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MOLECULAR AND CELLULAR BIOLOGY, Sept. 1997, p. 5023–5032 Vol. 17, No. 9
0270-7306/97/$04.00+0

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

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Received 3 February 1997/Returned for modification 19 March 1997/Accepted 23 June 1997

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 A_0, A_1, and A_2, separate the 20S pre-rRNA from the $27SA_2$ pre-rRNA. 20S pre-rRNA is the precursor to the 18S rRNA which is incorporated into the 40S ribosomal subunit, while $27SA_2$ 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 have therefore called this locus *RRP7* (for ribosomal RNA processing).

MATERIALS AND METHODS

Strains, media, and microbiological methods. *S. cerevisiae* W303-1B (*MAT***a** *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 *URA3-pGAL10::rrp7* construct, which is integrated at the *RRP7* locus. For the purpose of selecting multicopy suppressors, BMA59-1B was UV irradiated (λ = 256 nm) for 40 s (80% killing rate), grown on complete galactose medium for 24 h, and harvested. A total of 5×10^6 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 (*MAT***a** *ura3-1 ade2-1 leu2-3,112 his3-11,15 tp1*Δ *can1-100 rrp7::TRP1*/pFL38-RRP7), BMA67-1B (*MAT***a** *ura3-1 ade2-1 leu2-3,112 his3-*Δ*200 trp1-1 can1-100 rpS27B::HIS3*), and BMA66-1B (*MAT***a** *ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15 rpS27A::kan*^{r}) 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,

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2% glucose, adenine [30 mg/liter] or YPGALA [1% yeast extract, 1% Bacto Peptone, 2% galactose, adenine [30 mg/liter]) or selective medium (WO [2% glucose, 0.7% yeast nitrogen base, nutriments) or WOGal [2% galactose, 0.7% yeast nitrogen base, nutriments]). Yeast diploids were sporulated by growing the cells on SP1 plates (1% yeast extract, 2% Bacto Peptone, 1% potassium acetate, 2% agar). Cell growth at 30°C was monitored by measuring the optical density (OD) at 600 nm. Cells were diluted during growth with prewarmed medium to maintain them in early exponential phase. For Rrp7p depletion, cells growing exponentially in YPGALA were harvested by centrifugation, washed in water, and resuspended in YPGA. Transformation was achieved by the lithium acetate method (13). The following plasmids were used in this study. pRPS27B consists of the pFL44L plasmid (8) containing a *Sau*3AI fragment of 5.3 kb, including ORF *RPS27B* cloned at the *Bam*HI site. pRPS27A consists of the pFL44L plasmid containing a *Sau*3AI fragment of 5.5 kb, including ORF *RPS27A* cloned at the *Bam*HI site. A 2.7-kb *Eco*RI fragment containing *RPS27A* was cloned into the polylinker of pFL38, a centromeric *URA3*$^+$ vector, and a 3.1-kb *Xho*I-*Hin*dIII fragment including *RPS27B* was cloned into the same pFL38 vector. Finally, pFL38-RRP7 consists of a 2.4-kb *Asp*718-*Bam*HI fragment harboring the *RRP7* gene cloned into the polylinker of pFL38.

