**RRP20, a component of the 90S preribosome, is required for pre-18S rRNA processing in Saccharomyces cerevisiae**

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Received February 12, 2003; Revised and Accepted March 21, 2003

**ABSTRACT**

A strain of Saccharomyces cerevisiae, defective in small subunit ribosomal RNA processing, has a mutation in YOR145c ORF that converts Gly235 to Asp. Yor145c is a nucleolar protein required for cell viability and has been reported recently to be present in 90S pre-ribosomal particles. The Gly235Asp mutation in YOR145c is found in a KH-type RNA-binding domain and causes a marked deficiency in 18S rRNA production. Detailed studies by northern blotting and primer extension analyses show that the mutant strain impairs the early pre-rRNA processing cleavage essentially at sites A₁ and A₂, leading to accumulation of a 22S dead-end processing product that is found in only a few rRNA processing mutants. Furthermore, U3, U14, snR10 and snR30 snoRNAs, involved in early pre-rRNA cleavages, are not destabilized by the YOR145c mutation. As the protein encoded by YOR145c is found in pre-ribosomal particles and the mutant strain is defective in ribosomal RNA processing, we have renamed it as RRP20.

**INTRODUCTION**

The synthesis of ribosomes is a major indispensable cellular activity as ribosomes are one of the key components of translation. In eukaryotes, ribosomal subunit biogenesis is a highly conserved process that occurs mainly in the nucleolus, a specialized compartment of the nucleus (1). The process starts with transcription of rDNA by RNA polymerases I and III and is followed by maturation of the transcripts through a complex pre-rRNA processing pathway (2). Processing events do not take place on 'naked rRNAs' but occur within dynamic pre-ribosomal particles containing various cis-acting and trans-acting factors. Thus, the formation of these particles is important for the production of mature cytoplasmic ribosomal subunits. To date, most of the current knowledge on eukaryotic ribosomal biogenesis comes from studies on the yeast Saccharomyces cerevisiae. This organism is well-suited for the study of ribosomal biogenesis as it allows the combined use of genetic and biochemical tools. In addition, a large number of ribosome processing factors characterized in yeast have homologs in many other eukaryotes (recent reviews in 3,4).

In yeast, the large ribosomal subunit of 60S contains three rRNA species (25S, 5.8S and 5S) plus 46 r-proteins, while the small ribosomal subunit of 40S is composed of one rRNA (18S) and 32 r-proteins (5). The 18S, 5.8S and 25S rRNAs are co-transcribed as a single large 35S precursor where the mature RNA sequences are separated by two internal spacers (ITS1 and ITS2) and are flanked by two external spacers (5'-ETS and 3'-ETS) (Fig. 3). The primary transcript precursor undergoes covalent modifications such as base and ribose methylation and pseudouridinylation (4), as well as a series of endo- and exonucleolytic cleavages. The maturation process of pre-rRNAs is intimately linked to the assembly of ribosomal proteins to generate the 60S and 40S pre-subunits and finally mature ribosomes.

Although the pre-rRNA processing pathway is fairly well understood, the pathway of ribosomal assembly has been a 'black box' for a long time. However, successful large-scale purifications of distinct pre-ribosomal particles have recently provided several important insights into ribosomal assembly (6–11, reviewed in 12,13). Additionally, a number of novel proteins potentially associated with ribosomal biogenesis have been discovered. The study of purified 90S pre-ribosome particle has shown that besides 35S pre-rRNA precursor, it contains more than 30 non-ribosomal proteins, including components required for 18S synthesis, 40S biogenesis, U3-specific proteins and a few pre-60S processing factors (7). Among the newly identified proteins, Yor145c, an essential protein of unknown function, has also been isolated.

In the present study, we provide evidence that Rrp20p (Yor145c) is an additional factor involved in the processing of the 18S rRNA precursor. We isolated, by a screen for synergistically lethal mutations after the elimination of mtDNA, a point mutant, rrp20-1, that still permits growth.

