Mrd1p Is Required for Processing of Pre-rRNA and for Maintenance of Steady-state Levels of 40 S Ribosomal Subunits in Yeast*

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Ribosome biogenesis is a conserved process in eukaryotes that requires a large number of small nucleolar RNAs and trans-acting proteins. The Saccharomyces cerevisiae MRD1 (multiple RNA-binding domain) gene encodes a novel protein that contains five consensus RNA-binding domains. Mrd1p is essential for viability. Mrd1p partially co-localizes with the nucleolar protein Nop1p. Depletion of Mrd1p leads to a selective reduction of 18 S rRNA and 40 S ribosomal subunits. Mrd1p associates with the 35 S precursor rRNA (pre-rRNA) and U3 small nucleolar RNAs and is necessary for the initial processing at the A0–A2 cleavage sites in pre-rRNA. The presence of five RNA-binding domains in Mrd1p suggests that Mrd1p may function to correctly fold pre-rRNA, a requisite for proper cleavage. Sequence comparisons suggest that Mrd1p homologues exist in all eukaryotes.

In eukaryotes, three of the four ribosomal RNAs are derived from a single RNA polymerase I transcript, the precursor rRNA (pre-rRNA).1 Maturation of the pre-rRNA involves a series of sequential events that occur in the nucleolus (1, 2). These include extensive post-transcriptional modifications of many of the nucleotides and several endo- and exonucleolytic cleavages (3, 4) (see also Fig. 6A). The final maturation of the 18 S rRNA in the small 40 S subunit takes place in the cytoplasm (5), whereas the maturation of the rRNAs in the large 60 S subunit (5.8 and 25 S or 28 S rRNA from the pre-rRNA and 5 S rRNA separately synthesized by RNA polymerase III) occurs in the nucleus (3). The two ribosomal subunits are believed to be exported separately from the nucleus, and export involves the Ran cycle and specific nucleoporins (6–8).

Processing of the pre-rRNA takes place in a multicomponent complex that is assembled co-transcriptionally (9, 10) and requires the coordinated folding of the pre-rRNA as well as precise positioning of processing factors (3). This process is accompanied by the assembly of ribosomal proteins on the successive intermediates in an ordered manner to generate the two ribosomal subunits. In Saccharomyces cerevisiae, about 60 trans-acting proteins and 100 small nucleolar RNAs ( snoRNAs) are involved in ribosome biogenesis. The snoRNAs are present in the form of snoRNA-protein (snoRNP) complexes in cells and can be divided into the box (C/D) snoRNAs, the box (H/ACA) snoRNAs, and the RNase MRP (11–13). The U3 snoRNA, one of the most abundant and best characterized snoRNPs, is essential for processing at the three early cleavage sites A0, A1, and A2 that leads to the 18 S rRNA formation (9, 14, 15). In yeast, the U3 snoRNA shares several common protein components, Nop1p, Nop5p/58p, sno5p, and Sno1p (16–20), with other box (C/D) snoRNPs. In addition, a set of proteins that are associated specifically with the U3 snoRNA have been identified, including Soflp1, Mpp10p, Imp3p, Imp4p, Lcp5p, Rcl1p, Rrp9p, and Dhr1p (21–27). Depletion of any of these proteins or of U3 snoRNA leads to inhibition of the early pre-rRNA cleavages at the A0–A2 cleavage sites (in the case of Dhr1p, only the A1 and A2 sites) and reduction of the synthesis of 18 S rRNA.

Among the identified trans-acting nucleolar proteins, some are endo- and exonucleases and RNA helicases, whereas the role of others remains unknown (3). A shared feature of some nucleolar proteins that bind to pre-rRNA or snoRNA is that they contain a consensus RNA-binding domain (RBD), also called the RNA recognition motif. RBDS, which are also present in many proteins involved in different aspects of gene expression (28), have a typical βαββαββ-fold (29) and mediate specific RNA binding. Two proteins essential for ribosome biogenesis, Nop4p (30) and nucleolin (31), each contain four RBDs. Nucleolin is involved in several steps in ribosome biogenesis (32). Recent NMR spectroscopy studies have shown that the first two N-terminal RBDs in nucleolin bind to an RNA stem loop present at several sites in the pre-rRNA, and it has been suggested that nucleolin may play a role as an RNA chaperone (33).

