5). A total of 4 μg of RNA was used for each sample.

The nucleotide probes for Northern blots and primer extension are depicted in Fig. 6 and are as follows: probe A, CATGCTTAACTTGTGAGAC; probe B, CCC AGATACTATCTTTAAAG; probe C, CGGTTTTATAATGTCTCCTA; probe D, TTGTTAATCCGTGGCGCC; probe E, CTAGTCTGAAAAATTTCTC; and probe F, TGAGAAGGAAATGAGCCT.

Pulse-chase labeling. Aliquots (5 μl) of cells growing in galactose or glucose minimum medium at an OD of ~0.4 were labeled with 250 μCi of [methyl-3H]thymidine for 2 min at 30°C. Unlabeled methionine was added at a final concentration of 5 mM. Samples (1 ml) were taken at various times, and the reaction was stopped by addition of 2 volumes of ethanol. Samples were immediately frozen and stored at –20°C until used for RNA extraction.

Preparation and gradient analysis of yeast polysomes. Yeast polysomes were grown in 100 ml of YPGA or YPGAL medium at 30°C to an OD of 0.7. Polysome extraction and polysome profile analysis were done according to the method of Petitjean et al. (29) using a density gradient fractionator (ISCO s600).

Nucleotide sequence accession numbers. The GenBank accession numbers for the complete sequences of RRP7, RPS27A, and RPS27B are X99720, Z26156, and U10399, respectively.

**RESULTS**

**RRP7 encodes a 35-kDa protein that is essential for cell viability.** YCL031C (here referred to as RRP7) is an ORF located on chromosome III (27) that consists of 891 bp (297 amino acids) and is predicted to encode a 35-kDa protein. The codon bias is low (0.06), suggesting that the corresponding protein is in low abundance. Neither known motifs nor clear homology to other proteins were detected by computational analysis.

To determine the function of RRP7, we used a PAP approach to perform gene deletion (3). The ORF for RRP7 was deleted by inserting the TRP1 marker gene between the 5′ and 3′-flanking sequences of RRP7. After transformation of a diploid strain deleted for TRP1 and inaccurate replacement of the RRP7 marker gene between the 5′ and 3′-flanking sequences of RRP7, RRP7 was demonstrated by Southern analysis of two transformants (see Segregation of Materials and Methods). Tetrad analysis showed a 2:2 segregation of cell viability at 30°C, with all viable spores being Trp+. The diploid strain heterozygous for the RRP7::TRP1 deletion was transformed with plasmid bearing the RRP7 gene and the URA3-selective marker (pFL38-RRP7) and then sporulated. Haploid rrp7::TRP1 strains were then recovered in the progeny. These haploids were unable to lose the pFL38-RRP7 plasmid on medium containing 5-fluoro-orotic acid, which counterselects the URA3 plasmid (7), and were therefore unable to grow. The inability of rrp7::TRP1 cells to lose pFL38-RRP7 demonstrates that RRP7 is essential for cell viability.

**Construction of a conditional null RRP7 allele.** To create a conditional RRP7 allele, the RRP7 ORF was fused to a protein-destabilizing cassette and placed under the control of the GAL10 promoter. This promoter is active when grown on galactose, and strongly repressed on glucose. The cassette consisting of the GAL UAS-ubiquitin-lacI-hemagglutin (HA)-encoding sequence was fused to the 5′ end of the RRP7 gene. Thus, the Rrp7p fusion protein should be degraded via the ubiquitin-dependent protein degradation pathway, leading to its rapid depletion after transfer...
For this purpose, a new set of vectors based on the pYeF1 vector were constructed (see Materials and Methods). The entire construct (Fig. 1A) flanked by the URA3 marker gene was integrated at the RRP7 locus by homologous recombination. Correct replacement was demonstrated by Southern analysis in the integrated strain (data not shown). The growth defect due to the depletion of RRP7 following a shift from galactose to glucose medium appears rapidly; the doubling time increases progressively from the second generation after transfer to glucose medium (Fig. 1B), and cell growth is undetectable after seven generations. This growth inhibition is reversible, however, since cells grown for 24 h in glucose are still able to form colonies on galactose medium and not on glucose medium. The same phenomenon was observed upon depletion of the ribosomal protein L16 (23) and of Nop1p (37).