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Nucleic Acids Research, Vol. 31 No. 10 © Oxford University Press 2003; all rights reserved
The rrp20-1 mutant exhibits a marked decrease in the level of mature 18S rRNA due to the inhibition of pre-rRNA early cleavages at sites A₀, A₁ and A₂. Interestingly, all sites are not affected to the same magnitude; the major inhibition seems to occur at sites A₁ and A₂ leading to the accumulation of the atypical 22S precursor.

**Table 1. Strains of S.cerevisiae used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>BCD26/5A</td>
<td>MATa, ade2, leu2, ura3, met6, rrp20-1</td>
<td>This study</td>
</tr>
<tr>
<td>M2915-6A</td>
<td>MATa, ade2, ura3, leu2</td>
<td>Chen and Clark-Walker (49)</td>
</tr>
<tr>
<td>M9/B</td>
<td>MATα, his4, leu2, ura3</td>
<td>Zuo (50)</td>
</tr>
<tr>
<td>M9/B-RRP20</td>
<td>M9/B, RRP20::pURA-RRP20</td>
<td>This study</td>
</tr>
<tr>
<td>CS5</td>
<td>MATa/MATα, leu2/leu2, ura3/ura3, his4+/+, ade2/+</td>
<td>X. J. Chen, unpublished results</td>
</tr>
<tr>
<td>CS3Srp20</td>
<td>CS5, RRP20/rrp20 kan</td>
<td>This study</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**Strains and general methods**

*Saccharomyces cerevisiae* strains shown in Table 1 are derived from the same isogenic background. All strains were grown at 30°C. Complete medium, GYP, contains 0.5% Bacto yeast extract, 1% Bacto peptone and 2% glucose. EB and G418 media are GYP plus ethidium bromide at 25 µg/ml and G418 at 200 µg/ml, respectively. Other yeast media and genetic manipulation were followed from standard protocols (14). Microbiological methods were performed according to established procedures (15).

**Isolation of rrp20-1**

*Saccharomyces cerevisiae* M2915-6A was mutagenized by ethyl methanesulfonate (EMS) as described elsewhere (15). Mutants unable to grow on EB (25 µg/ml) at 30°C were selected. Candidate strains, failing to survive in the absence of mitochondrial DNA (ρ⁰) (growth in the presence of EB converts cells to ρ⁰), were crossed to wild type. Subsequent crosses to wild type were performed to ascertain persistence of the EB⁺ phenotype in segregants. A 2:2 segregation of EBs/− strains shown in Table 1 are derived from the same isogenic background. All strains were grown at 30°C. Complete medium, GYP, contains 0.5% Bacto yeast extract, 1% Bacto peptone and 2% glucose. EB and G418 media are GYP plus ethidium bromide at 25 µg/ml and G418 at 200 µg/ml, respectively. Other yeast media and genetic manipulation were followed from standard protocols (14). Microbiological methods were performed according to established procedures (15).

**Cloning of RRP20 by complementation and further subcloning**

Cloning of the RRP20 wild-type allele was performed as follows. The rrp20-1 mutant strain produced very small colonies even after a long period of incubation on solid media (rich or minimal) at all temperatures. The mutant strain was transformed with a genomic DNA library in the LEU2 multicopy vector YEp13-m4 (kindly donated by B. Stillman) and after incubation at 30°C for 3 days, approximately 4300 Leu⁺ transformants were obtained. Subsequently, three relative larger colonies were selected. After testing on GYP and EB media, only one clone showed complementation of both the slow growth phenotype and EB⁺. Plasmid rescue and back transformation into the rrp20-1 strain confirmed that complementation of both mutant phenotypes was linked to the library plasmid. The complementing plasmid yBCD26 was partially sequenced. Subclones shown in Figure 1A were generated from the complementing plasmid as follows. A 2.4 kb Sacl-Xbal and a 1.4 kb Kpnl-NSI restriction fragment from yBCD26 were respectively subcloned into Sacl-Xbal and Kpnl-NSI restricted pCXJ15 plasmids (X. J. Chen, unpublished results), yielding plasmids pEFD1 and pRRP20, respectively. The plasmid pRRP20 was further digested with Nhel and HindIII restriction enzymes, blunt-ended and then religated to remove the YORF46w region, which also deleted 84 amino acids from the N-terminus of the Rrp20 protein, yielding pRRP85-274. pRRP1-110 was generated by digestion of pRRP20 with EcoRI and BstEII, blunt-ended and religated to remove 111 amino acids from the C-terminus of Rrp20p. To delete both termini of Rrp20p, plasmid pRRP85-274, containing 5’-terminal truncated RRP20, was digested with EcoRI and XbaI, blunt-ended and religated to delete eight amino acids from the C-terminus, creating pRRP85-266. Plasmid pMonoRRP85-266 was made by subcloning a 553 bp XbaI-Nhel fragment from plasmid pRRP20 into the XbaI-digested low copy plasmid pCXJ24 (16).