Gene deletion. Gene deletions were performed according to the method of Baudin et al. (3). The oligonucleotides used to delete *RRP7*, *RPS27B*, and *RPS27A* were, respectively, CGAACGAACTAGGTAACAGATAACAACCTC AGATAGAGTGTTACGGGGGCCAAGAGGGAGGGC and TATAGATAT ATTAGATATGTTGAATATGATGACGAGGATGGTGGCTTAAATAAAT ACTACTC for oligopro and oligoterm, respectively; AGAGAATACGAAAA CACACCAGATAATTAGTGCATATATATTAGATCAATCTCTTGGCCT CCTCTAG and ATATTTAGATAAAAGAAGATGATTTTAACTACACAAA AAAATAAATTAACTCGTTCAGAATGACACG for oligopro and oligoterm, respectively; and GCAACTTCATCGTAACACCTTCCAACAAAGCAAAGA TAGATATCCCAAACCAGCGACATGGAGGCCC and TAATGCTCTTCTT TTGAAGTAAATTTCGTTTCATCTGTCTAGTAATGAAACATTCACAT ACGATTGACGC for oligopro and oligoterm, respectively; they allowed the amplification of the *TRP1*, *HIS3*, and *kan*^{r} markers, respectively (39). The recipient strains were, respectively, BMA64 (*ura3-1 ade2-1 leu2-3,112 his3-11,15 tp1D can1-100*) (4), BMA36 (*ura3-1 ade2-1 leu2-3,112 his3-Δ200 trp1-1 can1-100*) (replacement of the *his3-11,15* allele by the *his3-Δ200* allele in strain W303 [unpublished result]), and W303. To analyze the insertion of *rrp7::TRP1*, genomic DNA was digested with *Eco*RV and the filter was probed with a 0.8-kb fragment containing the *TRP1* gene. Two *Eco*RV fragments of 4.71 and 2.64 kb were detected in the strains carrying the deletions, due to the presence of an internal *Eco*RV site within the *TRP1* gene, whereas no signal was detected in the BMA64 parental strain as expected. Two bands of 2.28 and 8.9 kb, corresponding to the *TRP1* locus, were present in the W303-1B strain used as control. A strain showing the correct profile was kept and named BMA55. The replacement of *RPS27B* was controlled by digestion of the genomic DNA with *Eco*RI. The 1.1-kb PCR product was used as a probe. A single band of 3.8 kb was detected in the strains carrying the deletions, whereas no signal was detected, as expected, in the BMA36 parental strain with the *HIS3* gene deleted. A strain showing the correct profile was kept and named BMA67. For the control of the *rpS27A::kan*^{r} allele, genomic DNA with *Eco*RI and the filter were probed with the 1.4-kb PCR fragment. A 3.52-kb fragment is detected in the strains carrying the deletions, and no signal is detected in the W303 parental strain, as expected. A strain showing the correct profile was kept and named BMA66 (data not shown).

Construction of the *GAL::rrp7* strain. To construct the pF1URLF and pF1UMLF vectors, the *Xho*I-*Bam*HI fragment of pBaBa4 (28) containing the UAS-Gal-ubiquitin encoding sequence was inserted between the *Xho*I-*Bam*HI sites of pYeF1 (10) to generate pF1UBI. A *lacI-flu* fragment was then PCR amplified by using pBaBa4 as a matrix with two oligonucleotides that introduce a *Bam*HI and a *Not*I site at each end. This fragment was cloned between the *Bam*HI-*Not*I sites of pF1UBI to generate plasmids pF1URLF and pF1UMLF. A series of centromeric vectors were designed the same way (10a). The *RRP7* ORF was amplified by PCR with two oligonucleotides which introduce *Not*I and a *Bsu*36I restriction sites at the 5′ and 3′ ends of the gene. The resulting cassette was cloned into the *Not*I-*Bsu*36I sites of pF1URLF, leading to the pF1URLF-*RRP7* plasmid. pF1URLF-*RRP7* (a multicopy *URA3*$^+$ plasmid) was used to transform the heterozygous *RRP7*/*rrp7::TRP1* strain. After dissection, the Trp$^+$/Ura$^+$ spores were viable, indicating that the ubiquitin-lacI-hemagglutin (HA)-fused Rrp7p could fully complement the lethal deletion. Unfortunately, these strains were viable on plates containing either galactose or glucose. This indicated that the residual production of Rrp7p on glucose medium, due to the leakage of the *GAL10* promoter when expressed from a multicopy plasmid, was sufficient for cell viability. In order to integrate the *URA3-pGAL::rrp7* construction at the *RRP7* locus, the plasmid pF1URLF was digested with *Spe*I and filled in with the T4 polymerase. A blunt-ended 834-bp fragment which lies 152 nucleotides upstream of the *RRP7* ATG between two *Bst*B1 and *Pvu*II sites was inserted into the filled *Spe*I site. The resulting plasmid was digested with *Bsu*36I, and the linear fragment was used to transform the strain W303, with selection for uracil prototrophy. The correct replacement was controlled by Southern analysis (data not shown). After dissection, one haploid strain that was able to grow on galactose- but not on glucose-containing medium was chosen and named BMA59-1B.

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, GATGTTCAAACAACCTGGGC, or 1834 (29) was 5′ end labeled with 25 μCi of g-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 PhosphorImager 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, CATGGCTTAATCTTTGAGAC; probe B, CC AGATAACTATCTTAAAAG; probe C, CGGGTTTTAATTGTCCTA; probe D, TGTTACCTCTGGGCCC; probe E, CCAGTTACGAAAATTCTTG; and probe F, TGAGAAGGAAATGACGCT.

