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18S rRNA processing requires the RNA helicase-like protein Rrp3

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ABSTRACT

We report the identification of a new gene, *RRP3* (rRNA processing), which is required for pre-rRNA processing. Rrp3 is a 60.9 kDa protein that is required for maturation of the 35S primary transcript of pre-rRNA and is required for cleavages leading to mature 18S RNA. *RRP3* was identified in a PCR screen for DEAD box genes. DEAD box genes are part of a large family of proteins homologous to the eukaryotic transcription factor eIF-4a. Most of these proteins are RNA-dependent ATPases and some of them have RNA helicase activity. This is the third yeast DEAD box protein that has been shown to be involved in rRNA assembly, but the only one required for the processing of 18S RNA. Mutants of the two other putative helicases, Spb4 and Drs1, both show processing defects in 25S rRNA maturation. In strains where Rrp3 is depleted, 35S precursor RNA is improperly processed. Cleavage normally occurs at sites A_0, A_1 and A_2, but in the Rrp3 depletion stain cleavage occurs between A_2 and B_1. Rrp3 has been purified to homogeneity and has a weak RNA-dependent ATPase activity which is not specific for rRNA.

INTRODUCTION

Ribosomes in *Saccharomyces cerevisiae* are composed of a small 40S subunit (containing 18S rRNA) and a large 60S subunit (containing 5S, 5.8S and 25S rRNAs). The 5.8S, 18S and 25S RNAs of yeast are transcribed as a single 35S RNA polymerase I transcript (1,2). This 35S pre-rRNA is interrupted by internal transcribed spacers (ITS1 and ITS2) and is bounded by external transcribed spacers (5′- and 3′-ETS) that must be precisely and efficiently processed for mature RNA formation.

The endonucleotytic cleavages leading to mature yeast rRNA are outlined in Figure 1A. Aberrations in any of these steps usually results in defective maturation of the transcript. The 3′-ETS is most quickly processed and is not detectable *in vivo*. The primary transcript is separated into two intermediate precursors by cleavage at A_0, followed immediately by processing at A_1 and A_2 to release the 20S and 27SA intermediates (2). Cleavages at A_1 and A_2 are stepwise and appear to be linked. Failure to process at A_1 results in a loss of the A_2 cleavage. The 20S intermediate is further processed to 18S rRNA in the cytoplasm (3), while the 27SA intermediate can be processed by two alternative pathways which regulates the ratio of the short and long forms of the 5.8S RNA subunit. Cleavage can occur at A_3 followed by exonucleolytic cleavage to the B_1(S) site to give a 27SB(S) product (4). Processing of 27SB(S) gives rise to the 5.8(S) and the mature 25S RNA. Alternatively, processing directly at B_1(L) generates the 27SB(L) and hence 5.8(L) and 25S products.

Though the pathway of rRNA maturation is well characterized in both yeast and mammals, the biochemical mechanisms involved in rRNA maturation are still under active study. rRNA processing, like that of pre-mRNA processing, requires a number of RNPs, the snoRNPs (for reviews see 5,6). Many snoRNAs have been characterized in both the yeast and mammalian systems. They are associated with nucleolar proteins and, quite strikingly, many of them contain significant regions of homology to mature and flanking sequences in the pre-rRNA. Crosslinking studies have shown that these interactions occur and therefore must be meaningful. In several cases the snoRNAs have been directly implicated in processing events through *in vitro* studies. U3 snoRNA has been shown to be required for a cleavage event in the 5′-ETS in both mouse and frog *in vitro* systems (reviewed in 5,6). Recently it has been conclusively shown that the purified yeast MRP ribozyme can carry out precise cleavage at A_3 leading to maturation of 5.8S RNA (7). It seems likely that this is a RNA-mediated catalytic event (8), because MRP is related in structure to the catalytic RNA in RNase P and in yeast and mammals MRP and RNase P interact with the same protein (the Th/To antigen in mammals and the Pop1 protein in yeast), leading to the suggestion (8) that they share a common evolutionary ancestor. Depletion studies in yeast have implicated U3, U14 and snR30 snoRNPs in the early cleavages of pre-rRNA leading to maturation of 18S rRNA(3,9,10).