**Disruption of RRP20**

Disruption of RRP20 was carried out by the one-step gene replacement procedure (17). Plasmid pRRP20 was opened at the unique BstEII site within RRP20 and a 1.4-kb BglII–XhoI fragment containing the kan expression module was inserted using blunt-ended ligation (Fig. 1A). A 2.1 kb Kpnl–Nhel fragment obtained from this construct was transformed into the diploid strain CS5. G418r transformants were selected and Southern blotting analysis confirmed the correct disruption of one genomic copy of the RRP20 gene.

**In vitro mutagenesis of the unidentified ORF**

The ORF finder program (NCBI) revealed an unidentified ORF of 123 amino acids lying opposite the 3’ region of RRP20 (Fig. 1A). This ORF is not included in the Saccharomyces Genome Database (SGD) but it shows some identity to proteins or hypothetical proteins from several organisms. For example, the unidentified ORF shares 36% identity (17 out of 46 residues) to a DNA mismatch repair protein of Streptococcus mutans (data not shown). To eliminate the possibility that the unidentified ORF was responsible for complementation, in vitro mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Stratagene) was carried out to introduce stop codons at nucleotides 6, 27 and 132 of this ORF. These mutations were silent for the reading frame on the opposite strand encoding Rrp20p. The pRRP20 plasmid was the starting substrate for site-specific mutagenesis. The forward oligonucleotides: W2-F, GCCATTTCTGATGTGATCTAACCCGCC; W9-F, CCCGGCCAAAAATGTGATTTAGAGTACG; and W44-F, CCCCCCCAAAATGTG-
AATTTAGAGTCAGC (bold nucleotides represent mutations), with their respective reverse primers: W2-R, GGG-CGGGTTTCACTCACAGATGTC; W9-R, GCTGACTCTAAATTCACATTTTGGG; and W44-R, CTC-TAACAGGTGATCATTTTGCAGAGCC were used in PCRs to produce plasmids pRRP-W2, pRRP-W9 and pRRP-W44, respectively. Each mutated plasmid from two independent clones was isolated and the sequences verified using the sequencing primer. Sequence analysis revealed that the desired mutations were obtained (data not shown).

**Integration of the URA3 marker at the RRP20 locus**

To confirm that the complementing fragment contains RRP20, a genetic linkage analysis was carried out. A 1.4 kb SacI-HindIII fragment from pRRP20 containing the entire RRP20 ORF was cloned into the integrative plasmid pUC-URA3/4.
(18) carrying the yeast URA3 gene. The resulting construct was linearized by BsrEII located inside the RRP20 ORF and transformed into a wild-type haploid strain, M9/B. Correct integration was verified by Southern blotting. After crossing the newly constructed strain, M9/B-RRP20, to the rrp20-1 mutant, segregation of the URA3 marker and growth on EB were analyzed.

