MOLECULAR AND CELLULAR BIOLOGY, Oct. 2006, p. 7437–7450 Vol. 26, No. 20
0270-7306/06/$08.00+0 doi:10.1128/MCB.00664-06

The Helicase Has1p Is Required for snoRNA Release from Pre-rRNA▽

Xue-hai Liang and Maurille J. Fournier*

Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, Massachusetts 01003

Received 17 April 2006/Returned for modification 2 June 2006/Accepted 4 August 2006

Synthesis of rRNA in eukaryotes involves the action of a large population of snoRNA-protein complexes (snoRNPs), which create modified nucleotides and participate in cleavage of pre-rRNA. The snoRNPs mediate these functions through direct base pairing, in many cases through long complementary sequences. This feature suggests that RNA helicases may be involved in the binding and release of snoRNPs from pre-rRNA. In this study, we determined that the DEAD box helicase Has1p, a nucleolar protein required for the production of 18S rRNA, copurifies with the snR30/U17 processing snoRNP but is also present with other snoRNPs. Blocking Has1p expression causes a substantial increase in snoRNPs associated with 60S-90S preribosomal RNP complexes, including the U3 and U14 processing snoRNPs and several modifying snoRNPs examined. Cosedimentation persisted even after deproteinization. This effect was not observed with depletion of two nonhelicase proteins, Esf1p and Dim2p, that are also required for 18S rRNA production. Point mutations in ATPase and helicase motifs of Has1p block U14 release from pre-rRNA. Surprisingly, depletion of Has1p causes a reduction in the level of free U6 snRNP. The results indicate that the Has1p helicase is required for snoRNA release from pre-rRNA and production of the U6 snRNP.

Among the *trans*-acting factors required to make eukaryotic ribosomes are scores of small nucleolar RNA-protein complexes (snoRNPs) that modify rRNA nucleotides, several modifying enzymes that alter additional rRNA nucleotides, approximately 80 ribosomal proteins, a few "processing" snoRNPs required directly or indirectly for pre-rRNA cleavage reactions, several endo- and exonucleases for cutting and trimming pre-rRNAs, at least 19 putative RNA helicases, ribosome assembly factors, and other proteins (8, 11, 13, 30, 37). The snoRNPs and a particular helicase associated with these complexes are the focus of the present study.

The snoRNP complexes are classified along both structural and functional lines but named on the basis of conserved structural features of the snoRNA component. In particular, there are two major families of snoRNAs and snoRNPs that account for all but one snoRNP involved in ribosome synthesis. These groups are designated the box C/D and box H/ACA families (2, 4, 9, 25); the exception is the MRP snoRNP. Each snoRNP in the major families contains a single C/D or H/ACA snoRNA and a set of four core proteins that are also family specific. Some snoRNPs, including the MRP species, contain additional proteins. The core C/D proteins are Nop1p/fibrillarin, Nop58p, Nop56p, and Snu13p/15.5K, and the H/ACA proteins include Cbf5p/dyskerin, Gar1p, Nop10p, and Nhp2p (2, 14).

Nearly all of the snoRNPs in the two major families function in nucleotide modification, 2′-O-methylation of ribose moieties (Nm) in the case of the C/D snoRNPs (26, 50), and conversion of uridine to pseudouridine (Ψ) by the H/ACA snoRNPs (15, 38). The number of modifying snoRNPs in a cell reflects the number of rRNA modifications but not precisely, as many snoRNPs modify two sites and other RNAs. Both of the major snoRNP families include members needed for endonucleolytic reactions, in most cases for production of 18S rRNA. The MRP snoRNP has this function, too, for cleavage at a site between the small- and large-subunit rRNAs (34, 44). While snoRNPs are referred to as being of the modifying or processing variety, a few have both functions. In the yeast *Saccharomyces cerevisiae*, the organism featured here, approximately 75 snoRNPs participate in ribosome biogenesis.

In mediating their functions, both the modifying and processing snoRNPs interact directly with pre-rRNA through sequences complementary to the snoRNA. The snoRNAs present in the modifying snoRNPs target the snoRNP to the rRNA segment to be modified. Methylation is guided by single sequences of 10 to 21 nucleotides (nts) (26) and pseudouridylation by two short elements of 4 to 8 nucleotides that flank the uridine target (15, 38). Both types of modification are catalyzed by a core protein, Nop1p/fibrillarin in the case of methylation and Cbf5p/dyskerin for pseudouridine formation (14). Guide snoRNAs that act at two (or more) sites contain guide sequences in two different snoRNA domains.

The complementarities identified thus far for the processing snoRNAs range from 7 to 13 nts (33, 37, 46). Five processing snoRNPs in yeast are known: U3, U14, snR30/U17, snR10, and MRP. All but one of these snoRNPs (snR10) are present in animals and other organisms; vertebrates contain two additional processing snoRNPs (U8 and U22) (40, 49). Genetic depletion in yeast has shown the four conserved snoRNPs to be essential for ribosome synthesis and cell growth. Depleting these individually blocks particular cleavages in pre-rRNA and, in some cases, the same cleavage, indicating that subsets of snoRNPs act in concert in certain reactions. Among the processing snoRNPs, the U3 species has a special role. It binds to pre-rRNA early during transcription and corresponds to the terminal "knob" or "ball" observed at the 5′ end of growing transcripts. Affinity-isolated yeast U3 complexes have been shown to be associated with more than 30 proteins (10). These

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, Lederle Graduate Research Center, University of Massachusetts, Amherst, MA 01003. Phone: (413) 545-2732, ext. 0566. Fax: (413) 545-3291. E-mail: 4nier@biochem.umass.edu.

▽ Published ahead of print on 14 August 2006.

include transcription factors, indicating links between the processing and transcription machineries (18).