In pre-mRNA splicing, assembly of the spliceosome involves a series of RNA isomerization events in which interactions between snRNAs and the pre-mRNA or between snRNAs form and in turn are replaced by new interactions (reviewed in 11). Each of these steps in assembly requires ATP and are thought to be mediated by a set of RNA-dependent ATPases. These proteins, all related in sequence to the first member of the family, the translation initiation factor eiF-4A, share a number of sequence motifs, including a characteristic ATP binding site, which includes the sequence DEAD, giving rise to one name for the family, the DEAD box proteins (12). DEAD box proteins are ubiquitous in nature. They have been found in all three major lines of descent, the bacteria, the archaebacteria and the eukaryotes, and usually a species has many members of the gene family,

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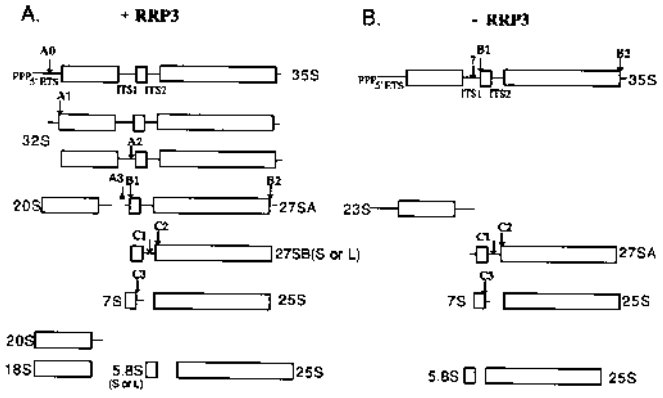

Figure 1. (**A**) The major processing pathway of *S.cerevisiae* to generate mature 18S, 5.8S and 25S rRNA. Either cleavage at A_3 or at B_1 can occur to generate the short or long form of 5.8S RNA. (**B**) The major processing products of the 35S RNA precursor in the Rrp3-depleted strain. Cleavage occurs at site B_1 or somewhere between sites A_2 and B_1 as denoted by the question mark.

especially in eukaryotes (13–15). Biochemical studies of the DEAD box proteins almost invariably reveal that they are RNA-dependent ATPases and in a few cases they have been shown to be RNA helicases, i.e. they can separate the strands of double helical RNA in an ATP-dependent reaction (16–19).

The requirement for RNPs in pre-rRNA processing, interactions of the snoRNAs with the substrate and the documented involvement of at least one of the snoRNPs in a specific cleavage suggest assembly of a large processing complex for pre-rRNA maturation similar to the spliceosome. The involvement of similar proteins to mediate the assembly of that complex might be expected. Indeed, cleavage and processing of the 25S RNA is dependent on ATP and at least two DEAD box proteins, Drs1 and Spb4, are required for that event (20,21).

In this paper, we report the identification of a new member of the DEAD box family of proteins, Rrp3. Rrp3 was identified in a PCR screen for proteins homologous to the family of DEAD box proteins. These proteins contain eight regions of homology in a 300 amino acid domain (12,22,23). Using degenerate oligonucleotides to regions complementary to those of the conserved regions in DEAD genes, we isolated three new genes. One of these, *RRP3* encodes a 60.9 kDa protein. Depletion of Rrp3 leads to a processing defect of 35S pre-rRNA and blocks 18S rRNA maturation. Unlike Drs1 and Spb4, which are required for processing of 25S RNA, Rrp3 is required for 18S rRNA maturation. The protein has been purified to homogeneity and has an extremely weak RNA-dependent ATPase activity. Recently it has been discovered that the RNA-dependent ATPase activity of the *Escherichia coli* DEAD box protein DBPA is specifically activated by 23S rRNA. The activity of Rrp3 is not stimulated by yeast rRNA, but further studies need to be completed to determine if any other RNAs, e.g. the snoRNAs, the ITS or the ETS, could activate this enzyme.

MATERIALS AND METHODS

Materials

Taq DNA polymerase was obtained from Perkin Elmer Cetus (Norwalk, CT). Restriction enzymes were obtained from New England Biolabs (Beverly, MA). Oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) DNA synthesizer and purified by urea–polyacrylamide gel electrophoresis. GeneScreen Hybridization Transfer Membrane was from NEN Research Products (Boston, MA). *Hin*dIII linkers were obtained from New England Biolabs. Sequenase was purchased from IBI. Exo-mung deletion kit was obtained from Stratagene. Oligonucleotides used for hybridization were obtained from J.Warner. Quantitation was carried out on an LKB ultrascan XL densitometer.