**Sequencing and in silico analyses**

To examine mutations of the rrp20-1 allele, two PCR amplifications of genomic DNA, isolated from the BCD26/5A mutant, were performed using two pairs of primers (RRP-F, CCGGCTCCGCTAAAGTTAGCGTTT; RRP-R, GGCTGCAAGATGACACCTTGAGGAAC; and RRP-1F, GCCGCTGAGAGATACATATTGCATC; RRP-1R, GGC- GGATCCGCTAACGTTACGCGTTTG; RRP-R, RRPR-G, regions, as well as 86 bp upstream and 124 bp downstream. The resulting PCR products were digested with *Bam*HI and *Pst*I and cloned into pTZ19U digested with the same enzymes. The entire nucleotide sequences of rrp20-1 from both plasmids were determined using sequencing primers to obtain data from both DNA strands. In the same way, the RRP20 allele was isolated from the parental strain and the sequences were compared to that of the mutant allele. DNA sequencing was carried out by the dideoxy-chain termination method of Sanger et al. (19), using a fluorescence-based method. DNA and protein analyses were carried out using the LASERGENE software package (DNASTAR, Inc). ORF mapping of DNA sequences was performed by means of the ORF Finder (NCBI). Sequence alignments of predicted proteins (retrieved from GenBank or SGD) were carried out using the CLUSTAL W program (20). For investigation of related sequences, the BLAST program (21) was utilized. An analysis for protein domains was accomplished from InterPro database (22). ImageQuant 5.0 software (Molecular Dynamics) was used to analyze phosphorimages.

**RNA analysis**

Isolation of total RNAs from yeast was performed as described previously (23). Large molecular weight RNAs were separated on 1.2% agarose–formaldehyde gels and transferred to Hybond-N+ nylon membranes by capillary blotting. Low molecular weight RNAs were separated on 7% polyacrylamide–8 M urea gels and transferred to Hybond-N+ nylon membranes by electroblotting. Northern hybridization analyses were performed in Church buffer (24). Primer extension was carried out according to Venema et al. (25). The oligonucleotides used for northern analysis and primer extension were previously described (26), except for probe H, which is CATGGOCTTAATCTTGAGAC. For localization of the oligonucleotides see Figure 3. For identification of the 22S intermediate, a riboprobe G complementary to sequences between the A0 and A1 sites was used. A PCR product containing the A0to-A1 sequence and a T7 promoter was synthesized using primers OBS330, CTATAACGACTC CACTATAGGACTACTCTAAAAGAAGAAG (underlined sequence corresponds to T7 promoter) and OBS331, ATCTTCTAGCAAGAGGG, primers with W303 rDNA subcloned in pUC19 plasmid as DNA template. To assess the steady-state levels of snoRNAs, oligonucleotide probes U3, U14, snR10 and snR30 were used as previously described (27).

**RESULTS**

**Isolation of rrp20-1 mutant and identification of RRP20**

A screen for mutants that die on loss of mtDNA (p0-lethality) was performed after EMS mutagenesis of M2915-6A. Strains that could grow on complete medium but failed to survive after EB elimination of mtDNA were chosen and crossed with a wild-type strain (see Materials and Methods). Upon tetrad analysis, one mutant exhibiting a 2:2 segregation of EB:EB' phenotypes, indicative of a single nuclear mutation, was isolated and subsequently referred to as rrp20-1. In addition to the EB' phenotype, the rrp20-1 strain exhibits a severe growth rate reduction on rich medium at 30°C (Fig. 1B). Both phenotypes were recessive, thus cloning of the corresponding wild-type gene was performed by functional complementation after transformation with a yeast DNA library in YEp13-m4. A complementing plasmid, yBCD26, carrying a fragment from chromosome XV containing the entire YOR145c (renamed RRP20) and YOR146w ORFs lying on the opposite strand as well as part of EFD1 and YOR147w, was recovered. In addition, the ORF finder program (NCBI) revealed another possible ORF lying opposite to the 3'-region of the RRP20 gene (Fig. 1A).