The mechanisms by which the snoRNPs enter into and leave the pairings with rRNA are unknown. Making and breaking the snoRNA-rRNA bonds pose both energetic and topological challenges, especially so for the methylation guide snoRNAs, in which perfect complementarity usually exists over the full-length of the 10- to 21-nt guide sequence. Results from mutational analysis of a vertebrate snoRNA indicate that methylation activity in vivo requires a minimum complementarity of 12 bp (7), slightly more than the length of the smallest guide sequences known. In yeast, the average methylation guide sequence is 13 nts. The nature of the actual interaction of the guide sequence with the substrate is not known precisely; however, the conserved sizes of the complementarities infer that substantial base pairing occurs during the modification process.

Consistent with the strong binding predicted, the complexes formed by both the modifying and processing snoRNAs with pre-rRNA persist during fractionation of cell extracts by ultracentrifugation (19, 22, 53). Taken together, these properties support the view that RNA helicases likely play important roles in the binding or release of snoRNPs from pre-rRNA substrates. Indeed, one DEAD box helicase (Dbp4) required for processing of 18S rRNA was recently found to be involved in the release of U14 and a methylation snoRNA (snR41) from pre-rRNA complexes (27).

Here, we report similar but more-extensive effects for another DEAD box helicase required for processing of 18S rRNA, called Has1p. Our attention was drawn to Has1p because we found it present in affinity-isolated preparations of the snR30/U17 processing snoRNP that is also required for processing of 18S rRNA. In addition, we found Has1p in an affinity-selected modifying snoRNP that acts on 25S rRNA. To determine whether Has1p has a role in snoRNP release from preribosomal RNP (pre-rRNP) complexes, we blocked its expression and found that dissociation of all eight snoRNAs examined was blocked as well. The strongest effects were observed for the U3 and U14 processing snoRNPs, which persisted in 60S-90S pre-rRNP complexes and were enriched ~6-fold more than wild-type complexes. Surprisingly, depletion of Has1p also impaired the accumulation of U6 snRNA.

MATERIALS AND METHODS

Strains. The test strains used are as follows: JDY10-3B[pAS24-Has1] (for Has1p), *MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* Has1::*HIS3* [pAS24-HAS1, *LEU2*, Gal promoter] (a gift from Patrick Linder [11]); TH_2070 (for Esf1p), *MAT***a** *URA3*::CMV-tTA *his3-1 leu2-0 met15-0* Esf1::Kan^r-tetO$_7$-TATA promoter (41); R1158 (wild-type control for Esf1p), *MAT***a** *URA3*::CMV-tTA *his3-1 leu2-0 met15-0* (a gift from Timothy R. Hughes); YO595 (for Dim2p), *MAT*α *ura3-52 leu2*Δ*1 trp1 his3*Δ*200* Dim2::*KANMX4* (pGal::ZZ-DIM2P *LEU2*) (a gift from Denis L. J. Lafontaine [51]); YS602 (wild-type control strain), *MAT*α *ade2-101 his3-11,15 trp1901 ura3-52 leu2-3,112*; YXL025, *MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* Has1::*HIS3* [pAS24-HAS1, *LEU2*, Gal promoter] [PXL025, *URA3*]; YXL024, *MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* Has1::*HIS3* [pAS24-HAS1, *LEU2*, Gal promoter] [PXL024, *URA3*]; and YXL028, *MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* Has1::*HIS3* [pAS24-HAS1, *LEU2*, Gal promoter] [PXL028, *URA3*].

Hybridization probes and targets. The sequences of the hybridization probes and targets used in this study are as follows: XL002, 5′-ATCTCTTTGTAAAA CGGTTCATCC-3′ (for U6 snRNA); XL007, 5′-TAGTTCAATGATAAATGC TTAACG-3′ (for U1 snRNA); XL017, 5′-GTAATGAGCCTCATTGAGGT-3′ (for U2 snRNA); XL018, 5′-CATAGTCTTGAAGTATTCAA-3′ (for U4 snRNA); XL019, 5′-AGTAACGGACAGCTTTACCT-3′ (for U5 snRNA); BLO308, 5′-AGATGTCTGCAGTATGGTTTTACCCAAATG-3′ (for snR30); BLO370, 5′-ACTGTCTAATGCGGTGGATTAGTACTTTAG-3′ (for snR3); BLO234, 5′-ACTCAGACATCCTAGGAAGGTC-3′ (for U14); BLO293, 5′-T CAACCTCGACGAGATACTGTACGTGTTAC-3′ (for snR10); E225, 5′-ACG ATACCTGAGCTTGAATCAGGC-3′ (for $tRNA_3^{Leu}$); QL008, 5′-CAGTGTA ACTATGACTATAAGG-3′ (for snR63); QL004, 5′-GAATAATGTGTCTCTT TGAGTCATGTTCCTTAGCTTCTTTTTTAAAAAAACTGTGCGGTATTT CACACCG-3′ (for snR84); QL018, 5′-CACTAAAGAACCATAATGCC-3′ (for snR70); QL022, 5′-AAACGTATCAACATATACTCG-3′ (for snR77); DPO10, 5′-AGGAGAGCTCTGGTTAACTTGTCAGACTGCCATTTGTACC CACCCATAG-3′ (for U3); E227, 5′-GTAAAAAAGAAAGAAACCGAAAT C-3′ (for pre-rRNA); BLO46, 5′-CAGTAAAAGCTCTTTGCTCTTGCC-3′ (for pre-rRNA, used for reverse transcription [RT]-PCR); and BLO48, 5′-CGT CGCTAGTACCGATTGAATGGC-3′ (for rRNA, used for RT-PCR).

Construction of Has1p mutations. Point mutations of Has1p were created by a two-step PCR approach. A sense oligonucleotide specific to a sequence upstream of Has1p (position −500) and an antisense oligonucleotide complementary to a sequence downstream of the Has1p coding region (position +300) were used to amplify the wild-type Has1p gene from the genome. The product was cloned into XhoI and SacI sites of the pYes2 plasmid, with a Myc tag at the N terminus of Has1p, resulting in plasmid PXL025. Point mutations K92A (AAA to GCT) and H375E (CAT to GAA) were created in motifs I and VI of Has1p, using PXL025 as a template. The final PCR product was inserted into XhoI and SacI sites of pYes2, resulting in plasmids PXL024 and PXL028, respectively. The plasmids PXL025, PXL024, and PXL028 were transformed into yeast strain JDY10-3B[pAS24-Has1] (11) and selected with the *URA3* marker, generating strains YXL025, YXL024, and YXL028, respectively. In these test strains, the chromosomal Has1 gene is disrupted, but two copies of the gene are on separate plasmids. One expresses HA-tagged wild-type Has1p under galactose control and can be shut off; the other constitutively expresses either mutant or normal Has1p, with a Myc tag under the control of its own promoter.