We have isolated and characterized a novel yeast gene MRD1 (multiple RNA-binding domain). MRD1 encodes a protein with five consensus RNA-binding domains that is essential for viability. Depletion of Mrd1p leads to a decrease in the synthesis of 18 S rRNA and a decrease in the steady-state level of 40 S ribosomal subunits. Mrd1p associates with the 35S pre-rRNA and the U3 snoRNA. We have found that Mrd1p is required for the initial A0–A2 cleavages during processing of the 35 S pre-rRNA. Mrd1p is the founding member of a conserved family of proteins found in all eukaryotes, each with multiple RNA-binding domains. The high degree of conservation suggests that the multiple RNA-binding domains play an important structural role in organizing specific rRNA processing events.
Mrd1p Is Essential for Pre-rRNA Processing

### TABLE I

| Strain   | Genotype Source | Source |
|----------|-----------------|--------|
| HKDY8    | MATa ura3-3::ura3-3 ura3-2::lea2-3,112 MATa HIS3/hisG::200 lys23201/lys23201 ade2/ADE2 | This work |
| LWDY1    | MATa ura3-3::ura3-3 ura3-2::lea2-3,112 MATa HIS3/hisG::200 lys23201/lys23201 ade2/ADE2 | This work |
| LWDY3    | MATa ura3-3::ura3-3 ura3-2::lea2-3,112 MATa HIS3/hisG::200 lys23201/lys23201 ade2/ADE2 MRR1A1::hisG-URA3-hisG/MRD1 | This work |
| LWDY5    | MATa ura3-3::ura3-3 ura3-2::lea2-3,112 MATa HIS3/hisG::200 lys23201/lys23201 ade2/ADE2 MRR1A1::hisG-URA3-hisG/MRD1 | This work |
| LWY001   | MATa ura3-3 ura3-2,112 his333200 lys2320 lysd131::hisG-LEU2-hisG | This work |
| LWY004   | MATa ura3-3::ura3-3 ura3-2::lea2-3,112 MATa HIS3/hisG::200 lys23201/lys23201 ade2/ADE2 MRR1A1::hisG-URA3-hisG/MRD1 | This work |
| LWY005   | MATa ura3-3::ura3-3 ura3-2::lea2-3,112 his333200 lys23201/lys23201 ade2/ADE2 MRR1A1::hisG-URA3-hisG/MRD1 | This work |
| LWY007   | MATa ura3-3::ura3-3 ura3-2::lea2-3,112 his333200 lys23201/lys23201 ade2/ADE2 MRR1A1::hisG-URA3-hisG/MRD1 | This work |
| LWY008   | MATa ura3-3::ura3-3 ura3-2::lea2-3,112 MATa HIS3/hisG::200 lys23201/lys23201 ade2/ADE2 | This work |

### EXPERIMENTAL PROCEDURES

**Strains**—S. cerevisiae strains are listed in Table I. Standard yeast methods and media are described within Guthrie and Fink (34). YPGal is similar to YPD medium except that 2% galactose replaces glucose. Yeast cells were transformed using the lithium acetate method (35). Transformants were selected on solid complete synthetic dextrose media lacking uracil, leucine, or histidine as required. Strain HKDY8, an isogenic diploid derivative of haploid strain AA255 (36), was transformed using the NotI/SalI fragment of pS002 (see Table II; plasmids are described in the following section) containing the mrd1A1::hisG-URA3-hisG allele resulting in the Ura" strain LWDY1. The URA3 marker in the mrd1A1 allele of LWDY1 was converted to LEU2 resulting in strain LWDY3. This was accomplished by transforming LWDY1 to Leu" with a HindIII fragment from plasmid pUC4-ura3::LEU2 (obtained from Y. Kasir). LWDY1 was propagated on media containing 5-fluoroorotic acid (5-FOA); strain LWDY5 with transforming LWDY1 to Leu" was verified to be Leu". The wild-type GAL2 promoter region (up to position –389) was inserted into NotI SalI restricted pRS143 (41). Plasmid pS005 contains the MRD1 ORF, amplified by PCR using primer pairs of the MRD-GFP-5 and MRD-GFP-3 (Table II) using plasmid pS005 as a template, inserted into NotI and PstI-restricted pBFG1/HA-GFP (42). A PstI- and NotI-flanked fragment encoding N-terminal HA-tagged Mrd1p, amplified by PCR using primer pair Gal-MRD-5 and Gal-MRD-6 (Table II) and pS001 as template, was inserted into PstI-NotI restricted B2202, creating plasmid pS006.