To investigate the location of complementation activity, multicopy subclones, pEFD1, pRRP20 and pRRP85-274 from yBCD26, were generated and transformed into the rrp20-1 mutant. As shown in Figure 1B, plasmids pRRP20 (row 3) and pRRP85-274 (row 4) were able to complement p0-lethality, whereas a strain carrying plasmid pEFD1 (row 2) could not grow on EB. In addition, the transformants containing the complementing plasmids exhibited a better growth on GYP almost resemble the wild type. As the pRRP85-274 plasmid contains not only the truncated RRP20 but also an uncharacterized ORF (marked by a question mark in Fig. 1A), the question arises as to which region is required for survival of p0 cells and recovery of slow growth. To answer this question, in vitro mutagenesis was carried out to introduce stop codons at nucleotides 6, 27 and 132 of the unidentified ORF that are silent for RRP20 (see Materials and Methods). In parallel, simultaneous disruption of RRP20 and the unidentified ORF in the diploid strain CS5 was done by insertion of a kan marker into the BsrEII site (Fig. 1A), generating the heterozygous strain CS5arrp20. This disruption was lethal as tetrad dissection yielded a 2:2 segregation of viable to non-viable spores where all viable spores were G418+. After transformation with a yeast DNA library in YEp13-m4, the mutated constructs, pRRP-W2, pRRP-W9 and pRRP-W44 (rows 5, 6, 7, respectively), were transformed into the CS5arrp20 diploid strain. After sporulation and ascus dissection, rescue of lethality by the three mutated plasmids is apparent (Fig. 1C). The identified clones, indicating that they harbor the complementing pCXJ15 recombinant plasmids (data not shown). These results not only reveal that RRP20 is responsible for complementation but they also indicate that the lethal phenotype is caused by disruption of this gene. Finally,
genetic linkage analysis using the integration of a RRP20 allele associated to the marker URA3 at the RRP20 genomic locus also confirmed that an ability to restore the EB^ phenotype of rrp20-1 was in fact due to RRP20 (data not shown).

Rrp20p is a putative RNA-binding protein conserved within eukaryotes

The RRP20 gene encodes a protein of 274 amino acids with a predicted molecular weight of 30.3 kDa and a predicted pl of 9.87. A BLAST search (21) demonstrated that Rrp20p shows strong similarity with potential RNA-binding proteins of at least six other organisms, including the fission yeast Schizosaccharomyces pombe, Arabidopsis thaliana, Caenorhabditis elegans, Drosophila melanogaster, Mus musculus and human (Fig. 2). The functions of these homologs have not previously been investigated. There is 53–72% sequence identity and 73–84% sequence similarity between Rrp20p and these six proteins. The sequence alignment reveals a high similarity at the C-terminal region. The conserved Gly235, which was changed to an Asp235 in the rrp20-1, is indicated by asterisks. KH domains reported from the InterPro database are underlined. Sequences were aligned using the MegAlign program, DNASTAR, Inc.
revealed that the N-terminal regions of these proteins lack sequence conservation, whereas the C-terminal regions are more similar and contain a well conserved K homology (KH) domain as reported in the InterPro database (22) (Fig. 2). In Rrp20p, this domain extends from residue 179 to residue 252. The KH domain was first identified in the human hnRNP K and is an evolutionarily conserved sequence of ~70 amino acids found in a wide range of RNA-binding proteins from various organisms (28,29). The presence of this domain suggests that Rrp20p is a potential RNA-binding protein.
The C-terminal part of Rrp20p containing the KH domain is essential for its function

To further characterize Rrp20p, truncations of RRP20 were subcloned into low copy or multicopy plasmids to identify the essential region. All Rrp20p truncations (Fig. 1, rows 4 and 8–10) were introduced to the CS5Δrrp20 heterozygous diploid strain followed by sporulation and tetrad dissection. As shown in Figure 1C, pRRP85-274 (row 4), carrying a N-terminally truncated Rrp20p missing the first 84 amino acids, can complement the lethal phenotype to a similar extent as plasmids carrying the full-length protein (yBCD26 and pRRP20). This indicates that the N-terminal part of Rrp20p is dispensable for its function and is in a good correlation with the low similarity of the N-terminal extensions shown in the sequence alignment (Fig. 2). In contrast, pRRP1-110 (row 8), expressing a truncated Rrp20p lacking the last 174 residues and thus the KH domain, failed to abolish lethality as only a 2:2 segregation of viable to non-viable spores was obtained. Interestingly, the smallest truncated version of Rrp20p, which was just able to complement the lethal phenotype of the RRP20 disruption when overexpressed, contains amino acids 85–266 (pRRP85-266; row 9). However, when this truncated protein was expressed at lower level (pMonoRRP85-266; row 10) there was no complementation. All viable spores were G418® and microscopic examination of the non-viable spores showed no evidence of any germination. Interestingly, sequence analysis revealed that rrp20-1 has a single point mutation that converts codon 235 from GGC to GAC resulting in the substitution of Gly235 by an Asp (data not shown). This glycine residue is conserved among the six orthologs and belongs to the KH domain (Fig. 2).