Isolation of two multicopy suppressors that can suppress the lethality of GAL::rrp7 and rrp7::TRP1 mutations. In order to gain information about the function of RRP7, a search for multicopy suppressors was undertaken. The GAL::rrp7 strain was grown on galactose medium and transformed with a yeast genomic library in pFL44L, a URA3 multicopy vector (34). A total of 20,000 uracil prototrophy transformants were replica-plated onto glucose medium twice to decrease the background of residual growth. After a 6-day incubation at 30°C, 15 growing transformants were obtained and further analyzed. After extraction and retransformation, 14 plasmids that were able to restore growth on glucose medium at different rates were recovered. A first class was composed of five independent plasmids conferring strong suppression. These included four plasmids bearing the ORF YHR21C and four plasmids bearing the ORF YKL156W (Fig. 2A). These two ORF were identified during the sequencing of the S. cerevisiae genome and map onto chromosomes VIII and XI, respectively. Based on data described below, we have assigned the designation RPS27B (for ribosomal protein S27) to YHR21C and the designation RPS27A to YKL156W.

To test whether the RPS27 genes are also able to suppress in the complete absence of Rrp7p, the diploid strain rrp7::TRP1/RRP7 was transformed with multicopy plasmids bearing either pRPS27A or pRPS27B, and the tetrads were dissected. The rrp7::TRP1 haploids were viable, although with a low growth rate, in the presence of either of the plasmids (Fig. 2B). pRPS27B supports marginally better growth (doubling time of about 7 h for the strain carrying the deletion when suppressed weak suppression. These included four plasmids bearing the ORF YHR21C and four plasmids bearing the ORF YKL156W (Fig. 2A). These two ORF were identified during the sequencing of the S. cerevisiae genome and map onto chromosomes VIII and XI, respectively. Based on data described below, we have assigned the designation RPS27B (for ribosomal protein S27) to YHR21C and the designation RPS27A to YKL156W. To test whether the RPS27 genes are also able to suppress in the complete absence of Rrp7p, the diploid strain rrp7::TRP1/RRP7 was transformed with multicopy plasmids bearing either pRPS27A or pRPS27B, and the tetrads were dissected. The rrp7::TRP1 haploids were viable, although with a low growth rate, in the presence of either of the plasmids (Fig. 2B). pRPS27B supports marginally better growth (doubling time of about 7 h for the strain carrying the deletion when suppressed weak suppression. These included four plasmids bearing the ORF YHR21C and four plasmids bearing the ORF YKL156W (Fig. 2A). These two ORF were identified during the sequencing of the S. cerevisiae genome and map onto chromosomes VIII and XI, respectively. Based on data described below, we have assigned the designation RPS27B (for ribosomal protein S27) to YHR21C and the designation RPS27A to YKL156W. To test whether the RPS27 genes are also able to suppress in the complete absence of Rrp7p, the diploid strain rrp7::TRP1/RRP7 was transformed with multicopy plasmids bearing either pRPS27A or pRPS27B, and the tetrads were dissected. The rrp7::TRP1 haploids were viable, although with a low growth rate, in the presence of either of the plasmids (Fig. 2B). pRPS27B supports marginally better growth (doubling time of about 7 h for the strain carrying the deletion when suppressed weak suppression. These included four plasmids bearing the ORF YHR21C and four plasmids bearing the ORF YKL156W (Fig. 2A). These two ORF were identified during the sequencing of the S. cerevisiae genome and map onto chromosomes VIII and XI, respectively. Based on data described below, we have assigned the designation RPS27B (for ribosomal protein S27) to YHR21C and the designation RPS27A to YKL156W. To test whether the RPS27 genes are also able to suppress in the complete absence of Rrp7p, the diploid strain rrp7::TRP1/RRP7 was transformed with multicopy plasmids bearing either pRPS27A or pRPS27B, and the tetrads were dissected. The rrp7::TRP1 haploids were viable, although with a low growth rate, in the presence of either of the plasmids (Fig. 2B). pRPS27B supports marginally better growth (doubling time of about 7 h for the strain carrying the deletion when suppressed weak suppression. These included four plasmids bearing the ORF YHR21C and four plasmids bearing the ORF YKL156W (Fig. 2A). These two ORF were identified during the sequencing of the S. cerevisiae genome and map onto chromosomes VIII and XI, respectively. Based on data described below, we have assigned the designation RPS27B (for ribosomal protein S27) to YHR21C and the designation RPS27A to YKL156W. To test whether the RPS27 genes are also able to suppress in the complete absence of Rrp7p, the diploid strain rrp7::TRP1/RRP7 was transformed with multicopy plasmids bearing either pRPS27A or pRPS27B, and the tetrads were dissected. The rrp7::TRP1 haploids were viable, although with a low growth rate, in the presence of either of the plasmids (Fig. 2B). pRPS27B supports marginally better growth (doubling time of about 7 h for the strain carrying the deletion when suppressed weak suppression. These included four plasmids bearing the ORF YHR21C and four plasmids bearing the ORF YKL156W (Fig. 2A). These two ORF were identified during the sequencing of the S. cerevisiae genome and map onto chromosomes VIII and XI, respectively. Based on data described below, we have assigned the designation RPS27B (for ribosomal protein S27) to YHR21C and the designation RPS27A to YKL156W.
by pRPS27B and about 7 h 15 min when suppressed by pRPS27A. The ability of the two genes to suppress the deletion of RRP7 when cloned into a centromeric vector was tested. Fragments harboring either RPS27A or RPS27B were cloned into pFL38, and the corresponding plasmids were transformed into the diploid strain rps27::TRP1/RRP7. After sporulation and dissection, no Trp+ Ura+ spores were obtained in either case, showing that suppression was dosage dependent.