**Immunoelectron Microscopy**—Strain LWY005 was grown to late log phase and treated with 50 μg/ml Zymolyase 100T (Seikagaku Corporation). Spheroplasts were fixed in 2% parafomaldehyde and 0.2% glutaraldehyde in PBS buffer (43). Sections were postfixed in 2% paraformaldehyde and 0.2% glutaraldehyde in PBS buffer and embedded in 70% ethanol for 1 h and immersed in LR White Resin (London Resin) at 50 °C for 3 days. The thin sections were blocked with 3% bovine serum albumin in PBS for 45 min followed by incubation with primary antibodies (anti-HA (12CA5), diluted 1:1, and anti-Nop1p, diluted 1:100) with 1% bovine serum albumin in PBS for 1 h. The sections were washed in PBS and incubated for 30 min with secondary antibody conjugated to 10-nm gold (AbD Serotec). Sections were postfixed in 2% osmium tetroxide, stained with 5% uranyl acetate, and photographed in a Zeiss electron microscope at 80kV.

**In Vivo Depletion of Mrd1p**—The effect of diminished MRD1 expression was monitored in strain LWY008 expressing pGAL-HA-MRD1 (pS005) as described (43). Cell growth was monitored by measuring the optical density at 600 nm over a period of 36 h. Preparations of protein and RNA were obtained from cells harvested at the times indicated after the shift to glucose-based medium. For each time point, an equal amount of cells was used.

**Analysis of Pre-rRNA Processing**—Pulse-chase analysis of rRNA processing was done according to Ref. 44. Briefly, strain LWY008 expressing pGAL-HA-MRD1 (pS006) was grown in SGal to an A600 of 1. Half of the cells were resuspended in SGal, and the other half were resuspended in SD medium, and the cultures were incubated for 24 h at 30 °C. The cells were concentrated and pulse-labeled for 2 min with 250 μCi of [methyl-3H]methionine, (PerkinElmer Life Sciences) and chased for 1, 3, and 10 min by addition of cold methionine to a final concentration of 5 mM. RNA was extracted using the hot phenol method (45), and aliquots containing 40,000 cpm were electrophoresed through 1% agarose gels.
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**TABLE II**

| Name       | Description or sequence                  |
|------------|------------------------------------------|
| pS001      | MRD1 in pRS316                           |
| pS002      | Not::SalI fragment containing mrd1::HisG-URA3-kan::HisG in pRS413 |
| pS003      | Not::SalI fragment containing mrd1::HIS3 in pRS413 |
| pS004      | HA-MRD1 in pRS413                        |
| pS005      | HA-MRD1-GFP in pRBS::I3A::GFP           |
| pS006      | pGal1::HA-MRD1 in RPL202                 |