PCR and cloning

Yeast genomic DNA was prepared as described by Sherman (24). Amplification of DNA was done according to Chang *et al.*, except for the difference in primers (14). We used the primers IIA used by Chang and the newly constructed oligonucleotide 5′-ACGG-GATCC(T/G)NCCNGT(A/G)CG(A/G)TGNAT(A/G)TA-3′. DNA was denatured for 1 min at 92°C, the primer was annealed for 45 s at 47°C and primer extension was carried out for 75 s at 72°C. PCR reactions were performed on a Perkin Elmer Thermocycler. After 30 rounds of amplification, the reaction was allowed to extend for 10 min at 72°C. The cloning and sequencing of PCR products was also done as described except that the PCR product was first blunt end ligated with a 100-fold excess of phosphorylated *Hin*dIII linkers (14). The mixture was then digested with *Bam*HI and *Spe*I at 37°C for 2 h. The digests were phenol/$CHCl_3$ extracted, ethanol precipitated and ligated into the Bluescript KS vector. The resulting transformants were sequenced using T3 and T7 primers. Sequence analysis of a number of clones revealed three unique genes. The PCR clone was then used as a probe to further clone the genomic copy of the gene. One of these genes, termed *RRP3* was found in a YCP50B library (25). Northern and Southern analysis was performed according to Chang *et al.* (14).

The genes were further mapped and 4 kb fragments were inserted into the Bluescript KS vector (Stratagene). The fragments were then sequenced by performing exo-mung deletions according to the Stratagene kit and sequenced with Sequenase according to the manufacturer's instructions (IBN).

Strains and plasmid construction

Yeast cells were grown in YPD or synthetic media supplemented with 2% dextrose or galactose as a carbon source. Yeast were transformed by the lithium acetate method (26). A 4 kb *Bgl*II fragment from the YCP50 genomic clones was cloned into pUC18 and termed pCO93.

The haploid strains SS330 (MAT**a** *his 200 tyr1 ade2-101 ura3-52 GAL*$^+$ *suc2*) and SS328 (MAT**a** *his 200 lys2-801 ade2-101 ura3-52 GAL*$^+$ *suc2*) were crossed to derive the diploid strain (SS329X) for disruption experiments. Construction of the disrupted gene was by ligating a blunt end *HIS3* gene fragment into the *Xho*I site of pCA093 to create the disrupted plasmid pCO09HS. The *Eco*RI–*Xba*I fragment of pCO09HS was then used for integration into the genome as described by Struhl (27). The disruption was verified by Southern analysis. This strain was designated CM101.

The disrupted diploids were sporulated by growing the cells in YPA to 1 OD_{600} (2% Bacto-peptone, 1% yeast extract, 1% potassium acetate). They were then inoculated into 1% potassium acetate supplemented with 0.004% adenine, uracil and histidine. They were allowed to grow at 30°C for 2–3 days. Tetrad analysis was performed by incubating the sporulated diploids for 15 min

with glusalase and dissecting the ascus into the four spores on YPD plates.

A plasmid containing the *RRP3* gene controlled by a galactose-inducible promoter was constructed by placing the *Eco*RV–*Ssp*I fragment of pCO093 into the *Bam*HI site of vector pSEY68 by blunt end ligation. The *Eco*RV restriction site is 97 bp upstream of the ATG start codon of *RRP3* and the *Ssp*1 site is 58 bp downstream of the stop codon. This plasmid was transformed into the diploid disruption strain, sporulated and dissected on YP galactose for identification of a haploid cell containing both the genomic disruption and the galactose-inducible plasmid. This haploid strain is designated CM201.

Depletion and pulse–chase experiments

Depletion analysis was performed as described by Hughes and Ares (9) except that the cells were grown in minimal medium (2% galactose) to an OD_{600} of 1.0. The cells were then washed and inoculated into minimal medium (2% glucose). The cells were kept in logarithmic phase by dilution. CM201 and SS330 were grown for 5 h in glucose and JH44 for 12 h before pulse–chase analysis. Pulse–chase analysis was performed following the method of Sachs and Davis (20). An aliquot of 3 ml logarithmically growing cells was pulsed with 60 µCi/ml [^{3}H-methyl]methionine (75 Ci/mmol; Amersham) at room temperature for 2.5 min. The cells were then chased with 50 µg/ml cold methionine. Samples were taken at 0, 3 and 12 min after the chase, centrifuged, washed and recentrifuged. The samples were then frozen at –70°C.