Pulse-chase labeling. Aliquots (5 ml) of cells growing in glucose or galactose minimum medium at an OD of ~0.4 were labeled with 250 μCi of [*methyl*-^{3}H]methionine 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 strains were grown in 100 ml of YPGA or YPGALA 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 model 640).

Nucleotide sequence accession numbers. The GenBank accession numbers for the complete sequences of *RRP7*, *RPS27A*, and *RPS27B* are X59720, Z28156, 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 was detected by computational analysis.

To determine the function of *RRP7*, we used a PCR 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*, accurate replacement of *RRP7* was demonstrated by Southern analysis of two transformants (see 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 on galactose and strongly repressed on glucose. The cassette consisting of the GAL UAS-ubiquitin-lacI-HA-encoding sequences 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

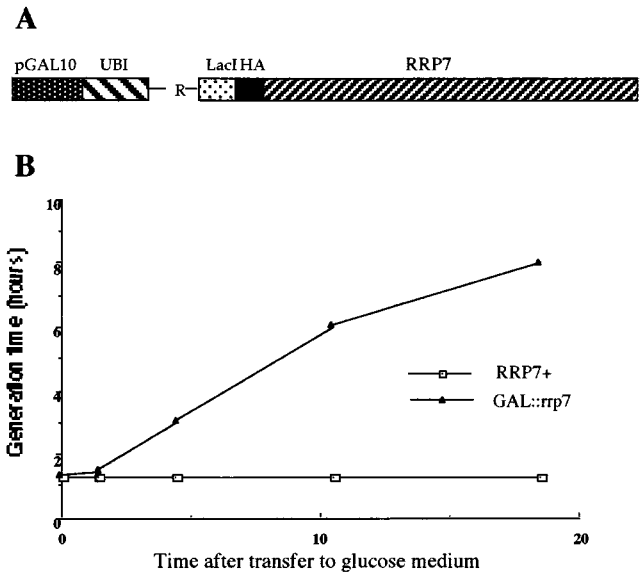

FIG. 1. (A) Schematic representation of the *RRP7* conditional null allele. The *RRP7* ORF is fused in frame to the HA epitope, the LacI fragment beginning with the destabilizing amino acid arginine (R), and the ubiquitin (UBI) sequence. This cassette is under the control of the *GAL10* promoter. (B) Growth curve of a *GAL::rrp7* strain after transfer to glucose medium. The doubling time increases as early as the second generation from 1.45 to 3 h, and the growth is progressively inhibited.

to glucose medium (28). 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 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 were found to contain the wild-type *RRP7* gene. A second class of suppressor, found only once, exhibited a medium level of suppression and was composed of a plasmid harboring the *GAL4* gene. This was likely due to an increase of expression of the *GAL::rrp7* construct on the glucose medium mediated by the high level of Gal4p. The last class, of eight independent plasmids, conferred 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

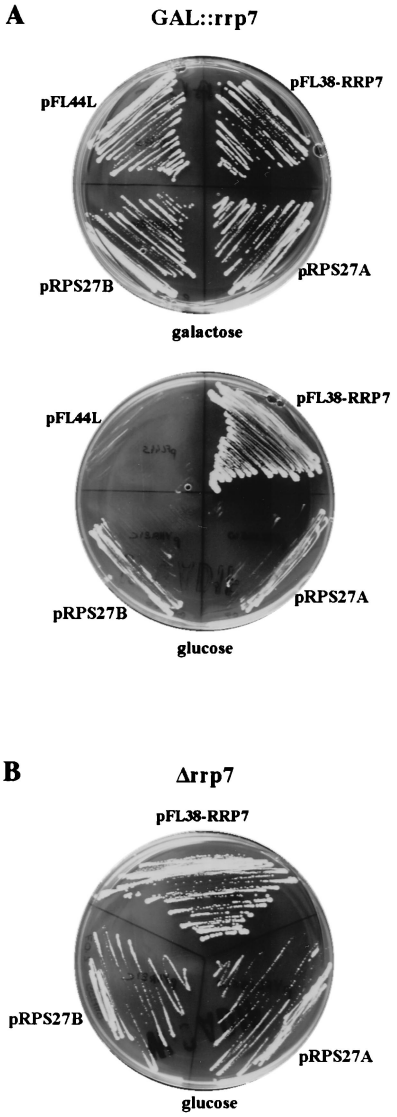

FIG. 2. Suppression of the growth defect of a strain depleted for *RRP7* by plasmids pRPS27B and pRPS27A. (A) Strain *GAL::rrp7* harboring either pFL44L (negative control), pFL38-RRP7 (positive control), pRPS27B (pFL44L carrying *RPS27B*), or pRPS27A (pFL44L carrying *RPS27A*) was streaked on galactose medium and incubated for 48 h at 30°C (top) or streaked on glucose medium and incubated for 48 h at 30°C (bottom). (B) *rrp7* deletion strain transformed by pFL38-RRP7, pRPS27B, or pRPS27A.