Preparation of cell extracts. For affinity selection, cells expressing tandem affinity purification (TAP)-tagged Gar1p, Myc-tagged U1A protein, and a specific snoRNA tagged with the U1 snRNA hairpin II were grown in galactose medium (to an optical density at 600 nm of ~2), harvested, washed twice with ice-chilled water, and resuspended in buffer A (25 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 100 to 150 mM KCl [as indicated], 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, and one tablet of protease inhibitor cocktail/50 ml [Roche]). Glass beads were added, and cells were broken by vigorous vortex mixing. Debris was removed by centrifugation at 2,570 × *g* for 10 min, and the crude extract was further cleared by centrifugation at 30,000 rpm for 1 h using a Beckman 60 TI rotor. This extract was used for affinity selection of snoRNP complexes.

In vivo protein depletions were performed as described elsewhere (11, 41, 51). Briefly, for the Has1p and Dim2p test strains, cells were grown in yeast extract-peptone-galactose medium for 14 h and washed with distilled water. To deplete the target protein, cells were shifted to yeast extract-peptone-dextrose medium and incubated at 30°C for 20 h; previous work showed that a significant amount of Has1p and Dim2p could be detected 10 h after the shift to glucose medium, and strong processing defects were observed 12 to 24 h after this shift (11, 51). As a control, a portion of the cells was maintained in yeast extract-peptone-galactose medium under the same condition. The Esf1p test strain, TH_2070, and a control wild-type strain (R1158) were first grown in yeast extract-peptone-dextrose medium and then diluted to an optical density at 600 of 0.1, and 10 μg/ml doxycycline (Sigma) was added to shut off expression of Esf1p. Incubation was continued at 30°C for 20 h. Cells were harvested and washed twice with ice-cold water. Buffer A without glycerol was then added to the cell pellet to adjust the concentration to 1 g/ml. Prechilled glass beads were added, and the cells were broken by vigorous vortex mixing. After centrifugation at 2,570 × *g*, 4°C, for 10 min, the supernatant was further clarified by centrifugation at 12,000 × *g*, 4°C, for 20 min. These extracts were used for subsequent RNA and protein analyses. For deproteinization experiments, extracts were prepared with buffer A lacking glycerol and containing 100 mM NaCl instead of KCl. This material was treated with proteinase K (2-mg/ml final concentration) and sodium dodecyl sulfate (SDS) (1% final concentration) at 16°C for 15 min and then stored on ice for 5 min, followed by centrifugation at 11,000 × *g* for 1 min, as described previously (48). This supernatant was used for ultracentrifugation analysis.

Affinity selection. A two-step affinity selection approach that will be described in detail elsewhere was used to select specific snoRNP complexes (D. Pieknа-Przybylska, B. Liu, and M. J. Fournier, unpublished data). Briefly, a core snoRNP protein was tagged with the TAP sequence and the snoRNA was tagged

with the human U1 hairpin II sequence. The entire family of snoRNPs was selected through the TAP-tagged protein, and the specific snoRNP was subsequently isolated using the snoRNA tag. Extracts prepared from 20 g of cells with buffer A containing 100 mM KCl were incubated with 400 μl immunoglobulin G beads (Amersham) at 4°C for 4 or 14 h. After being washed with 20 ml buffer B (25 mM Tris-Cl, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 0.5 mM EDTA), the beads were resuspended in 1 ml buffer B, and 160 units of tobacco etch virus protease (Invitrogen) and 600 units of RNasin (Promega) were added. Cleavage of TAP-tagged Gar1 was performed at 20°C for 2 h, and the supernatant portion was collected from a Flex-Column (1.0 by 5 cm) (Kontes). The released material was then incubated with 50 μl anti-Myc beads (Sigma) at 4°C for 2 h. The beads were washed with 10 ml buffer B, and the bound material was released from the beads by incubation with 200 μl C-Myc peptides (0.5 mg/ml) at 4°C for 30 min. Isolated proteins were precipitated with acetone and analyzed by 12% SDS-polyacrylamide gel electrophoresis (PAGE). The gel was silver stained, and bands of interest were excised and identified by mass spectrometry (LC/MS/MS; Midwest Bio Services, LLC).

Sucrose gradient fractionation. Cell extract was loaded onto a 10% to 30% continuous sucrose gradient buffered with 150 mM KCl, 5 mM $MgCl_2$, 25 mM Tris-Cl, pH 8.0, and 5 mM β-mercaptoethanol. For deproteinized materials, the sucrose gradient was buffered with 100 mM NaCl, 5 mM $MgCl_2$, and 20 mM Tris-Cl, pH 7.5. Fractionation was performed by centrifugation at 33,000 rpm for either 3 or 18 h using a Beckman SW-41 rotor, as indicated in the figure legends, and 400-μl portions were sampled from top to bottom. RNA was prepared from fractions using TRI Reagent (Sigma) according to the manufacturer's instructions.

Northern analysis. Five micrograms of total RNA or RNA prepared from sucrose gradient fractions was separated on gels of 10% polyacrylamide-7 M urea and transferred onto nylon membranes (GeneScreen Plus; PerkinElmer Life Sciences, Inc.). Prehybridization and hybridization were performed at 42°C with specific oligonucleotides. To analyze rRNA or pre-rRNA, 10 μg total RNA or RNA prepared from sucrose gradient fractions was separated on gels of 1.2% agarose-formamide. Hybridization was performed at 42°C with an oligonucleotide specific to the 5′ ETS region of pre-rRNA by using Rapid-hyb hybridization buffer (Amersham). Hybridization results were visualized with a Fuji PhosphorImager.