**Plasmids and oligonucleotides**

- **MRD1-** 5′-CCACGCCGCCCAAGAAGCATTGTCGCCAGTAC-3′
- **MRD1** 5′-CCAGTACGCAAGTTGATGCTGACCTACG-3′
- **mrd1** 5′-CCACGCCGCCCAAGAAGCATTGTCGCCAGTAC-3′
- **mrd1** 5′-CCAGTACGCAAGTTGATGCTGACCTACG-3′
- **HA-MRD1** 5′-CCACGCCGCCCAAGAAGCATTGTCGCCAGTAC-3′
- **HA-MRD1** 5′-CCAGTACGCAAGTTGATGCTGACCTACG-3′
- **mrd1** 5′-CCACGCCGCCCAAGAAGCATTGTCGCCAGTAC-3′
- **mrd1** 5′-CCAGTACGCAAGTTGATGCTGACCTACG-3′
- **HA-MRD1** 5′-CCACGCCGCCCAAGAAGCATTGTCGCCAGTAC-3′
- **HA-MRD1** 5′-CCAGTACGCAAGTTGATGCTGACCTACG-3′
- **HA-MRD-C** 5′-ATCGCCGTCACGGATGCGTACATG-3′
- **p-rNA-1** 5′-GCCGCTCTCGGTGGCGG-3′
- **p-rNA-2** 5′-GGCCGCTCTCGGTGGCGG-3′
- **p-rNA-3** 5′-ATGGGAAATCCACCTAGTG-3′
- **p-rNA-4** 5′-CCAGTACGCAAGTTGATGCTGACCTACG-3′
- **p-rNA-5** 5′-CCAGTACGCAAGTTGATGCTGACCTACG-3′
- **p-rNA-6** 5′-CCAGTACGCAAGTTGATGCTGACCTACG-3′
- **U5** 5′-TAGCTTCTGGCTCGG-3′
- **U14** 5′-CCACGATGCTGTCACGGG-3′
- **U18** 5′-GCCGCTCTCGGTGGCGG-3′
- **snR11** 5′-GGCCGCTCTCGGTGGCGG-3′
- **snR190** 5′-GGCCGCTCTCGGTGGCGG-3′

Agarose-formaldehyde gels, transferred to nylon membranes, sprayed with ENHANCE (PerkinElmer Life Sciences), and exposed to x-ray film.

Northern blot hybridizations were performed as described (46). For analysis of snoRNAs, 5 μg of total RNA was electrophoresed through 6% polyacrylamide-7M urea gels. The RNA was electroblotted to Zeta/probe membranes and probed with 32P-labeled oligonucleotides U3, U4, U12, U20, U31, and U44 films.

**RESULTS**

**The MRD1 Gene Is Essential for Cell Viability**—We have recently identified a gene in the dipteran *Chironomus tentans* (CTE314912) called Ct-RBD-1, which contains six conserved RNA-binding domains. The presence of these RBDS is unusual, and Ct-RBD-1 appears to be involved in ribosomal synthesis and/or function. Based on sequence similarity with Ct-RBD-1, we have identified a gene in *S. cerevisiae*, ORF YPR112c on chromosome XVI, which encodes a protein with five RBDS (Fig. 1A). We have named this previously uncharacterized gene *MRD1*. Mrd1p is comprised of 887 amino acid residues, and in addition to the five RBDs, Mrd1p contains several putative nuclear localization sequences. Computer searches identified Mrd1p homologues, each with a similar domain architecture, in *Saccharomyces pombe* (O13620), *Caenorhabditis elegans* (Q9XU67), *Drosophila melanogaster* (Q9VTF9), and human (Q9UFN5). The *S. pombe* homologue contains five RBDS, whereas the *C. elegans*, *D. melanogaster*, *C. tentans*, and human proteins all have six RBDS (Fig. 1B).

To characterize the function of Mrd1p, we constructed a *mrd1* null allele by replacing the ORF in one of the chromosomal copies of *MRD1* with *URA3* in the diploid strain HKDY8. Subsequent tetrad analysis showed a 2:2 segregation for viability, and no *Ura* spore-derived colonies were recovered, indicating that the *MRD1* gene is essential. Microscopic analysis revealed that the *mrd1Δ* spores germinated, but cell division stopped after three to four generations. To confirm the essential nature of *MRD1*, the heterozygous LWYD3 strain containing one wild-type *MRD1* gene and one disrupted allele marked with *LEU2* was transformed with a *URA3* centromeric plasmid (pS001) carrying the *MRD1* gene under its cognate promoter. This strain was sporulated, and tetrad analysis showed that plasmid-borne *MRD1* was able to support wild-type growth in the cells containing the *mrd1 null* allele; *Leu* "Ura" spores were recovered at the expected frequency.