rrp20-1 has a severe deficit in 18S ribosomal RNA and accumulates aberrant 22S and 23S intermediates

Previously, RRP20 (YOR145c) has been shown to be an essential gene whose GFP-fusion protein is localized in the nucleolus (30), an organelle dedicated to ribosomal biosynthesis. To determine if Rrp20p plays a role in this process, we first compared the mature rRNA content of rrp20-1 mutant (lane 2) and wild-type (lane 1) grown wild-type and rrp20-1 mutant cells were annealed with oligonucleotides H or F. Oligonucleotide H hybridizes to the 5′ end of 18S rRNA which allows detection of processing sites A0 and A1, while oligonucleotide F binds to a region in ITS2 which allows A2, A3, B1L and B1S sites to be detected. In the mutant strain (lane 2), there is an increase of the signals of the primer extension stops at sites A0 and A1 associated with a decrease of the signals of the primer extension stops at sites A2 and A3 (Fig. 4). This is in good agreement with the detection of a 22S RNA extending from A0 to A3 sites. It also confirms the accumulation of the 33S pre-rRNA and the depletion of the 20S and 27SA2 pre-rRNAs, which results in a defect of mature 18S rRNA. The levels of 27SB1 and 27SB5, as shown by the primer extension stops at sites B1L and B1S, appears slightly increased in the rrp20-1 mutant without affecting the intermediate ratio between the two strains. Finally, processing at all sites of intermediate products tested were accurate at the nucleotide level (Fig. 4). The results of both northern hybridization and primer extension analyses indicate that in the rrp20-1 strain, the deficiency in 18S rRNA production is due to an inhibition of the pre-ribosomal RNA early cleavages at sites A0, A1 and A2.

rrp20-1 does not affect the steady-state levels of snoRNAs U3, U14, snR10 and snR30

Four snoRNAs have been previously shown to be necessary for the early A0- to A2 cleavages. These are snoRNAs U3, U14, snR10 and snR30 (reviewed in 31). It has been reported that genetic depletion of these snoRNAs inhibits early cleavages at sites A0, A1 and A2. To ascertain whether the processing deficiency in these cleavages is a direct consequence of the mutation in RRP20, steady-state levels of the snoRNAs U3, U14, snR10 and snR30 were assessed. Total RNAs from both wild type and rrp20-1 strains were separated in an agarose-formaldehyde gel, transferred to a nylon membrane and detected by serial hybridizations with specific probes. Prior to hybridization, the membrane was stained with methylene blue to visualize and mark the location of the major 18S and 25S rRNA species (Fig. 3A'). The location of the probes used to detect the various processing intermediates is shown on the structure of 35S pre-rRNA. As seen in Figure 3B, probing with oligonucleotide B, which hybridizes upstream of the A0 cleavage site, shows that the rrp20-1 mutant (lane 2) accumulates the 35S pre-rRNA and a 23S aberrant processing product. While 35S precursor can be detected by all probes used, the aberrant product is only detected by probes B, C, D and G, confirming that this atypical intermediate corresponds to the previously described 23S, which extends from the 5′ end of the 5′-ETS to the A0 site. Traces of the 23S RNA species are also detected in the wild type. As shown in Figure 3C, hybridization with probe C, which binds on the 5′ side of the A2 cleavage site, reveals a reduced amount of the 20S pre-rRNA, which is the direct precursor of the 18S rRNA. In addition, there is an accumulation of a pre-rRNA species below 35S, which presumably corresponds to the 33S precursor since it was also detected by the riboprobe G, delimiting the A0–A1 spacer fragment (Fig. 3G). Furthermore, an unusual precursor migrating between the 25S and the 20S was detected in the mutant (lane 2) but not in the wild type (lane 1). As it was also detected with probe D and the riboprobe G but not with probes B, E and F (Fig. 3B, D–G), it may correspond to a 22S RNA extending from the A0 to the A3 sites. Consistent with the production of 22S and 23S RNAs, we observed that the 27SA2 pre-rRNA is strongly depleted whereas the level of 27SB intermediates as well as the amount of mature 25S rRNA remains similar to that of the wild type strain (Fig. 3A, A′ and D–F).