Immunoprecipitation. Cell extracts prepared in buffer A with 150 mM KCl as described above were incubated with antihemagglutinin (anti-HA) beads (Roche) at 4°C for 4 h. After seven washes with wash buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 0.05% SDS), the coselected RNAs were prepared directly from the beads using TRI Reagent (Sigma) and subjected to Northern or RT-PCR analysis.

RESULTS

Has1p is affinity selected with snR30/U17 snoRNP complexes. With the aim of identifying novel proteins associated with specific H/ACA snoRNPs, our laboratory developed a new two-tag affinity approach for preparing these and other snoRNP complexes (D. Piekna-Przybylska, B. Liu, and M. J. Fournier, unpublished). In the present scheme, the core protein Gar1p carries the TAP tag at its C-terminal end, and this tag is used to enrich the entire family of H/ACA snoRNPs. To isolate a specific snoRNP, a tagged variant of the snoRNA component is expressed that contains a unique protein-binding signal, specifically the human U1 hairpin II sequence. The hairpin sequence was inserted in the 5′ portion of the target snoRNA. The natural ligand for this signal is the U1A snRNP protein (3, 20), which is expressed in the same cells and carries a Myc tag sequence that is used in the second stage of enrichment. The tagged snoRNA is expressed conditionally from a plasmid-carrying *GAL1* allele in cells that lack the normal chromosomal snoRNA gene. Using this strategy, we affinity selected the snR30 complex initially and then, for comparison, another H/ACA snoRNP complex known to be involved only in Ψ modification. The latter complex is the snR3 snoRNP, which creates three Ψs in 25S rRNA (Ψ2128, Ψ2131, and Ψ2265) (15, 38, 43).

Extracts were prepared from cells expressing TAP-tagged Gar1p, Myc-tagged U1A, and the snoRNA of interest outfitted with the U1 hairpin domain. Affinity selection was performed first with immunoglobulin G beads to enrich all H/ACA snoRNPs. The bound material was released from the beads by cleavage with tobacco etch virus protease and subsequently incubated with anti-Myc beads to enrich the desired specific complex. Following recovery of the material selected in this step, the RNA and protein compositions of the preparations were analyzed. The RNA was examined by polyacrylamide gel electrophoresis, using both staining with ethidium bromide and Northern hybridization analysis with probes specific for snR30 or snR3 (Fig. 1A). The hybridization patterns demonstrate excellent levels of enrichment for each snoRNP preparation, with little or no cross-contamination apparent; the RNA staining patterns also support this conclusion. Substantial degradation is evident for the tagged RNA species, however, and analysis of RNA prepared directly from cells indicates that the damage occurs primarily in vivo (data not shown). While cells expressing the tagged snoRNAs have normal growth rates, it is possible that the tagged variants are not active and that activity is provided by snoRNPs lacking the snoRNA tag. Despite this uncertainty, we proceeded with the comparative protein analysis.

The selected proteins were fractionated by SDS-PAGE and visualized by silver staining, and the dominant bands were identified by mass spectrometry analysis (LC/MS/MS). The results indicate that along with Gar1p, the Cbf5p and Nhp2p core proteins are highly enriched as expected (Fig. 1B). The small Nop10p protein (<10 kDa) was not evident, perhaps because of comigration with the loading dye or loss during precipitation because of its small size; however, it has been observed in other preparations made in the same way (Fig. 1B, lower panel). The presence of the four core proteins indicates that the snoRNA tag does not block snoRNP formation.

To identify proteins that may weakly or transiently associate with the snoRNPs, prolonged silver staining was performed. The protein patterns are largely the same for the snR30 and snR3 RNP preparations but with differences in relative abundance. One such difference is a protein of ~56 kDa that is substantially and reproducibly more enriched in the snR30 preparation than in the snR3 preparation (Fig. 1B). This band was excised and identified as the RNA helicase Has1p.

snoRNAs coimmunoprecipitate with HA-tagged Has1p. To further evaluate the specificity of the Has1p-snoRNP associations, we immunoprecipitated HA-tagged Has1p from cell extracts and examined the coprecipitating RNAs by Northern analysis. The results verify that Has1p associates with the snR30 snoRNA and other H/ACA snoRNAs as well, such as snR10 and snR3, with somewhat more affinity for snR30 RNA (Fig. 1C). The immunoprecipitate also contains the C/D snoRNAs U3, U14, and snR63 at levels comparable to that of snR3, indicating that Has1p association is not unique to the H/ACA RNAs. Importantly, only trace amounts of snoRNAs were detected in a control reaction with cell extract from a wild-type strain, and no tRNA was coprecipitated in either case. Interestingly, the spliceosomal U1 and U6 snRNAs were also coselected with Has1p, al-