Extraction of RNA

RNA was extracted from cells essentially as described by Elion and Warner (28). Cells were harvested by centrifugation at 4°C, washed in TE buffer and resuspended in 20 µl SB3 buffer (50 mM Tris–HCl, pH 8.0, 1 M sorbital, 10 mM $MgCl_2$, 3 mM DTT). Zymolase was added to 1 µg/ml and incubated for 30 min at 30°C. The cells were then diluted in 350 µl 50 mM NaOAc, pH 5.3, 10 mM EDTA, 0.5% SDS and immediately mixed with an equal volume of hot phenol (equilibrated in the above buffer). The samples were vortexed at 65°C for 15 min and centrifuged to separate the phases. The aqueous phase was further extracted with $CHCl_3$ and ethanol precipitated.

Northern blotting and hybridization

Yeast RNA (5 µg) was denatured with glyoxal and separated on a 1.2% agarose gel containing 10 mM sodium phosphate, pH 7.0 (29). RNA was transferred onto GeneScreen membrane by pressure blotting and crosslinked.

Hybridization with oligonucleotides complementary to pre-rRNA was carried out at the calculated T_m of the oligonucleotide in 6× SSPE, 1% SDS, 10× Denhardt's, 50 µg/ml tRNA and 50 µg/ml denatured salmon sperm DNA. The filters were washed at room temperature twice for 10 min in 6× SSPE, 0.1% SDS and once at the calculated T_m.

Expression and purification of Rrp3

An *Nde*I site was introduced, by site-directed mutagenesis, into the *RRP3* gene at the initiating methionine codon. The corresponding *Nde*I–*Xba*I fragment was then ligated into vector pet19 (Novagen). The vector contains a series of 10 histidine residues followed by the thrombin protease cleavage site. The plasmid was placed in *E.coli* strain Bl21(DE3) and grown to an OD_{600} of 0.4. At this point the cells were induced with 0.5 mM IPTG and grown for 4 h more. Cells were harvested and lysed in 50 mM Tris, pH 8.0, 500 mM NaCl, 5 mM imidizole, 10% glycerol, 5 mM β-mercapatoethanol. The extract was loaded onto a 1.5 ml NTA column, washed with a similar buffer containing 50 mM imidazole and eluted with 200 mM imidazole. The eluate was dialyzed against 100 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 10% glycerol, 1 mM DTT. The protein was loaded onto a Sepharose S FPLC column and eluted with a gradient of 0.1–1 M NaCl.

RESULTS AND DISCUSSION

Proteins involved in the rRNA processing pathway have been difficult to identify by conventional mutation screens (20,30,31). Recently, one processing protein, Drs1, was identified as a cold-sensitive mutation (21). Study has been further complicated by the fact that very few mutants have been found to accumulate intermediates in the processing pathway (possibly due to the instability of incorrectly processed intermediates). Only rrp1 was found to accumulate an intermediate to 25S rRNA (32,33). For the mutant clp8 the efficiency of processing of 18S rRNA from the 20S intermediate is reduced, but this is believed to be caused by a defect in export, since final processing takes place in the cytoplasm (34). Therefore few *trans*-acting factors have been identified and most by means other than their biochemical function. Several, processing proteins were identified by localization in the nucleolus (35,36).

PCR identification and cloning of *RRP3*

We have cloned a new gene by PCR analysis in a search for additional putative RNA helicases in *S.cerevisiae*. Degenerate oligonucleotides complementary to the conserved regions IV and VIII of DEAD box genes were synthesized and used as primers to identify new genes. A PCR product of the appropriate size was isolated, cloned and 45 clones were sequenced, revealing three new genes. A YCP50 library was then screened to detect genomic clones. The genes were then mapped and sequenced. These new genes have all the highly conserved motifs characteristic of the 'DEAD' family of RNA helicases (Fig. 2). One of these genes, *RRP3*, codes for a 60.9 kDa protein and contains a large (130 amino acid) N-terminus prior to the helicase domain. Furthermore, Rrp3 has several GR repeats that are common to nucleolar proteins in the C-terminus between amino acids 524 and 532.

RRP3 is an essential gene

A rrp3 null allele was created by inserting the *HIS3* gene into the helicase domain. The clone containing the disrupted gene was excised and integrated into the chromosome to disrupt one allele. Southern analysis verified the disruptions. Upon sporulation the tetrads segregated 2:0, indicating that *RRP3* is an essential gene. Northern and Southern analysis also verified that *RRP3* is a single copy gene (data not shown). A haploid disruption containing the rrp3 disruption was created by sporulating from a heterozygous diploid containing the plasmid pCO09HS. This plasmid contains the wild-type *RRP3* gene controlled by the Gal1 promoter (pCO09HS). This strain was termed CM201 and grows on galactose but is inviable on glucose.