**Mrd1p Co-localizes with the Nucleolar Protein Nop1p and Is Also Present in the Nucleoplasm**—To determine the intracellular localization of Mrd1p, we constructed an allele of *MRD1* (pS005) that is under the control of the phosphoglycerate kinase promoter and that encodes a Mrd1p fusion protein with an N-terminal HA and a C-terminal GFP. The HA-Mrd1p-GFP protein complemented *mrd1* null alleles and thus is functional. The HA-Mrd1p-GFP fusion protein is predominantly in the nucleus and excluded from both the vacuoles and the cytoplasm (Fig. 2A). In contrast, in cells with the plasmid pBFG-1/HA-GFP alone, the green fluorescence was spread throughout the cytoplasm (Fig. 2C), consistent with previous reports (42). As compared with DAPI staining (Fig. 2B), it was evident that HA-Mrd1p-GFP was present throughout the nucleus but that a more strongly stained crescent pattern was present within the fluorescent nucleolus. This pattern suggests that Mrd1p localizes to the nucleolus.

To more precisely determine the subcellular localization of Mrd1p, a functional N-terminal HA-tagged Mrd1p was expressed under its cognate promoter in the CEN vector pRS413 (pS004). The HA-Mrd1p was localized by immunoelectron mi-

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*P. Björk, G. Banrén, S.-B. Jin, Y.-G. Tong, T. R. Bürglin, U. Hellman, and L. Wieslander, in preparation.*
The immunolabeling was found throughout the nucleus including the nucleolus (Fig. 2D). Little if any labeling was detected in the cytoplasm. Double labeling confirmed that Mrd1p is present in the nucleolus with an overlapping distribution of the nucleolar marker Nop1p (Fig. 2E). Combined, our data show that Mrd1p is a nuclear protein that is enriched in the nucleolus.

**In Vivo Depletion of Mrd1p Impairs Growth and Decreases the Level of Mature 18 S rRNA**—To determine the function of the *MRD1* gene, we analyzed the effect of depletion of Mrd1p by using a conditional system for phenotypic analysis. The N-terminal HA-tagged *MRD1* ORF was placed under the control of a galactose promoter (pS006). On YPGal, the pGal-HA-Mrd1p allele complements the *mrd1* null mutation in strain LWY008, indicating that the HA-Mrd1p is functional.

In YPGal liquid medium, strain LWY008 showed a doubling time of 2.2 h. Following transfer to YPD medium, in which HA-MRD1 expression is repressed, the growth rate remained similar to that of control cells in YPGal for the first 12 h. Thereafter the doubling time began to increase significantly. After 30 h in YPD, the generation time increased to 6 h (Fig. 3A). Concomitant with the decrease in the growth rate, the amount of HA-Mrd1p was clearly diminished after 6 h of incubation in the YPD medium, became very low after 9 h, and was undetectable after 12 h (Fig. 3B). In contrast, the expression level of Nop1p remained constant during growth in YPD medium for the indicated times, resolved by electrophoresis, blotted to a nylon membrane, and stained with 0.03% methylene blue.

The cessation of growth caused by reduced levels of Mrd1p was reversible. The viability of cells grown in YPD media for 20, 30, or as long as 40 h was examined. Around 95, 90, and 80% of the cells, respectively, began dividing again and formed...
When plated on YPGal media. No colonies formed when cells were plated on YPD media.

We determined the steady-state levels of rRNAs following Mrd1p depletion and found that the amount of 18 S rRNA was reduced dramatically, whereas the abundance of the 25 S rRNAs was not affected (Fig. 3D). It is unlikely that the decreased steady-state level of 18 S rRNA was due to the effect of cell death because (a) at least more than 90% of cells grown in YPD medium were still viable at the relevant time points based on the cell viability test and (b) the 5.8 S rRNA levels were not affected (data not shown). Further, the levels of Nop1p did not change during the experiment (Fig. 3C). These observations indicate that Mrd1p could have a direct role in 18 S rRNA metabolism.

Mrd1p Depletion Leads to a Deficiency of 40 S Ribosomal Subunits—Since Mrd1p is located in the nucleolus and its depletion leads to a decrease in cell growth and a reduction in the level of 18 S rRNA, we explored the possible function of Mrd1p in ribosome biogenesis. In Fig. 4, it is demonstrated that depletion of Mrd1p leads to drastic changes in the polysome profile. As compared with the profile in cells expressing Mrd1p (Fig. 4A), there is a gradual decrease in polyribosomes and 80 S monosomes with time (Fig. 4, B and C). Simultaneously, there is a decrease in the amount of 40 S ribosomal subunits and a relative increase in 60 S ribosomal subunits. The 40 S ribosomal subunit deficit was further examined by analysis of the total amount of ribosomal subunits (Fig. 4D). The A260 60S:40S ratio for the LWY008 strain increased drastically after 24 h in YPD medium (Fig. 4E). Taken together, these data
show that depletion of Mrd1p leads to a decrease of 40 S ribosomal subunits.