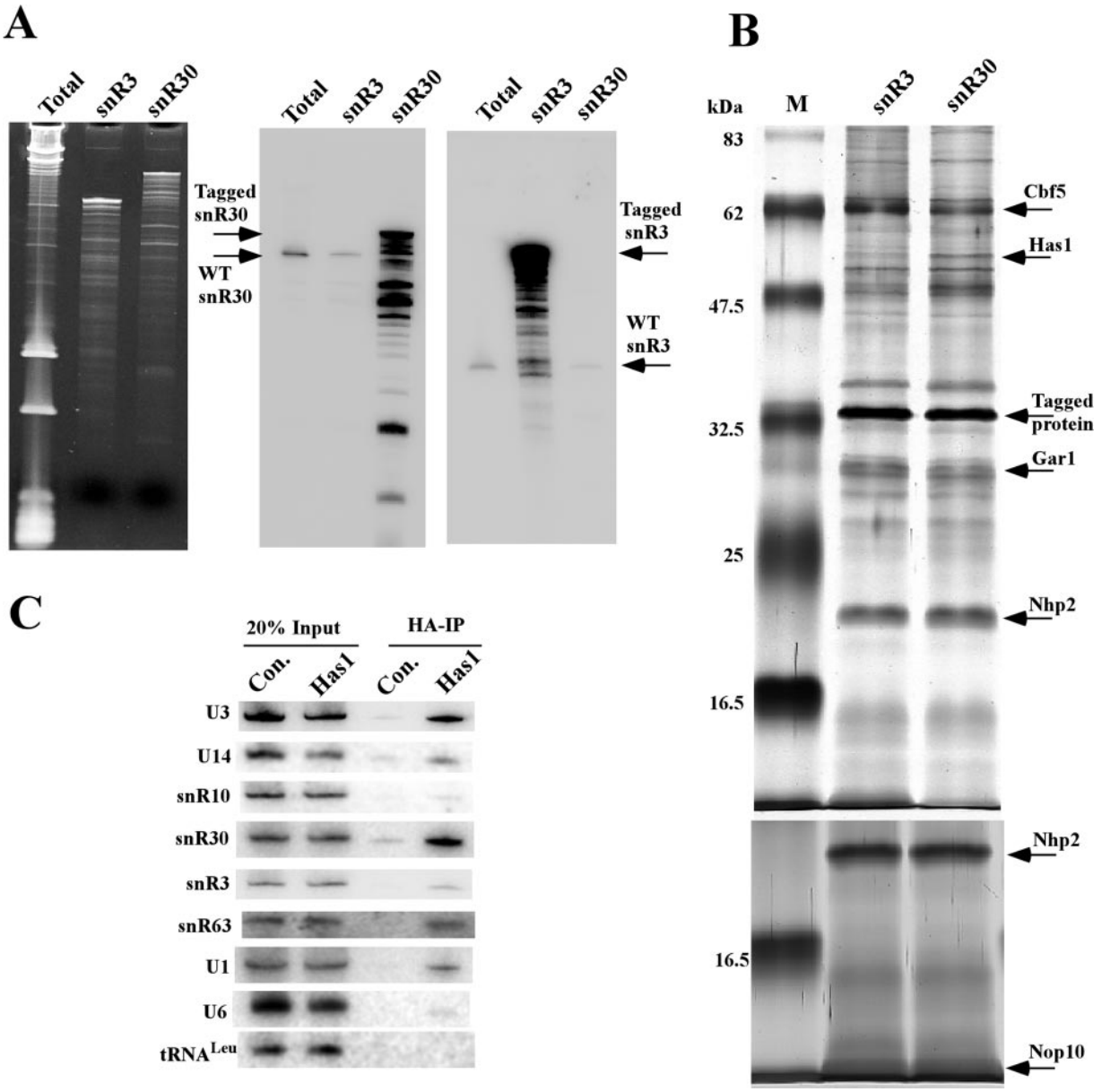

FIG. 1. Has1p is affinity selected with individual snoRNPs. A processing snoRNP (snR30) and modifying snoRNP (snR3) were enriched in a two-step strategy that features affinity tags in a core protein (Gar1) and the snoRNA in the snoRNP of interest. (A) Northern analysis of RNA present in the affinity-selected complexes. RNA from the preparations was examined with probes for snR30 and snR3. The patterns shown are for ethidium bromide staining (left) and hybridization of snR30 (middle) and snR3 (right). The lanes labeled snR3 and snR30 contain RNAs from the enriched RNP complexes. Total, total RNA from control cells. The tagged and wild-type (WT) snoRNAs are identified. Nucleolytic damage to the tagged RNAs is apparent. (B) Proteins present in the snoRNP preparations. Proteins were fractionated on 12% SDS-PAGE gels and detected by silver staining. Mass spectral analysis was used to identify dominant proteins, and the identities are given. M, marker proteins. (C) Northern analysis of RNA that immunoprecipitates with tagged Has1p. Con. and Has1, extracts from cells expressing control wild-type and HA-tagged Has1p, respectively; HA-IP, material precipitated using anti-HA beads.

though at different levels (see below). These results indicate that Has1p is not specific to the snR30 snoRNP but rather associates directly or indirectly with many snoRNAs. This interpretation is also supported by our finding that affinity selection of C/D snoRNPs coselects Has1p as well (data not shown). These results are consistent with earlier findings by others showing that Has1p is coselected with the H/ACA snoRNP core proteins Cbf5p and Nhp2p and the C/D snoRNP core protein Nop58p when each was used independently as bait in affinity selection analyses (16, 17).

To determine whether pre-rRNA is also selected during enrichment of snoRNPs, we carried out RT-PCR analyses on a preparation of affinity-isolated snR30 complexes, using oligonucleotides that span the pre-rRNA region (Fig. 2A). Material prepared from a strain expressing tagged Gar1p but no tagged snoRNA was used as a control. Although of low abun-