RPS27A and RPS27B are duplicated genes encoding an essential protein highly homologous to the rat ribosomal protein RS27. Comparison between the sequences of RPS27A and RPS27B shows that the predicted proteins differ by only 1 amino acid among 82. Computer analysis reveals high homology to the rat small subunit ribosomal protein RS27 (70% identity at the protein level), suggesting that the suppressors are the two copies of the corresponding yeast ribosomal protein gene. RPS27A and RPS27B contain introns of 550 and 350 nucleotides, respectively, that lie immediately after the first codon. They have high codon biases, 0.745 and 0.510, respectively, as expected for yeast ribosomal protein genes. The predicted proteins have a zinc finger-like motif of the C2-C2 type as do many other ribosomal proteins from eukaryotes, archaebacteria, eubacteria, and mitochondria (9).

The complete ORF for RPS27B was replaced by the kan' gene, while the RPS27A ORF was replaced by the HIS3 gene. Accurate gene replacement was verified by Southern blot analysis (see Materials and Methods). The deletion of RPS27A has no significant effect on growth on YPGA (the doubling time of 75 min being the same as that for the wild-type strain W303), whereas the deletion of RPS27B strongly affects growth on YPGA (doubling time of 4 h). The stronger phenotype of the RPS27B deletion correlates with its higher codon bias, suggesting a higher level of expression. It also correlates with the higher degree of suppression observed for RPS27B. A multiply plasmid bearing RPS27A can fully restore a wild-type growth rate to the strain with RPS27B deleted, indicating that the two proteins can functionally substitute for each other. To determine whether they are essential for growth, the two strains carrying the deletions were crossed and the resulting diploid cells were dissected. No His+ Kan' recombinants were recovered among the progeny. This shows that the double deletion is lethal. Viable His+ Kan' spores were obtained among the meiotic progeny of the same diploid cells following transformation by a plasmid harboring either RPS27A or RPS27B. We conclude that both genes are expressed, that they complement each other, and that the protein they encode is essential.

In order to test whether the slow growth of the rps27B deletion strain is a consequence of a higher level of expression of RPS27B, we measured the relative steady-state level of the RPS27A and RPS27B mRNAs in a wild-type haploid strain and in strains carrying the disrupted alleles. Total RNA was extracted, and the levels of the RPS27A and RPS27B mRNAs were quantified by Northern hybridization using an oligonucleotide probe that is 100% homologous to the nucleotide sequence of both RPS27A and RPS27B. Two transcripts, of about 550 and 400 nucleotides, that correspond to the RPS27A and RPS27B mRNA, respectively, were detected (Fig. 3). In the wild-type strain, RPS27B is expressed at approximately twice the level of RPS27A. Results were recorded as percentages of total RPS27 mRNA in wild-type cells. The results for RPS27A and RPS27B, respectively, for the indicated yeast strains were as follows: wild type, 36% ± 3% and 64% ± 6%; rps27A, 0% and 60% ± 2%; and rps27B, 33% ± 4% and 0%. No dosage compensation mechanism appears to exist, since the levels of the RPS27A and RPS27B mRNAs remain constant in the strains with the other gene deleted. We conclude that the growth defect observed in the rps27B deletion strain results from reduced production of rps27 due to the lower level of transcription of RPS27A.