18 S rRNA Synthesis Is Impaired upon Mrdp1 Depletion—To determine whether the reduction in the levels of 18 S rRNA and 40 S ribosomal subunits following the depletion of Mrd1p was due to defects in pre-rRNA processing, the pre-rRNA processing pathway was directly followed by using pulse-chase labeling analysis (Fig. 5; see also Fig. 6A for a schematic view of the pre-rRNA processing). In the cells expressing the MRD1 gene, the 35 S pre-rRNA was rapidly processed to yield 27 and 20 S pre-rRNA intermediates as detected after 1 min of chase (Fig. 5, $\text{SGal}^{-}$). After 3 min of chase, the 35 S precursor disappeared, and most of the 27 and 20 S intermediates were processed to 25 S rRNA and 18 S rRNA. The processing appeared to be completed within 10 min since all of the labeling was chased into mature 25 and 18 S rRNAs. However, in the Mrd1p-depleted cells, processing is slowed down with an increased amount of the 35 S precursor (Fig. 5, $\text{SD}^{-}$). After 3 min of chase, the 35 S precursor disappeared, and most of the 27 and 20 S intermediates were processed to 25 S rRNA and 18 S rRNA. The processing appeared to be completed within 10 min since all of the labeling was chased into mature 25 and 18 S rRNAs. However, in the Mrd1p-depleted cells, processing is slowed down with an increased amount of the 35 S precursor (Fig. 5, $\text{SD}^{-}$). After up to 3 min of chase, a significant amount of 35 S precursor was still present. Processing into 27 S pre-rRNA intermediates and 25 S rRNA was not affected. In contrast, processing into the 20 S pre-rRNA intermediate and 18 S rRNA was severely impaired. Thus, accumulation of 35 S pre-rRNA followed by reduced formation of the 20 S pre-rRNA intermediate leads to a net decrease and delay in 18 S rRNA production in the Mrd1p-depleted cells. This is consistent with our observations from the ribosomal gradient analysis that the amount of 40 S subunits decreased.

Mrd1p Is Required for the Initial Processing of Pre-rRNA at the A₀–A₃ Sites—To assess the role of Mrd1p in pre-rRNA processing in more detail, we analyzed the steady-state levels of the pre-rRNA processing intermediates upon depletion of Mrd1p. The yeast pre-rRNA processing pathway and the locations of the oligonucleotide probes used to detect 5′-ETS, -ITS1, and -ITS2 are shown in Fig. 6A. Equal amounts of total RNA prepared from the LWY008 strain (harvested after growth for 0, 16, 24, and 30 h in YPD medium) were subjected to Northern blot analysis (Fig. 6B). When probe 1, which hybridizes to a sequence upstream of the A₀ site, was used, little 35 S was detected in the control RNA extract (Fig. 6B, probe 1, lane 1; see also probes 2–4, lane 1), consistent with the known rapid processing of this precursor in wild-type cells. In the Mrd1p-depleted cells, the amount of the 35 S pre-rRNA increased. This accumulation was also detected with all probes that could detect the 35 S pre-rRNA precursor (Fig. 6B, probes 2–4, lanes 2–4), showing the inhibition of cleavage at site A₀. Meanwhile, we also detected an increase of the 23 S aberrant pre-rRNA intermediate (Fig. 6B, probe 1, lanes 2–4), which is the product of cleavage of the 35 S pre-rRNA at site A₂ in the absence of prior cleavage at sites A₀–A₃ (48). The 23 S aberrant pre-rRNA intermediate could also be seen when hybridizing with probes 2 and 3 at the 5′-side of A₂ and A₃ cleavage sites, respectively (Fig. 6B, probes 2 and 3, lanes 2–4). We also detected a decrease in the amount of 20 S (Fig. 6B, probe 2), which is consistent with the inhibition of cleavage at A₂. Hybridization with probe...
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**DISCUSSION**