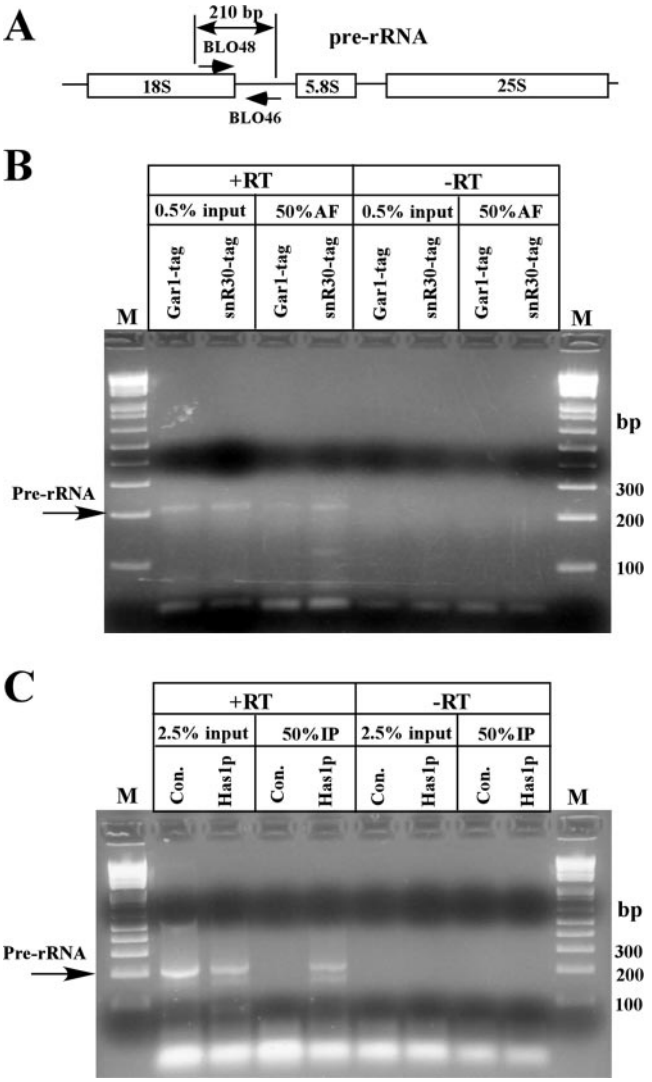

FIG. 2. Pre-rRNA is present in the snR30 snoRNP preparation and coimmunoprecipitates with Has1p. RT-PCR analysis was employed to test the presence of pre-rRNA. (A) Schematic representation of yeast pre-rRNA and oligonucleotides used for RT-PCR analysis. (B) RNA prepared from input material and affinity-selected (AF) material was either subjected to reverse transcription followed by PCR amplification (+RT) or used directly for the PCR analysis to detect potential ribosomal DNA contamination (−RT). M, DNA marker. (C) RNA prepared from input and immunoprecipitated materials (IP) using anti-HA beads was analyzed by RT-PCR for the presence of pre-rRNA as for panel B. Con., materials from control cells; Has1p, materials from cells expressing HA-tagged Has1p.

dance, pre-rRNA was indeed found in the snR30 preparation at a level higher than the background level from the control experiment (Fig. 2B), indicating that pre-rRNA was coselected with the snR30 snoRNP complex. We also asked whether pre-rRNA coprecipitates with Has1p by subjecting RNA from an immunoprecipitate to RT-PCR analysis. A significant amount of pre-rRNA was detected (Fig. 2C), consistent with previous data showing that Has1p is present in 90S and pre-60S ribosomal complexes (13, 21).

We do not know the nature of the Has1p association with pre-rRNA and snoRNPs. However, its coselection with both pre-rRNP and snoRNP complexes is consistent with the possibility that it interacts with both, perhaps at the level of pre-rRNA. Since Has1p depletion does not affect snoRNP stability (11), it seems unlikely that it is involved in snoRNP assembly. Thus, a role in snoRNP function is more likely.

Loss of Has1p causes snoRNPs to accumulate with pre-rRNPs. Has1p has been shown to be required for cleavage of pre-rRNA at sites A_0, A_1, and A_2 (11). Its loss also leads to a decrease in 18S rRNA production and a delay in processing of 25S rRNA. In the present study, it was observed to associate with the processing snoRNAs required for the A_0, A_1, and A_2 cleavages, i.e., U3, U14, snR10, and snR30 (23, 31, 36, 48). These properties suggest that Has1p may be involved in the binding or release of the corresponding snoRNPs from pre-rRNA.

We next tested the possibility that the Has1p depletion effects on pre-rRNA processing reflect malfunctions of the snoRNPs involved in those reactions. To this end, we examined the association of processing and other snoRNPs with pre-rRNP complexes for cells deficient in Has1p. The analysis was done with a test strain in which expression of Has1p is under the control of a Gal promoter, and depletion was achieved by shifting the carbon source from galactose to glucose (11). The Has1p used in the earlier experiments and throughout this study contains an HA or Myc tag. Both tagged variants support normal growth (11; this study). The essentiality of Has1p was previously shown with a Gal-dependent (tagged) allele (11), and our data are consistent (Fig. 3A). Extracts were fractionated on sucrose gradients, and RNA prepared from the gradient fractions was examined by Northern analysis following gel electrophoresis. The blot was probed with oligonucleotides specific for snoRNAs as indicated (Fig. 3).

The distribution of the RNAs from the control cells was as expected, based on previous fractionations of pre-rRNP and snoRNP complexes (5, 19, 53). The pattern of the U3 snoRNP is representative, with the U3 snoRNA present in three regions of the gradient (Fig. 3B). These regions correspond to (i) a free U3 RNP particle at about 12S (Fig. 3B, fractions 2 to 6), (ii) a bound snoRNP at about 40S (fraction 10) which comigrates with the 5′ ETS pre-rRNA that is likely cleaved at site A_2, and (iii) a peak that migrates with unprocessed 35S pre-rRNA at about 60S-90S (fractions 16 to 20) (see below) (5, 19, 53; this study). The other snoRNAs examined are evident in the 60S-90S pre-rRNP region as well, although at lower abundance than U3, also as expected. No apparent peak was detected in the 40S region for other snoRNPs; however, it is likely that the fraction contains bound snoRNPs which are less abundant and masked by free snoRNPs.

Interestingly, the distribution pattern of the snoRNAs changes dramatically with depletion of Has1p (Fig. 3C). The U3 and U14 species accumulate to a much higher extent (five- to sixfold) in the 60S-90S region (Fig. 3C, fractions 16 to 20) and also in the 40S region (fractions 10 to 14). For U14, the increased abundance in the large pre-rRNA fractions is accompanied by a decrease in the mono-snoRNP region (Fig. 3C, fractions 2 to 6), consistent with a reduced amount of free snoRNPs. The other snoRNAs examined also showed accumulation in the 60S-90S region. Enrichment was ~2-fold for two other processing snoRNAs (snR30 and snR10) and a modification guide snoRNA (snR63) and lower for another guide