Primer extension analysis was carried out to determine the precise steps at which pre-rRNA processing is blocked in the rrp20-1 mutant. Total RNAs isolated from exponentially grown wild-type and rrp20-1 cells were annealed with oligonucleotides H or F. Oligonucleotide H hybridizes to the 5′ end of 18S rRNA which allows detection of processing sites A0 and A1, while oligonucleotide F binds to a region in ITS2 which allows A2, A3, B1L and B1S sites to be detected. In the mutant strain (lane 2), there is an increase of the signals of the primer extension stops at sites A0 and A1 associated with a decrease of the signals of the primer extension stops at sites A2 and A3 (Fig. 4). This is in good agreement with the detection of a 22S RNA extending from A0 to A3 sites. It also confirms the accumulation of the 33S pre-rRNA and the depletion of the 20S and 27SA2 pre-rRNAs, which results in a defect of mature 18S rRNA. The levels of 27SB1 and 27SB5, as shown by the primer extension stops at sites B1L and B1S, appears slightly increased in the rrp20-1 mutant without affecting the intermediate ratio between the two strains. Finally, processing at all sites of intermediate products tested were accurate at the nucleotide level (Fig. 4). The results of both northern hybridization and primer extension analyses indicate that in the rrp20-1 strain, the deficiency in 18S rRNA production is due to an inhibition of the pre-ribosomal RNA early cleavages at sites A0, A1 and A2.
the other snoRNAs species, including U14, snR10 and snR30 are not altered in the rrp20-1 mutant. This supports a direct involvement of Rrp20p in pre-rRNA processing and possibly in 40S ribosomal subunit biogenesis.

DISCUSSION

Lethality of rrp20-1 on the loss of mtDNA

The rrp20-1 mutation causes a severe reduction in growth on a complete glucose medium as well as lethality after EB elimination of mtDNA (ρ0-lethality). Prior to the present study, nuclear genes that are required for viability of ρ0 cells have been found to encode proteins whose primary functions are restricted to mitochondria. For instance, ATP1 and ATP2 encode the α- and β-subunits of F1-ATPase (32), and RPM2 encodes a protein subunit of mitochondrial RNase P (33). Therefore, this is the first report that a mutant defective in pre-rRNA processing cannot tolerate the loss of mtDNA. The isolation of such a mutant is not unreasonable if the viability of ρ0 cells is dependent on an adequate level of ribosomes. Thus, the respiratory competent ρ+ rrp20-1 mutant can just survive on the small amount of 18S rRNA that it contains, but loss of mtDNA cannot be tolerated. Consequently, it might be expected that other leaky mutants for rRNA production could be identified by their failure to survive loss of mtDNA.