**Mrd1p Together with Several Other Components Contribute to the Initial Processing of Pre-rRNA**—The MRD1 gene is essential for cell growth. We demonstrated that Mrd1p is required for maintaining steady-state levels of the 40 S ribosomal subunit. Analysis of the effect of Mrd1p depletion showed that the 35 S pre-rRNA and the aberrant 23 S pre-rRNA intermediate increased and that the levels of the 20 S pre-rRNA intermediate and 18 S rRNAs were reduced. This is characteristic of a specific inhibition of processing at the A0–A2 sites. The aberrant 23 S pre-rRNA intermediate cannot be further processed to 18 S rRNA and is known to be rapidly degraded by the A0–A2 sites.

**Impaired pre-rRNA processing at the A0–A2 processing sites is also observed after depletion of the snoRNAs U3, U14, and snR10 and several nucleolar proteins.** We therefore used co-immunoprecipitation to assay whether Mrd1p is associated with some of these factors. Extracts from cells expressing the HA-Mrd1-GFP fusion protein were immunoprecipitated with anti-GFP antibodies. The immunoprecipitated RNA was separated on 6% polyacrylamide gels and probed with oligonucleotides specific to several box (C/D) (U3, U14, U18, snR190) snoRNAs and the box (H/A)CA (snR11) snoRNAs. Only U3 snoRNA specifically co-immunoprecipitated with the Mrd1 fusion protein (Fig. 7C). The same result was obtained when anti-HA antibody was used for immunoprecipitation (data not shown). We also showed that the U3 snoRNA protein Nop1 could be co-immunoprecipitated with Mrd1p (Fig. 7D).

**Finally, we tested the stability of the U3, U14, U18, snR11, and snR190 snoRNAs following Mrd1p depletion (Fig. 8). The hybridization signals from Mrd1p-depleted cells were not different from those seen from cells grown in galactose medium. This suggests that the effect of Mrd1p depletion is not due to a decrease in the amount of snoRNAs.**

**Fig. 8. Levels of U3 snoRNA and several other snoRNAs are not affected following depletion of Mrd1p.** Total RNA was extracted from LWY008 cells grown in YPD for the indicated times and separated on 6% polyacrylamide gels. After electro blotting to a nylon filter, the RNA was hybridized with oligonucleotide probes specific for the indicated snoRNAs.

**Fig. 7. Mrd1p is associated with 35 S pre-rRNA, Nop1p, and U3 snoRNA.** A, a nuclear extract from LWY008 cells, expressing HA-Mrd1p, was centrifuged through a 10–40% sucrose gradient. 35 S pre-rRNA and Mrd1p were both located in 80–90 S particles by Northern and Western blotting, respectively. In B–D, whole-cell extracts from LWY007 cells, expressing HA-Mrd1p-GFP, immunoprecipitated with or without anti-GFP antibodies. In panel B, 35 S pre-rRNA was detected by Northern blot analysis using oligonucleotide probe 2 (see the legend for Fig. 6) after immunoprecipitation with the anti-GFP antibody (lane 2) but not when the anti-GFP antibody was left out (lane 2). Total RNA is shown as a size control (lane 1). In panel C, the immunoprecipitated RNA was electrophore blotted and probed with oligonucleotide probes specific for the indicated snoRNAs. U3 snoRNA was specifically precipitated with the anti-GFP antibody (lane 2) but not in controls without anti-GFP antibodies (lane 3). The positions of the different snoRNAs are shown in total RNA (lane 1). In panel D, immunoprecipitated proteins were analyzed by Western blotting. Nop1p was detected when anti-GFP antibodies were used (lane 3) but not when anti-GFP antibodies were left out (lane 2). Nop1p in a total cell extract is shown in lane 1.

4, specific for the precursor containing 5.8 and 25 S rRNAs, showed that the cleavages at the A0 and downstream sites were not affected upon Mrd1p depletion (Fig. 6B, probe 4).

Based on these results, we conclude that depletion of Mrd1p leads to increased levels of 35 S pre-rRNA and the 23 S aberrant pre-rRNA intermediate. These effects are concordant with impaired pre-rRNA processing at sites A0, A1, and A2. Consistently, we observed decreased levels of the 20 S pre-rRNA intermediate and 18 S rRNA. Although our results are indicative of severely impaired processing, some 20 S pre-rRNA intermediate and 18 S rRNA were still produced, and 35 S and the aberrant 23 S pre-rRNA intermediate did not accumulate to very high levels.