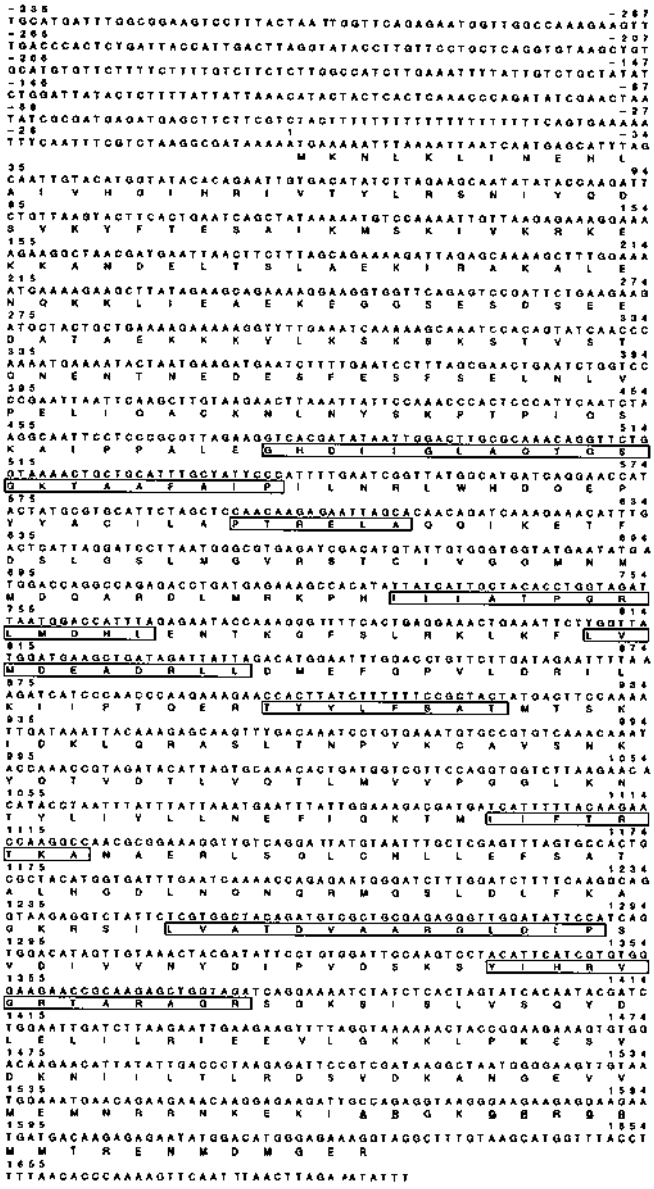

Figure 2. Nucleotide sequence of the *RRP3* gene and and amino acid sequence of its product. The boxes represent the eight conserved regions of DEAD box genes. GR domains are underlined.

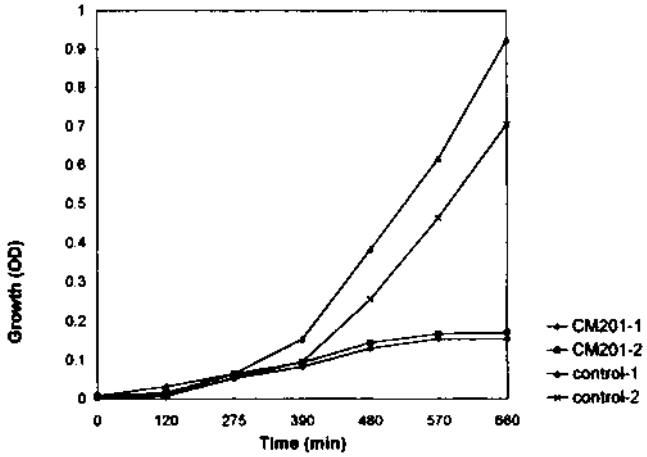

Figure 3. Growth curve for the CM201 and wild-type strains upon transfer to glucose. Optical densities were measured at 90 min intervals.