Depletion of either rps27A, rps27B, or Rrp7p affects the level of 40S ribosomal subunits. To test whether rps27 is required for ribosome synthesis, polyribosome profiles from the strains with RPS27A and RPS27B deletions were analyzed. The requirement for Rrp7p was assessed by comparing the GAL::rrp7 strain grown in galactose medium to that grown in glucose medium (Fig. 4A). The polysome profile of the GAL::rrp7 strain grown in galactose medium was identical to that obtained from the wild-type strain (data not shown). Compared to this strain, the RPS27A and RPS27B deletion strains both show a lowered accumulation of free 40S subunits. The effects of the RPS27B deletion are more pronounced than for RPS27A deletion, in agreement with the relative growth rates of the strains. This indicates that rps27 is required for the formation of 40S ribosomal subunits but not for the formation of 60S subunits. The GAL::rrp7 strain was grown to mid-log phase in galactose medium and transferred to glucose medium for 17 h. Following depletion, the GAL::rrp7 strain had no free 40S ribosomal subunits and a large excess of free 60S subunits. It also had reduced amounts of monoribosomes and polyribosomes. The drastic lack of 40S subunits should result in fewer initiation events and, consequently, in a shift in the distribution of polyribosomes to those associated with fewer ribosomes. This indicates that Rrp7p is required for the production of 40S ribosomal subunits but not for 60S subunits.

These observations suggested that the growth suppression of strains lacking Rrp7p by multiple copies of RPS27 is due to restoration of 40S subunit synthesis. To test this, ribosome profiles were compared following depletion of Rrp7p and in the rps27A or rps27B strain complemented by each of the RPS27 genes (Fig. 4B and C). In the suppressed rps27A or rps27B, a significantly higher level of 40S subunits, and consequently fewer free 60S subunits, was observed. Interestingly, there is a correlation between the profiles obtained for the suppressed rps27::TRP1 strains and the level of suppression seen for each gene. The peak of free 60S subunits is lower in the rps27::TRP1 strain carrying pRPS27B than in the strain carrying pRPS27A, indicating that more 40S subunits are available to assemble these
60S subunits into complete ribosomes. This correlates with the better growth rate in the rrp7::TRP1 strain carrying pRPS27B. We conclude that overexpression of rpS27 partially suppresses the 40S subunit synthesis defect in strains that lack Rrp7p.

**RRP7 is required for processing of pre-rRNA.** The observation that strains depleted of Rrp7p or with either of the RPS27 genes deleted were deficient in the production of 40S subunits led us to test whether this was due to defects in 18S rRNA synthesis.

The levels of mature 18S and 25S rRNA were determined by Northern hybridization (Fig. 5A). No differences in rRNA levels were seen between the wild-type strain, W303, and the GAL::rrp7 strain grown in galactose medium (Fig. 5A, lanes 1 and 2). Following transfer of the GAL::rrp7 strain to glucose medium for 14 h, the level of 25S rRNA was unaffected whereas the level of 18S rRNA was strongly reduced (Fig. 5A, lane 3). Reduced levels of 18S rRNA, but not of 25S rRNA, were also observed in the rrp7::TRP1 deletion strain suppressed by either pRPS27B (Fig. 5A, lane 4) or pRPS27A (Fig. 5A, lane 5). Consistent with the polysome gradient analyses, the levels of 18S rRNA were less reduced in the suppressed rrp7 deletion strain than in the GAL::rrp7 strain on glucose medium (Fig. 5A, compare lanes 4 and 5 with lane 3). A clearly reduced level of 18S rRNA was also observed in the strain with RPS27B deleted (Fig. 4A, lane 6), but only a slight reduction in 18S was seen in the strain with RPS27A deleted (Fig. 5A, lane 7). Again, these results are in good agreement with the polysome profiles from these strains. We conclude that the effects of the various mutations on the polysome profiles of the strains are due to alterations in the accumulation of the 18S rRNA.