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 *rrp7::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*^{r} 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 multicopy 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^r recombinants were recovered among the progeny. This shows that the double deletion is lethal. Viable His^+ Kan^r 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* mRNA 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 mRNA, which were measured as percentages of total *RSP27* 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%; Δ*rps27A*, 0% and 60% ± 2%; and Δ*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*.

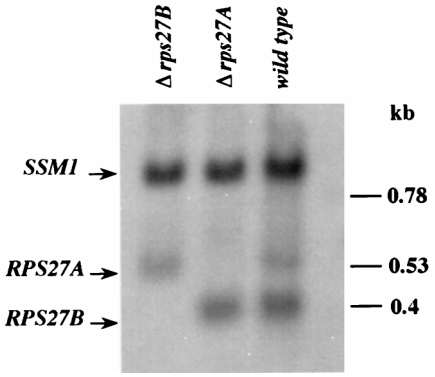

FIG. 3. Northern analysis of *RPS27A* and *RPS27B* mRNA from haploid wild-type or *rps27A* or *rps27B* deletion strains. Total yeast RNA was hybridized with the ^{32}P-labeled oligonucleotide (8238) complementary to nucleotides +110 to +129 of the *RPS27A* and *RPS27B* mRNA sequences. Each filter was subsequently hybridized to an SSM1 probe (1834) and to an actin probe (data not shown) to normalize the amount of mRNA loaded in each lane.

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 polyribosome 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 *rrp7::TRP1* strain complemented by each of the *RPS27* genes (Fig. 4B and C). In the suppressed *rrp7::TRP1* strain carrying either pRPS27A or pRPS27B, 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 *rrp7::TRP1* strains and the level of suppression seen for each gene. The peak of free 60S subunits is lower in the *rrp7::TRP1* strain carrying pRPS27B than in the strain carrying pRPS27A, indicating that more 40S subunits are available to assemble these