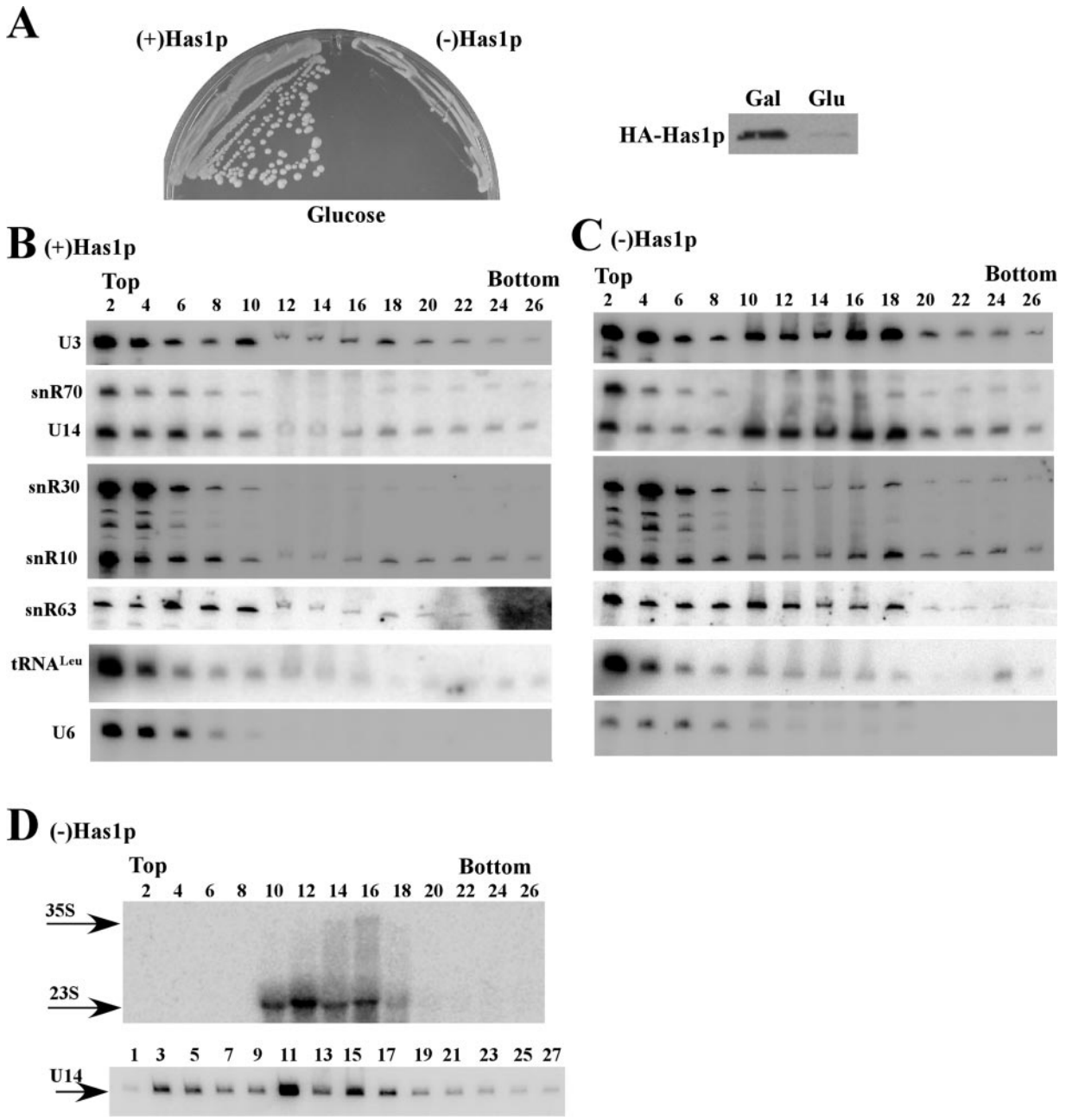

FIG. 3. Depletion of Has1p causes snoRNAs to accumulate with large pre-rRNP complexes. Effect of Has1p depletion on snoRNA release was analyzed by sucrose gradient fractionation. (A) Cell growth is inhibited by glucose repression of Has1p production. (Left panel) Cells grown on glucose medium. (Right panel) Western analysis of HA-tagged Has1p. (B to D) Extracts from Has1p-depleted cells and control cells were fractionated for 3 h in a 10 to 30% sucrose density gradient. RNA was prepared from alternate fractions and examined by Northern analysis. The blots were hybridized sequentially with 5′-end-labeled oligonucleotides specific to different snoRNAs or pre-rRNAs, as indicated. (B) Control cells. (C and D) Has1p-depleted cells. The numbers of the gradient fractions appear above the sample lanes.

snoRNA (snR70). In other experiments, a higher level of accumulation was seen for snR70 as well as for two additional guide snoRNAs, snR77 and snR3 (data not shown). Altogether, the snoRNAs analyzed include four species involved in processing (U3, U14, snR30, and snR10), two snoRNAs involved both in processing and in modification (U14/snR10 for Nm/Ψ, respectively), three species known only to guide 2′-O-methylation (snR63, snR70, and snR77), and one snoRNA that guides pseudouridylation only (snR3). The sites targeted for modification occur in both the small- and large-subunit rRNAs, as follows: 18S rRNA, U14 and snR70; and 25S rRNA, snR3, snR10, snR63, and snR77. Thus, the snoRNPs examined bind to different regions in the pre-rRNA transcripts.

The difference in snoRNA accumulation in the high-molecular-weight complexes is not because of differences in RNA loading. As can be seen from the control patterns with

$tRNA^{Leu}$, the abundance and migration behavior of the tRNA are not significantly different from those for the control and Has1p-depleted strains. These data indicate that Has1p depletion results in the accumulation of all eight snoRNPs tested, in the 60S-90S pre-rRNP fractions, albeit at somewhat different levels. The distribution patterns for wild-type cells and the Has1p test strain are similar for cells grown in galactose (inducing) medium, indicating that differences in Has1p expression levels or a spurious genetic difference is not the basis of the effects observed (data not shown). The U6 snRNA was also used as a control, and no difference in migration was observed. However, interesting differences in the relative abundances of U6 were apparent for the Has1p depletion condition (see below).