To determine whether the reduced levels of 18S are the consequence of defects in the pre-rRNA processing pathway, the steady-state levels of pre-rRNAs were also analyzed by Northern hybridization. The pre-rRNA processing pathway is depicted in Fig. 6. In the GAL::rrp7 strain grown on galactose medium (Fig. 5B to F, lanes 2), a mild effect on pre-rRNA levels was observed, probably indicating that the protein destabilization cassette causes a slight reduction in Rrp7p protein levels. Following transfer to glucose medium, pre-rRNA processing was strongly affected (Fig. 5B to F, lane 3). Some increase in the 35S pre-rRNA was observed accompanied by a drastic loss of the 27SA2 and 20S pre-rRNAs. Two aberrant pre-rRNA species, the 21S and 23S RNAs, were also accumulated. The 23S RNA is generated by direct cleavage of the 35S transcript at site A3 in the absence of cleavage at sites A0, A1, or A2, while the 21S RNA extends from site A1 to site A3 and is generated by cleavage of the 32S pre-rRNA at site A2. The levels of the 32S and 27SB pre-rRNAs were little altered by Rrp7p depletion. The almost complete absence of 20S and 27SA2 shows that cleavage of site A2 is strongly inhibited by depletion of Rrp7p. The mild accumulation of 35S and, particularly, the appearance of the 23S RNA show that cleavage at site A0 is strongly inhibited by depletion of Rrp7p. The mild accumulation of 35S and, particularly, the appearance of the 23S RNA show that cleavage of sites A0 and A1 are also at least delayed. However, the relatively small reduction in 32S pre-rRNA and the appearance of the 21S rRNA both indicate that cleavage at site A2 is most strongly affected. The 27SA3 pre-rRNA is not detected by Northern hybridization, but the appearance of the 23S and the 21S RNAs and the normal levels of the 27SB pre-rRNA and 25S rRNA all indicate that cleavage of site A3, as well as subsequent processing on the 25S pathway, is unaffected by depletion of Rrp7p. Similar but not identical effects were seen in the strains both with RRP7 deleted and suppressed by overexpression of pRPS27B (Fig. 5, lane 4) or pRPS27A (Fig. 5, lane 5). These strains had levels of the mature 18S rRNA higher than those in the strain depleted of Rrp7p, consistent with this being the basis of the observed growth suppression. The levels of 18S in the two complemented strains are similar, consistent with their similar
growth rates. Surprisingly, however, the levels of the 20S and 27SA₂ pre-rRNAs were not detectably elevated in the suppressed strains. The increased level of 18S rRNA in these strains, compared to the strain depleted of Rrp7p, might be due to an increased ability of the 21S RNA to function as a substrate for cleavage at site D to generate mature 18S. Alternatively, in strains lacking Rrp7p, a low level of residual 18S synthesis may continue, with this rRNA being stabilized by overexpression of rpS27.

Pre-rRNA processing in the \textit{rp7::TRP1} deletion strain carrying pRPS27B (Fig. 5, lane 4) closely resembled that in the strain depleted of Rrp7p but showed elevated levels of 35S pre-rRNA and 21S RNA. The \textit{rp7::TRP1} strain carrying pRPS27A (Fig. 5, lane 5) was rather different, with reduced accumulation of the 35S pre-rRNA and little accumulation of the 23S RNA but strong accumulation of the 32S pre-rRNA and 21S RNA; some accumulation of the 33S pre-rRNA was also detected. Our interpretation is that the strain complemented by pRPS27B remains strongly inhibited for cleavage at A₀, A₁, and A₂ (as shown by the accumulation of 35S and 23S), while in the strain complemented by pRPS27A, there is increased cleavage at A₀ and A₁ (as shown by the transfer to 32S and 21S). This was unexpected, since it appears to indicate that the expression of rpS27A better supports pre-rRNA processing in the absence of Rrp7p than does expression of rpS27B.