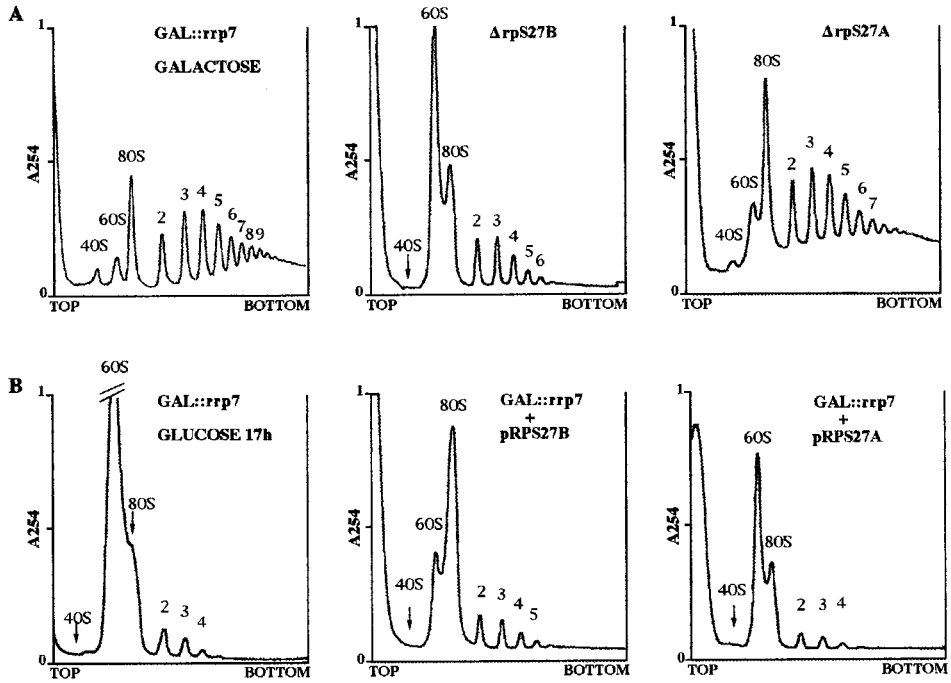

FIG. 4. Polyribosome profiles. Ribosomes and free ribosomal subunits were extracted, separated on a 7 to 47% sucrose gradient, and analyzed with an ISCO gradient fractionator. (A) Ribosomal profiles of the *GAL::rrp7* strain grown on glucose medium (left) and of the *rpS27B* or *rpS27A* deletion strains (center and right). (B) Ribosomal profiles of the *GAL::rrp7* strain after transfer to galactose medium for 17 h (left) and of the *rrp7* deletion strain harboring either pRPS27B or pRPS27A (center and right). The positions of the 40S and the 60S subunits are indicated as well as the number of ribosomes in the polyribosome peaks.

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 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 $27SA_2$ 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 A_3 in the absence of cleavage at sites A_0, A_1, or A_2, while the 21S RNA extends from site A_1 to site A_3 and is generated by cleavage of the 32S pre-rRNA at site A_3. The levels of the 32S and 27SB pre-rRNAs were little altered by Rrp7p depletion. The almost complete absence of 20S and $27SA_2$ shows that cleavage of site A_2 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 A_0 and A_1 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 A_2 is most strongly affected. The $27SA_3$ 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 A_3, 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 rpS27B (Fig. 5, lane 4) or rp27SA (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