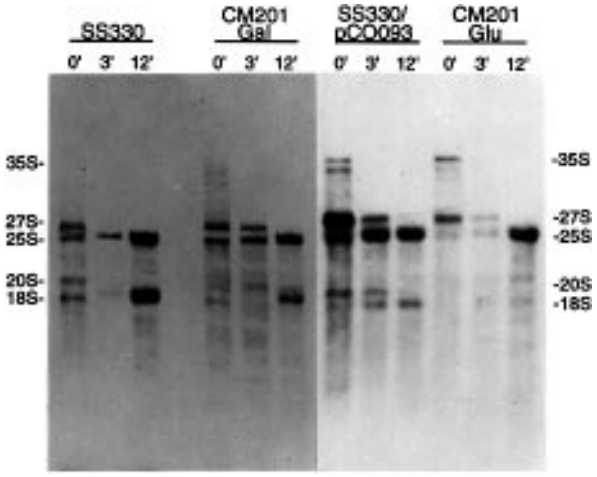

Figure 4. Pulse–chase analysis. Three milliliter log phase cultures of SS330 (containing the *RRP3* galactose-inducible plasmid) and CM201 strains growing at 30°C in galactose were shifted to glucose for 0, 6 and 12 h respectively and labeled with 60 μCi/ml [^{3}H-methyl]methionine for 2.5 min. Cold methione (500 μg/ml) was then added. Samples were taken at the indicated time points. 20 000 counts were glyoxylated and analyzed on a 1.3% agarose gel. The gel was transfered onto GeneScreen membrane, sprayed with EN^{3}HANCE and visualized by fluorography.

Depletion of Rrp3 by transcriptional repression

In order to deplete the cell of Rrp3, the strain CM201 was grown in minimal medium containing galactose. The cells were transferred to glucose-containing medium and grown until the doubling time deviated from the wild-type. After the shift to galactose the rate of growth of CM201 quickly decreases as the medium is changed and deviates from control after 5–6 h (Fig. 3). The control strain, SS330 and a wild-type containing the *RRP3* plasmid was used as a control and has a doubling time of ~90 min in glucose. The strain JH44 (a U3 depletion strain) was also subjected to depletion and pulse–chase analysis as another control (data not shown). After 5 h growth in glucose, both strains were pulsed with [^{3}H-methyl]methionine, chased and samples were taken at the indicated time points (see Fig. 4). The ability to observe the 35S precursor varied from experiment to experiment because of its instability. For the Rrp3-depleted cells, mature 18S rRNA and its 20S intermediate did not form and a faint 23S band was sometimes observed, as seen at the 3 min time point for CM201. The control strains showed normal processing of the 35S substrate. The U3-depleted strain showed a similar defect to that seen in depleted CM201, but the time course was slower (data not shown).

Ethidium gel analysis also indicated that processing of 18S rRNA is impaired. By depleting the cells for 6, 12, 24 and 36 h one can see a gradual decrease in the abundance of 18S rRNA (Fig 5). By 24 h very little 18S rRNA can be detected, but complete depletion was not observed. This also shows that Rrp3 affects 18S rRNA formation and not its ability to be detected by methylation.

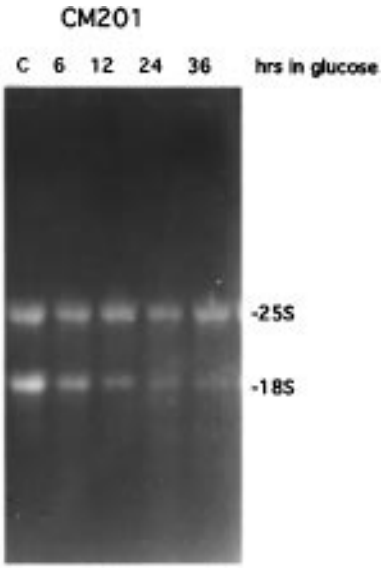

Figure 5. Agarose gel analysis of rRNA. C, wild-type SS330 strain grown in galactose and shifted for 6 h to glucose. Other lanes are samples taken at 6, 12, 24 and 36 h after strain CM201, initially grown in glucose, was shifted to glucose medium. RNA from 1 ml samples was prepared, denatured with glyoxal and analyzed on a 1.3% agarose gel. The gel was subsequently stained with ethidium bromide.

A_0, A_1 and A_2 cleavages do not occur in CM201

Many of the intermediates in rRNA processing shown in Figure 1A can be detected by Northern analysis. Some of these intermediates cannot be detected in the Rrp3-depleted strain. Processing, as determined by Northern analysis, of the RNA in Rrp3-depleted cells occurs as indicated in Figure 1B. A similar scheme was also determined for other genes involved in 18S rRNA processing (9,10,35). We were able to tell from the detectable intermediates that cleavages at A_0, A_1 and A_2 were lacking (Fig. 1A). Interestingly, this is the same phenotype seen when the three essential snoRNAs are depleted. Either cleavage at B_1 and/or A_3 separates the 18S precursor from the 27S RNA. The 27S intermediate is further processed to mature 5.8 and 25S rRNA as discussed below.