Pre-rRNA processing was also assessed in the strains deleted for the two ribosomal protein genes. The deletion of \textit{RPS27A} had little effect on pre-rRNA processing (Fig. 5, lane 7). The deletion of \textit{RPS27B}, which confers a slow-growth phenotype to the strain, led to some accumulation of the 35S pre-rRNA and the formation of the 23S species (Fig. 5, lane 6), with underaccumulation of the 32S, 27SA₂, and 20S pre-rR-
FIG. 6. Pre-rRNA processing pathway. (A) Structure of the rDNA operon. Cleavage sites within transcribed spacers are indicated above the bottommost line, and the positions of the oligonucleotides used for Northern hybridization are indicated below the bottommost line. (B) Processing pathway. The 35S primary transcript is cleaved at site A0 to give the 33S pre-rRNA. Cleavage at site A1, the 5′ end of the mature 18S rRNA, follows rapidly, producing the 32S pre-rRNA after cleavage at site A2, and this primer extension stops identified as 18S+1780 are due to the presence of dimethyladenine residues (A1779 and A1780) in the 18S rRNA sequence. The steady-state level of pre-rRNA cleaved at site A0 in the 5′ region of ITS1 (Fig. 7A). In the wild-type cells (Fig. 7B, lane 7) and GAL::rrp7 strain (Fig. 7B, lane 1) grown on galactose, similar levels of primer extension stops at sites A2, A3, B1L, and B1S were detected. In the GAL::rrp7 strain grown on glucose (Fig. 7B, lane 2) and the rrp7::TRP1 strain carrying pRPS27A and -B (Fig. 7B, lanes 3 and 4, respectively), cleavage at sites A0, B1L, and B1S was unaltered but the primer extension stop at site A1 was almost undetectable. The primer extension stops identified as 18S+1780 are due to the presence of dimethyladenine residues (A1779 and A1780) in the 18S rRNA. In the wild-type strain these modifications occur on the 20S pre-rRNA after cleavage at site A2, and this primer extension stop is, therefore, not detected by primers 3′ to site A2 (Fig. 7B, lane 7). In mutants in which cleavage at site A2 is delayed, dimethylation can occur prior to A2 processing, on the 32S pre-rRNA or larger precursors (16, 19), and this presumably is also the case for these strains.

The primer extension stop at A0 is also strongly reduced in the strain with RPS27B deleted (Fig. 7B, lane 5) but is little affected by deletion of RPS27A (Fig. 7B, lane 6), consistent with the results of Northern hybridization. Primer extension was also performed with oligonucleotide A within the 18S rRNA sequence. The steady-state level of pre-rRNA cleaved at site A0 in the 5′ ETS is unaltered in all the strains (data not shown). Cleavage at sites A0, A1, A2, A3, B1L, and B1S was accurate at the nucleotide level in all strains.

To assess the kinetics of rRNA synthesis, pulse-chase labeling experiments were undertaken (Fig. 8). Following growth in galactose medium, GAL::rrp7 cells were transferred to glucose medium for only 3 h prior to labeling to ensure good incorporation of the label. Cells were then pulse-labeled for 2 min at 30°C with [3H]methionine and chased with a large excess of unlabeled methionine for 0, 1, 2.5, 5, and 10 min. Even at this early depletion time point, GAL::rrp7 cells showed a strong accumulation of the 35S pre-rRNA. The 27SA2 processing intermediate was undetectable in the Rrp7p-depleted strain, and the levels of the 20S pre-rRNA and 18S rRNAs were greatly reduced. The 23S species is clearly detected, demonstrating that this is a major processing intermediate in the strain depleted of Rrp7p. Formation of the 27SB pre-rRNA was kinetically delayed, consistent with the accumulation of 35S, but the yields of the 27SB pre-rRNA and 25S rRNA were unaffected.

In the rrp7::TRP1 strain carrying pRPS27A or pRPS27B (Fig. 8), 35S processing was delayed, and, consistent with the results of Northern hybridization, neither the 27SA2 nor the 20S pre-rRNAs were detected. A clear signal was, however, normally processed into the 25S and 5.8S rRNAs. (D) Pre-rRNA processing that leads to the formation of the 21S RNA. Cleavage at site A0 occurs in the absence of cleavage at site A2.
seen at the position of the 21S RNA, consistent with residual 18S synthesis via this intermediate.