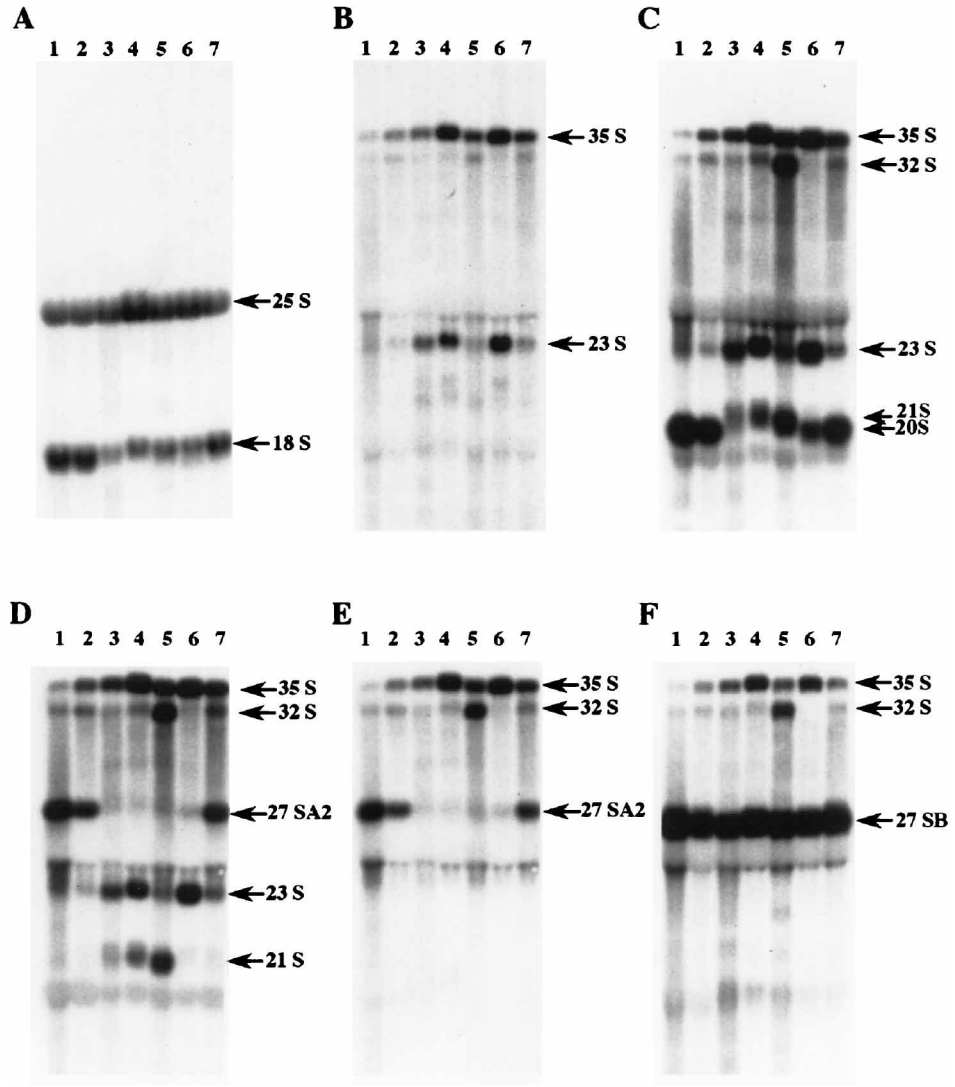

FIG. 5. Northern hybridization of rRNA and pre-rRNA. Hybridizations with the following are shown: oligonucleotides A and G, specific for the 18S and 25S rRNAs (A); oligonucleotide B, specific for the 5′ ETS (B); oligonucleotide C, specific for the 5′ region of ITS1, upstream of A_2 (C); oligonucleotide D, specific for the central region of ITS1, between sites A_2 and A_3 (D); oligonucleotide E, specific for the 3′ region of ITS1 (E); and oligonucleotide F, specific for the 5′ region of ITS2. Aliquots (10 μg) of total RNA were loaded on a 1.2% agarose gel and transferred to nitrocellulose membranes for Northern hybridization. Lanes contain RNA extracts from the following: the wild-type strain grown on galactose medium (lanes 1), the *GAL::rrp7* strain grown on galactose medium (lanes 2), the *GAL::rrp7* strain following growth for 14 h on glucose medium (lanes 3), the *rrp7* deletion strain harboring plasmid pRPS27B (lanes 4), the *rrp7* deletion strain harboring plasmid pRPS27A (lanes 5), and the *rpS27B* deletion strain (lanes 6), and the *rpS27A* deletion strain (lanes 7).

growth rates. Surprisingly, however, the levels of the 20S and $27SA_2$ 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 *rrp7::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 *rrp7::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 A_0, A_1, and A_2 (as shown by the accumulation of 35S and 23S), while in the strain complemented by pRPS27A, there is increased cleavage at A_0 and A_1 (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 *RPS27A* had little effect on pre-rRNA processing (Fig. 5, lane 7). The deletion of *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, $27SA_2$, and 20S pre-rR-