The oligonucleotides used for hybridization were obtained from J.Warner and are listed below (30; Fig. 6B). The oligonucleotide JW127 anneals to the 5′-ETS and weakly labels the 35S precursor band, only visible under prolonged exposure in both wild-type and depleted cells (Fig. 6A). As also observed by Warner, this oligonucleotide hybridizes poorly. In depleted CM201 cells, an additional band at 23S and one slightly smaller band appears, probably a 22S intermediate from the 5′-end of the primary transcript. It is unstable and does not accumulate. The length and presence of this intermediate indicates that cleavage at A_0 did not occur.

Failure to cleave at the A_1 and A_2 cleavage sites can be deduced by the hybridization patterns of JW127, JW144 and JW166. Hybridization of JW127 to a 23S RNA indicates that this intermediate contains part of the 5′-ETS, including the A_1 site. Hybridization of oligonucleotides within ITS1, JW144 or JW166 reveals very little of the normal 20S intermediate and the 23S intermediate accumulates. This also indicates that cleavage must not have occurred at A_2, because the 23S RNA contains that site. This 23S species is the major intermediate in the aberrant processing pathway. The wild-type also contains a trace amount of 23S RNA, but it does not accumulate as in the Rrp3 depletion strain. The 23S intermediate has also been observed after depletion of Nop, Gar, U3, snR30, snR128, Sof1 and deletion of the non-essential gene snR10 (3,9,10,36–39).

Oligonucleotide JW134, which is complementary to the 3′-end of ITS1, hybridizes to 27SA and 35S RNA in wild-type cells. In depleted CM201 cells, hybridization to 27SA was weak and depleted with time (Fig. 6A). We believe this is due to the fact that the exonuclease activity of Xrn1 and Rat1 is high in depleted CM201 cells (4). Detection of this intermediate indicates that cleavage must occur upstream of B_1. Hybridization to the 23S RNA should be apparent if B_1 is the primary cleavage site, as proposed by Hughes and Ares (9,10,36,38), but is absent. The apparent hybridization to 23S RNA is misleading, because of background hybridization to the abundant 25S RNA. However, close analysis shows there is little hybridization to 23S RNA. The short form of 5.8S RNA is the predominant form in yeast, so one would expect most of the JW134 oligonucleotide to hybridize to 27SA RNA and much less to 23S RNA.

Probing with oligonucleotide JW134 allowed crude mapping of the 3′-end of the 23S intermediate. Because JW134 does not hybridize to the 27S or 23S intermediates, it must extend roughly from the 5′-end of the primary transcript to within the limits of positions 2636–2760. There are two interpretations of these results. Either the 3′-end of 23S RNA is somewhat unstable and rapidly degrades when B_1 is the first cleavage event or initial cleavage occurs somewhat upstream of B_1. For this reason, a question mark remains in the Rrp3 processing scheme to denote uncertainty of the position of this cleavage. Morrissey and Tollervey found, in an snR30 depletion strain, that the 3′-end of 23S RNA was somewhere between A_2 and B_1 (subsequently termed A_3) (3). It is tempting to assume the same biochemistry for Rrp3, although it is certainly possible that similar sized intermediates detected in different depletion strains have different cleavage sites.

Hybridization outside of this region (5′-ITS2) with oligonucleotide JW145 indicates normal cleavage, but it does appear that there is a greater than normal accumulation of the 7S intermediate (Fig. 6A).

Characterization of purified Rrp3

As described in Materials and Methods, we have adapted Rrp3 for metalloaffinity chromatography and have purified the protein to near homogeneity. The ATPase activity of the recombinant enzyme was measured in the presence or absence of a number of RNA cofactors. There was a barely detectable level of ATPase activity in the presence of poly(A), but yeast rRNA did not affect the activity. Though more study of this enzyme is required, the low ATPase activity may indicate that the enzyme requires a quite specific RNA ligand. The activity of DbpA, for example, is nil with all RNA effectors other than its specific substrate (40).