Labeling was performed with \([\text{methyl-}^3\text{H}]\)methionine, which labels the methyl group that is added to the 35S pre-rRNA. The good efficiency of label incorporation therefore indicates that Rrp7p is not involved in the methylation of the pre-rRNA. We conclude that both Rrp7p and rpS27 are necessary for the early pre-rRNA processing events that lead to the synthesis of the 18S rRNA but are not required for subsequent processing on the 5.8S and 25S processing pathway.

**DISCUSSION**

The systematic functional analysis of ORF is a novel approach to genetic analysis in yeast. Here, we report that this approach has led to the characterization of a novel, essential component of the pre-rRNA processing machinery, Rrp7p. Since RRP7 is essential for viability, we constructed a conditionally expressed allele under the control of a GAL10 promoter that allows the induction or repression of gene expression depending on the carbon source. We added, however, a modification to this technique, in that the RRP7 ORF was fused to a protein-destabilizing cassette to lower the protein accumulation and allow its more rapid depletion after transfer to repressing conditions. It seemed likely that this strategy would be important to decrease the effect of the leakage of the GAL promoter in glucose medium, since the codon bias suggested that Rrp7p is normally expressed at a low level. In fact, this turns out to be an effective strategy, since the doubling time of the mutant strain under repressing conditions was increased as early as the second generation after transfer to glucose medium and the final growth inhibition was much tighter than is generally observed with GAL-regulated expression of pre-rRNA processing components. This growth inhibition was sufficiently stringent to allow the isolation of genes which can act as high-copy-number extragenic suppressors. The genes thus identified were RPS27A and RPS27B, the previously unrecognized, duplicated genes encoding a protein which shows 70% identity to the rat ribosomal protein S27. The presence of either of these genes on multicopy vectors can also suppress the lethality of an rrp7 deletion mutation. Cells with either RPS27 gene deleted are viable, but the double deletion strain is nonviable, showing that both genes are expressed and that the rpS27 protein is essential. The RPS27B gene is expressed at approximately twice the level of RPS27A and, consistent with this deletion of RPS27B, is much more detrimental to cell growth.

Using the conditional GAL::rrp7 allele, we found that strains depleted of Rrp7p are deficient in accumulation of 40S ribosomal subunits, as are strains lacking rpS27A and, to a lesser extent, rpS27B. Strains with RRP7 deleted and suppressed by overexpression of rpS27 show better production of 40S subunits than the Rrp7p-depleted strain, consistent with their improved growth rates. Northern analysis showed a good correlation between the levels of 40S subunits observed in the various strains and their ability to synthesize 18S rRNA, indicating that the deficiencies in 40S subunit levels are a direct consequence of impaired accumulation of the 18S rRNA.

Analyses of pre-rRNA processing revealed that depletion of

![FIG. 7. Mapping of 5' ends in ITS1 by primer extension. (A) Primer extension was performed with oligonucleotide F, which hybridizes within the 5' region of 25S rRNA. (B) Primer extension experiments. Lanes contain RNA extracts from the following: the GAL::rrp7 strain grown on galactose medium (lane 1), the GAL::rrp7 strain following growth for 14 h on glucose medium (lane 2), the rrp7 deletion strain harboring plasmid pRPS27B (lane 3), the rrp7 deletion strain harboring plasmid pRPS27A (lane 4), the rpS27B deletion strain (lane 5), the rpS27A deletion strain (lane 6), and the wild-type strain grown on galactose medium (7). The rDNA sequences obtained with the same primer are shown. The positions of the primer extension stops are indicated with arrows.](image)

![FIG. 8. Pulse-chase labeling of rRNA. Cells were labeled for 2 min with [methyl-\(^3\)H]methionine and chased with a large excess of unlabeled methionine for 0, 1, 2.5, 5, and 10 min. (A) GAL::rrp7 strain grown on galactose medium; (B) GAL::rrp7 strain following growth for 3 h on glucose medium; (C) rrp7 deletion strain harboring plasmid pRPS27A; (D) rrp7 deletion strain harboring plasmid pRPS27B. RNA was extracted, loaded on a 1.2% agarose-formaldehyde gel, and transferred to nitrocellulose membranes before being visualized by fluorography. The positions of the different species are indicated.](image)
Rrp7p inhibits the early pre-rRNA cleavages at sites A0, A1, and A2, which are required for the synthesis of the 20S pre-rRNA, preventing production of the 18S rRNA. Pulse-chase experiments confirmed that this deficiency is due not to the degradation of the 20S pre-rRNA but rather to impaired production of this species. Processing at sites A0, A1, and A2 is also impaired by deletion of RPS27A or RPS27B, presumably as a consequence of reduced rpS27 synthesis. Deletion of RPS27B more strongly inhibits pre-rRNA processing, consistent with their relative expression. Processing at site A3 and at sites further 3', which are required for the synthesis of the 5.8S and 25S rRNAs, are not affected by genetic depletion of Rrp7p or deletion of the genes encoding rpS27A or -B.