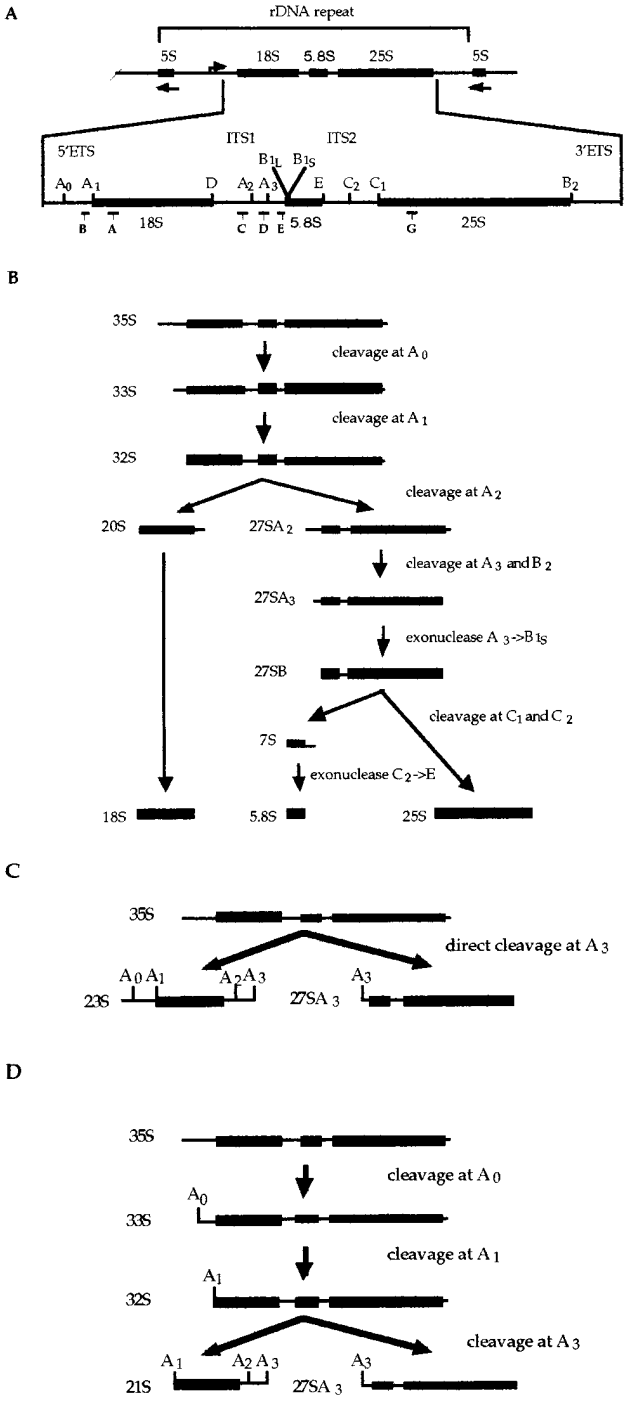

FIG. 6. Pre-rRNA processing pathway. (A) Structure of the rDNA operon. Cleavage sites within transcribed spacers are indicated above the bottommost line, and the positions of the oligonucleotides used for Northern hybridization are indicated below the bottommost line. (B) Processing pathway. The 35S primary transcript is cleaved at site A_0 to give the 33S pre-rRNA. Cleavage at site A_1, the 5′ end of the mature 18S rRNA, follows rapidly, producing the 32S species. A_2 cleavage separates the pre-rRNA destined to the small and the large subunits. It generates the 20S pre-rRNA, which is endonucleolytically cleaved at site D, yielding the mature 18S rRNA and the $27SA_2$ species. In the major processing pathway, cleavage at site A_3 occurs, giving the $27SA_3$ species, which is then degraded to site $B1_S$ by an exonuclease activity. In the minor pathway, $27SA_2$ is processed to site $B1_L$ by an unknown mechanism. In both cases, cleavages at sites C_1 and C_2 yield the 25S rRNA and the 7S pre-rRNA. Finally, the 7S pre-rRNA is matured to generate the 5.8S rRNA. (C) Pre-rRNA processing that leads to the formation of the 23S species. The 35S pre-rRNA is cleaved at site A_3, yielding the 23S RNA and the $27SA_3$ pre-rRNA, which is 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 A_3 occurs in the absence of cleavage at site A_2.

NAs. This indicates that processing at sites A_0, A_1, and A_2 is partially inhibited, consistent with the underaccumulation of the 18S rRNA (Fig. 5, lane 6). These data are consistent with the observation that RPS27B is more strongly expressed and its disruption is therefore predicted to more strongly reduce protein levels.

In no strains were 3′-extended forms of 35S or 27SB pre-rRNA detected, indicating that processing of the 3′ ETS is not affected, and no alteration in the ratio of the long and short forms of the 5.8S rRNA was detected (data not shown).

Primer extension was used to further analyze the pre-rRNA processing defects. An oligonucleotide located in the 5′ region of ITS2 (oligonucleotide F) was used to precisely map the sites of cleavage within 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 A_2, A_3, $B1_L$, and $B1_S$ 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 A_3, $B1_L$, and $B1_S$ was unaltered but the primer extension stop at site A_2 was almost undetectable. The primer extension stops identified as 18S+1780 are due to the presence of dimethyladenine residues (A_{1779} and A_{1780}) in the 18S rRNA. In the wild-type strain these modifications occur on the 20S pre-rRNA after cleavage at site A_2, and this primer extension stop is, therefore, not detected by primers 3′ to site A_2 (Fig. 7B, lane 7). In mutants in which cleavage at site A_2 is delayed, dimethylation can occur prior to A_2 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 A_2 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 A_0 in the 5′ ETS is unaltered in all the strains (data not shown). Cleavage at sites A_0, A_1, A_2, A_3, $B1_L$, and $B1_S$ 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 [*methyl*-^{3}H]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 $27SA_2$ processing intermediate was undetectable in the Rrp7p-depleted strain, and the levels of the 20S pre-rRNA and 18S rRNAs was 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 unaltered.