As previously stated, the pattern of cleavage events seen in Rrp3-depleted cells is identical to that seen when other processing factors are depleted (3,9,10,36–39). In each case the A_1 and A_2 cleavage events are blocked. This suggests that Rrp3 could mediate structural transitions between one of the essential

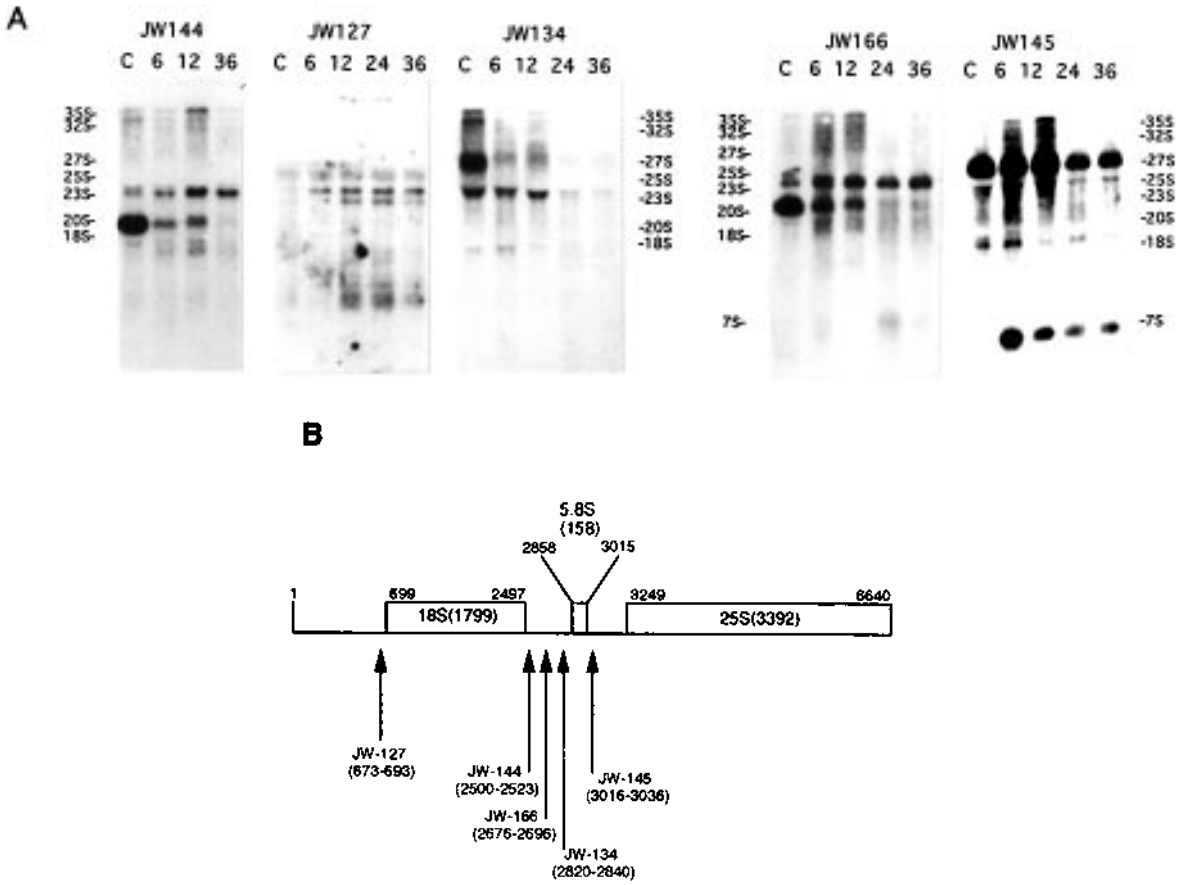

Figure 6. (**A**) Northern analysis of the CM201 depletion strain. The wild-type (C) and CM201 galactose depletion strains were grown in galactose in logarithmic phase and transferred to glucose for the indicated time in hours. The wild-type (C) was grown in glucose for 6 h. Longer periods of growth for the control did not affect the pattern of hybridization. RNA was harvested and prepared as described in Materials and Methods. Samples (20µl) were loaded and following electrophoresis the RNA was transfered to a membrane and hybridized to labeled probes as described. The 27SA and 27SB intermediates are not labeled because of their proximity, but are distinguished in the text. (**B**) Oligonucleotides used for hybridization, given to us by Dr J.Warner.

snoRNAs, U3, U14 or snR30, and the pre-rRNA substrate. Since U3 and U14 contain sequences complementary to the substrate, Rrp3 could be required as a helicase to mediate ATP-dependent changes in these interactions.

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