Overexpression of rpS27A or -B suppresses the lethality of the rrp7 deletion by increasing 18S rRNA synthesis. The suppressed strains cannot be directly compared to the deletion mutant, but they clearly have a higher level of 18S rRNA than strains which have been genetically depleted of Rrp7p. Strikingly, these strains lack any detectable 20S pre-rRNA, as strains which have been genetically depleted of Rrp7p. Strikingly, these strains lack any detectable 20S pre-rRNA, as judged by Northern hybridization or in vivo labeling. They do, however, have high levels of a 21S RNA species which has been processed at sites A0 and A1. We assume that in the absence of A1 cleavage, that has been shown to be dispensable (1, 2, 22), the 21S RNA is being directly cleaved at the 3' of the 18S rRNA to generate the mature rRNA. Such processing is believed to occur in strains lacking the small nuclear RNA (snRNA) snR10 (36), but no strain which is viable in the absence of detectable 20S rRNA synthesis has been described. While the rrp7-depleted strain has a similar level of 18S rRNA when complemented by pRPS27A or pRPS27B, the pathway of processing is strikingly different, with pRPS27A apparently supporting more efficient processing at sites A0 and A1. Whether this is a subtle effect of differences in expression levels or represents a functional difference between the two forms of rpS27 is currently unclear. rpS27A and -B differ at only one amino acid position, but other examples of functional differences between natural allelic variants have been reported, including the case of the nucleolar protein Nop4/77p (6, 35).

Cleavage of site A2 appears to be more sensitive to depletion of Rrp7p than processing at site A0 or A1. This is most clearly seen in the rrp7 deletion strain with growth supported by overexpression of rpS27A; in this strain, the RNA species cleaved at site A0, 32S and 21S, are very strongly accumulated. Many mutants defective in the processing of sites A0 and A1 have been identified, but this strong preferential inhibition of processing at site A1 has not previously been observed.

We conclude that Rrp7p is absolutely required for cleavage at site A2, with fewer strong requirements for Rrp7p in A0 and A1 cleavages. This resembles the situation in strains genetically depleted of the snoRNAs U14 (21) and snR30 (25) or the snoRNP protein Gar1p (14), in which cleavage at sites A0 and A1 is strongly inhibited while cleavage of site A2 is less inhibited. Since processing at sites A0 and A1 appears to be coupled (no mutation allowing cleavage of site A2 in the absence of prior cleavage of site A1 has been reported), the effects of these mutations may be primarily on A1 cleavage, in contrast to rrp7 mutants.

The simplest explanation for the suppression data is that Rrp7p is required for efficient association of rpS27 with the preribosomal particle. In the absence of Rrp7p, the efficiency of assembly may be improved by elevated levels of the ribosomal protein. If this is the case, Rrp7p must also play other roles in 40S subunit assembly. Overexpression of rpS27 in the rrp7 deletion strain allows cleavage of A0 and A1 with moderate efficiency, but processing at A2 remains blocked, presumably reflecting another requirement for Rrp7p.

In this model, the block in pre-rRNA processing would most likely be an indirect consequence of alterations to the structure of the preribosomal particle. It might be envisaged that, in the absence of correct assembly, processing of the subunit is aborted to prevent the accumulation of misassembled, non-functional ribosomes. The existence of such a quality control mechanism has recently been proposed to inhibit pre-rRNA processing in strains lacking the 18S rRNA dimethylase Dimlp. This acts to prevent the accumulation of nonmethylated 18S rRNA (19). The inhibition of processing in strains lacking Rrp7p or rpS27 may be a consequence of similar quality control mechanisms.

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