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 $27SA_2$ nor the 20S pre-rRNAs were detected. A clear signal was, however,

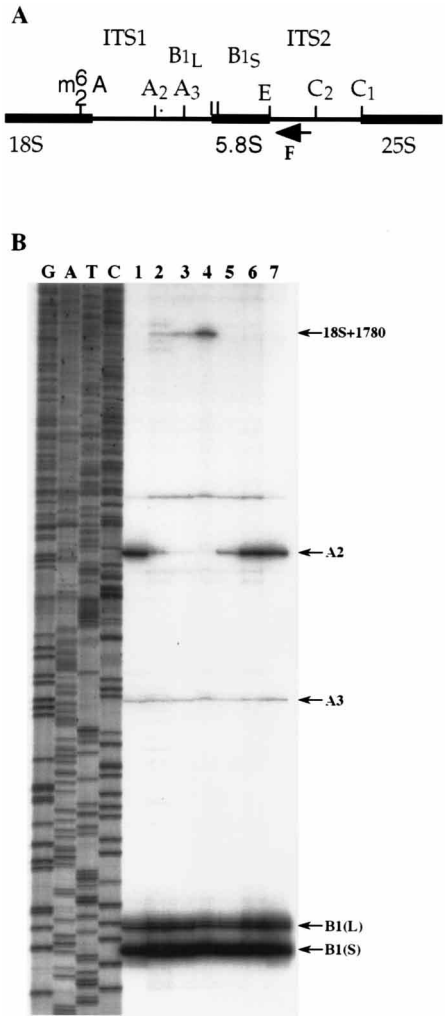

FIG. 7. Mapping of 5′ ends in ITS1 by primer extension. (A) Primer extension was performed with oligonucleotide F, which hybridizes within the 5′ region of 25S rRNA. (B) Primer extension experiments. Lanes contain RNA extracts from the following: the *GAL::rrp7* strain grown on galactose medium (lane 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.

seen at the position of the 21S RNA, consistent with residual 18S synthesis via this intermediate.

Labeling was performed with [*methyl*-^{3}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

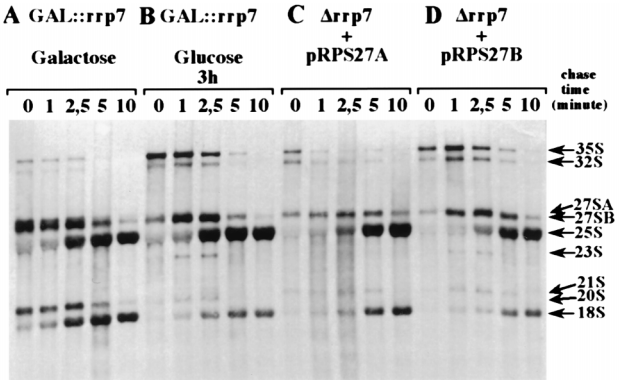

FIG. 8. Pulse-chase labeling of rRNA. Cells were labeled for 2 min with [*methyl*-^{3}H]methionine and chased with a large excess of unlabeled methionine for 0, 1, 2.5, 5, and 10 min. (A) *GAL::rrp7* strain grown on galactose medium; (B) *GAL::rrp7* strain following growth for 3 h on glucose medium; (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.

Rrp7p inhibits the early pre-rRNA cleavages at sites A_0, A_1, and A_2, 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 A_0, A_1, and A_2 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 A_3 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 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 A_1 and A_3. We assume that in the absence of A_2 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 nucleolar RNA (snoRNA) 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 A_0 and A_1. 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 A_2 appears to be more sensitive to depletion of Rrp7p than processing at site A_0 or A_1. 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 A_1, 32S and 21S, are very strongly accumulated. Many mutants defective in the processing of sites A_1 and A_2 have been identified, but this strong preferential inhibition of processing at site A_2 has not previously been observed.

We conclude that Rrp7p is absolutely required for cleavage at site A_2, with fewer strong requirements for Rrp7p in A_0 and A_1 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 A_1 and A_2 is strongly inhibited while cleavage of site A_0 is less inhibited. Since processing at sites A_1 and A_2 appears to be coupled (no mutation allowing cleavage of site A_2 in the absence of prior cleavage of site A_1 has been reported), the effects of these mutations may be primarily on A_1 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 A_0 and A_1 with moderate efficiency, but processing at A_2 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, nonfunctional 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 Dim1p. 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.

ACKNOWLEDGMENTS

We are grateful to M. E. Dufour for technical assistance and to C. Sassier for the design of the figures. Special thanks are due to F. Wyers for her helpful discussions, critical reading of the manuscript, and help on obtaining ribosomal profiles. We thank D. Menay for the synthesis of oligonucleotides.

A.B.-B. was supported by a grant from the Ministère de la Recherche et de l'Espace and a grant from the Association pour la Recherche sur le Cancer. This work was supported in part by the Groupement de Recherches et d'Etudes sur les Génomes, grant 503070, and by the Association pour la Recherche sur le Cancer, grant 6892.

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