**Mrd1p Is Associated with the 35 S Pre-rRNA and U3 snoRNA—**Our results show that Mrd1p is involved in the initial cleavages of the 35 S pre-rRNA. If so, Mrd1p is most likely physically associated with the pre-rRNA precursor. In Fig. 7A, it is shown that 35 S pre-rRNA and Mrd1p are both present in 80–90 S particles in the nucleus. As a further test, we immunoprecipitated cell extracts from cells expressing the HA-Mrd1p-GFP fusion protein with anti-GFP or anti-HA antibodies. In Fig. 7B, it is shown that 35 S pre-rRNA could be detected in the immunoprecipitated Mrd1p complex. These results suggest that Mrd1p interacts with the 35 S pre-rRNA and are consistent with the fact that Mrd1p is necessary for efficient cleavage at the A0–A2 cleavage sites.
exosome (49). The inhibition of cleavage at the A₀–A₂ sites was significant, but some 18 S rRNA could still be made. The fact that we did not detect any changes in the steady-state levels of the U3, U14, U18, snR11, and snR190 snoRNAs showed that the effect of Mrd1p depletion is not due to a defect in the synthesis or stability of these snoRNAs. Rather, our results showing that Mrd1p is associated with the 35 S pre-rRNA in the 80–90 S pre-rNP particle suggest a more direct role for Mrd1p in pre-rRNA processing.

Mrd1p is co-immunoprecipitated with U3 snoRNA (and the U3 snoRNP-associated Nop1p). It is established that U3 snoRNA base-pairs to the 35 S pre-rRNA at the 5′-ETS and also at the 5′-stem loop within the 18 S rRNA. These interactions are needed for the early A₀–A₂ cleavages and A₁ and A₂ cleavages, respectively (11). Our immunoprecipitation results therefore are in agreement with the interpretation that Mrd1p and the U3 snoRNA are both bound to the 35 S pre-rRNA precursor. Although it is likely that these interactions occur in a region of the 35 S rRNA precursor containing the 5′-ETS and 18 S rRNA, our data do not rule out other alternatives.

Several components have been shown previously to be necessary for processing at the A₀–A₂ sites. These include the U3, U14, snR30, and snR10 snoRNAs and the snoRNP proteins, Nop1p, Sof1p, and Gar1p (16, 21, 50–54), the putative helices F1p1p (44), Rrp3p (55), and Rok1p (56), the dimethylase Dim1p (57), and the nucleolin-like Nsr1p (58). Mutations in different components have shown that processing at the three sites is interconnected but not necessarily coupled (57, 59).

Many different components are involved in all three processing events, indicating that the A₀, A₁, and A₂ cleavages take place in a multi-snoRNP complex (60). Processing at the A₀–A₂ sites is also coupled to cleavage at the A₀ site, suggesting that contacts between processing factors can bridge longer distances in a large pre-rRNA-protein complex (61, 62). The structure of Mrd1p and its association with the 35 S pre-rRNA and U3 snoRNA, combined with its involvement in the processing of Mrd1p and its association with the 35 S pre-rRNA and U3 snoRNA are both bound to the 35 S pre-rRNA precursor. Therefore are in agreement with the interpretation that Mrd1p is associated with the 35 S pre-rRNA in a large pre-rRNA-protein complex (60). Processing at the A₀, A₁, and A₂ sites was shown to be necessary for the formation of specific rRNA tertiary structures; thus, these proteins inhibit other possible alternative base pairings that can kinetically trap rRNA molecules in inactive conformations (68). Based on the current understanding of Mrd1p, we suggest that Mrd1p binding to pre-rRNA facilitates the proper folding of rRNA for cleavage at the A₀–A₂ processing sites. Mrd1p has a similar sequence organization to proteins in several eukaryotic organisms (see “Results”), and it has a similar nuclear localization to proteins in Diptera and human. This suggests that Mrd1p is evolutionarily conserved in eukaryotes and that the functional properties of Mrd1p revealed here are of general importance.

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