A mutation of RRP20 affects pre-rRNA early cleavages mainly at sites A1 and A2

Recent work, using a large-scale purification of pre-ribosomal particles followed by identification of their protein content by mass spectrometry, has identified many additional factors associated with ribosomal biogenesis (6,7,9–11,34). Rrp20p (Yor145c) has been identified as one of the ‘19 additional factors of unknown function’ associated with the 90S pre-ribosome (7). Here, we demonstrate for the first time that Rrp20p participates in pre-rRNA processing. Assessment of steady-state levels of mature rRNAs revealed that the rrp20-1 mutant exhibits a strong deficiency in 18S rRNA production. Further analyses using northern hybridization and primer extension procedures clearly indicate that the rrp20-1 strain harbors an aberrant pre-rRNA processing pathway. The accumulation of the 35S pre-rRNA is a sign that cleavage at site A0 is impaired. The direct cleavage at site A3 of the 35S precursor generates 23S RNA and 27SA3 pre-rRNA. Nonetheless, it is noticed that a fraction of the 35S pre-rRNA still undergoes A0 cleavage. The resulting 33S pre-rRNA is, however, not further processed at sites A1 and A2. The 33S intermediate is instead cleaved directly at the A3 site in ITS1, and generates 22S RNA and 27SA3 pre-rRNAs. The appearance of 22S RNA is associated with the loss of 20S and 27SA2 pre-rRNAs. Despite the loss of the latter, subsequent processing of the 3′ region of the pre-rRNA is not affected. The 27SA3 pre-rRNA is processed normally leading to wild-type levels of 27SB pre-rRNAs and of mature 25S. On the other hand, the loss of 20S pre-rRNA results in the depletion of the mature 18S rRNA, indicating that the 22S RNA cannot be processed efficiently to 18S rRNA. In this regard, accumulation of 22S rRNA, extending from A0 to A3, clearly indicates that the rrp20-1 mutation has a mild effect on A0 cleavage while strongly affecting A1 and A2 cleavage.
Interestingly, in respect to the number of components responsible for ribosomal synthesis, there are surprisingly few mutants yielding the atypical 22S precursor. A similar phenotype to that shown by rrp20-1 can be found in strains carrying an inactivation of Rcl1p (35), Mpp10p (36,37), Dhr1p (38) and Dim1p (39,40). In relation to the latter protein, it has been reported that Rrp20p is found in two multiprotein complexes both containing Dim1p (41). In addition, among a large number of identified components of 90S-pre ribosome, Rrp20p has been co-purified together with Mpp10p, Dhr1p and Dim1p, which are known to be specifically required for 40S subunit synthesis. This finding supports the present study that Rrp20p is a bona fide factor involved in the small subunit rRNA processing. The possibility that defects in processing events might be due to destabilization of snoRNAs has been excluded since the levels of U3 or the other snoRNAs species, including U14, snR10 and snR30 are not affected in the rrp20-1 mutant.

Rrp20p and its orthologs contain KH domains at the C-terminus

It is likely that the function of Rrp20p is specific and evolutionarily conserved since its orthologs have been identified from yeast to humans. All of them possess a KH-type RNA-binding domain located at the C-terminus. Besides containing a KH motif, the deduced Rrp20p protein sequence is not found to carry any putative enzymatic domain. Since it has been reported that the early cleavage at sites A0, A1 and A2 require endonucleolytic activity (4), Rrp20p might mediate the cleavage of these sites by recruiting further catalytic proteins or stabilizing the functional complex. Interestingly, the missense mutation, Gly235Asp, which impairs the early cleavage ability of rrp20-1, falls in the KH domain. This suggests that the defective processing results from a decreased binding efficiency of Rrp20p to RNA. Experimental support that this domain binds RNA might shed light on the actual role of Rrp20p and its orthologs, including the protein from humans. Proteomic analyses of human nucleolar proteins have recently been investigated (42–45). Evidence of impaired rRNA modification associated with dyskeratosis congenital and Hoyeraal–Hreidarsson syndromes has been reported in mice (46). This observation raises the possibility that more diseases in other mammals, including humans, could be caused by defective rRNA processing.

YOR145c annotation

While this manuscript was being prepared, YOR145c annotation has been reported as PNO1 (47). Pno1p was identified as a protein partner of Nob1p which has been proposed to function in ubiquitin-specific proteasome assembly (47). It was also suggested that Pno1p was required for proteasome maturation. However, recently, Fatica et al. have demonstrated that Nob1p is instead involved in 40S ribosomal subunit biogenesis and, interestingly, that an accumulation of 22S RNA was also seen in strains depleted for Nob1p (48). Thus, this finding appears to rule out the previously proposed role of Yor145c in proteasome formation. However, it has also been reported that a Gly203Asp mutation at the KH domain of Yor145c caused a ts phenotype (47). It would be interesting to know if this mutant would lead to lethality on the loss of mtDNA, as well as showing defects in pre-rRNA processing as found in rrp20-1 carrying a Gly235Asp mutation. The present study, therefore, is the first report revealing early pre-rRNA processing defects of yor145c mutant and, thus, supports renaming the gene RRP20.

ACKNOWLEDGEMENTS

We are grateful to Dr B. Stillman (Cold Spring Harbor) for supplying a S.cerevisiae genomic library cloned in YEp13-m4 and to Dr D. Kressler (Biozentrum Basel) for providing pUC19-based plasmids containing 3′ and 5′ regions of W303 rDNA sequence. S.S. has been supported by a Royal Thai Government Scholarship.

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