The cosedimentation of snoRNA with pre-rRNA was verified by additional probing of gradient-fractionated extracts from Has1p-depleted cells, using oligonucleotides specific to U14 and the 5′ ETS region of pre-rRNA. The results show accumulated U14 in two peaks (Fig. 3D, fractions 11 and 15 to 17) that include 23S pre-rRNA (fractions 10 to 12) and 35S pre-rRNA (fractions 14 to 18). Both pre-rRNA species accumulate with Has1p depletion (11). These data confirm that the snoRNAs accumulate in pre-rRNP complexes upon depletion of Has1p.

The snoRNAs remain associated with pre-rRNA after deproteinization. In the absence of Has1p, the accumulated snoRNAs in high-molecular-weight complexes could be associated through base pairing with pre-rRNA or could simply be trapped. With the aim of distinguishing these possibilities, we examined the effect of treating extracts with proteinase K and SDS before fractionation. The procedure used has been shown to maintain snoRNA–pre-rRNA pairings (48). Equivalent amounts of treated and untreated samples were analyzed by SDS-PAGE. As expected, no protein was detected by Coomassie blue staining following treatment, except the 28.9-kDa proteinase K (Fig. 4A).

Analysis of the snoRNA migration pattern after deproteinization showed that for Has1p-depleted cells, U3 and U14 snoRNAs cosedimented with 23S and 35S pre-rRNAs (Fig. 4C, fractions 6 to 8 and 10 to 14, respectively), as seen earlier for untreated extracts. However, for normal cells, the U3 and U14 species were present mainly in the top regions of the gradients, presumably as unbound snoRNAs (Fig. 4B). These results argue that the snoRNAs that comigrate with pre-rRNA after depletion of Has1p are associated through base pairing rather than a trapping phenomenon. The pattern of small rRNAs also serves as a control for this analysis.

Widespread accumulation of snoRNAs with pre-rRNA does not occur with loss of other proteins involved in maturation of 18S rRNA. While the data are consistent with Has1p functioning in snoRNA release, the snoRNA accumulation effect could arise by other means that interfere with rRNA processing. Even less direct is the possibility of an effect triggered by ribosome deficiency. To test these possibilities, we examined the distribution pattern of snoRNPs in cells depleted of two nonhelicase proteins required for the production of 18S rRNA. The proteins are Esf1p, a conserved nucleolar protein required for cleavage at sites A_0, A_1, and A_2 (41), and Dim2p, a KH domain protein associated with Dim1p and required for cleavage at sites A_1 and A_2 (51). Depletion of either protein has been shown to result in a sharp decrease in 18S rRNA synthesis and simultaneous accumulation of 35S and 22/23S pre-rRNAs; these defects mimic those of Has1p depletion (11, 41, 51).

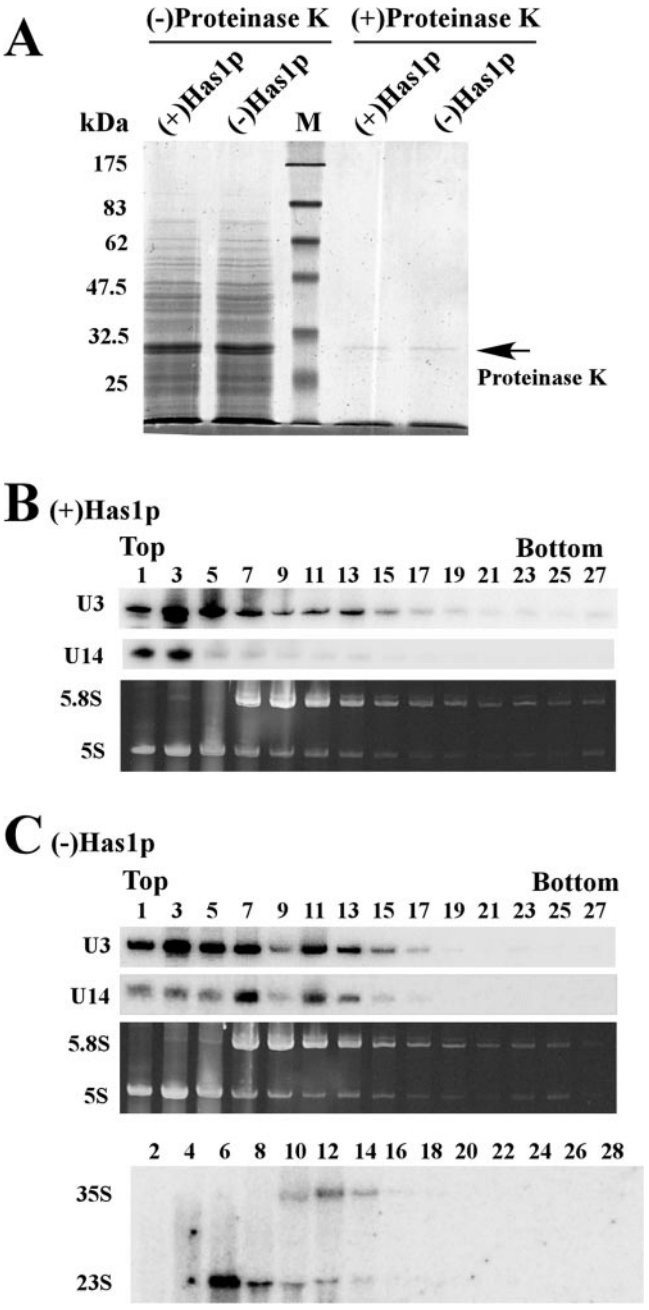

FIG. 4. Accumulated snoRNAs remain associated with pre-rRNA after deproteinization. Extracts from control or Has1p-depleted cells were treated by proteinase K and fractionated on a 10 to 30% sucrose gradient for 3 h. (A) Effects of protein removal were examined by 12% SDS-PAGE, followed by Coomassie blue staining. (B and C) RNA was prepared from gradient fractions for control and Has1p-depleted cells, respectively, and analyzed by ethidium bromide staining (rRNAs) or Northern probing (small RNAs and pre-rRNAs).

Both proteins were depleted in our study by blocking conditional expression. In the Esf1p test cells, expression is under

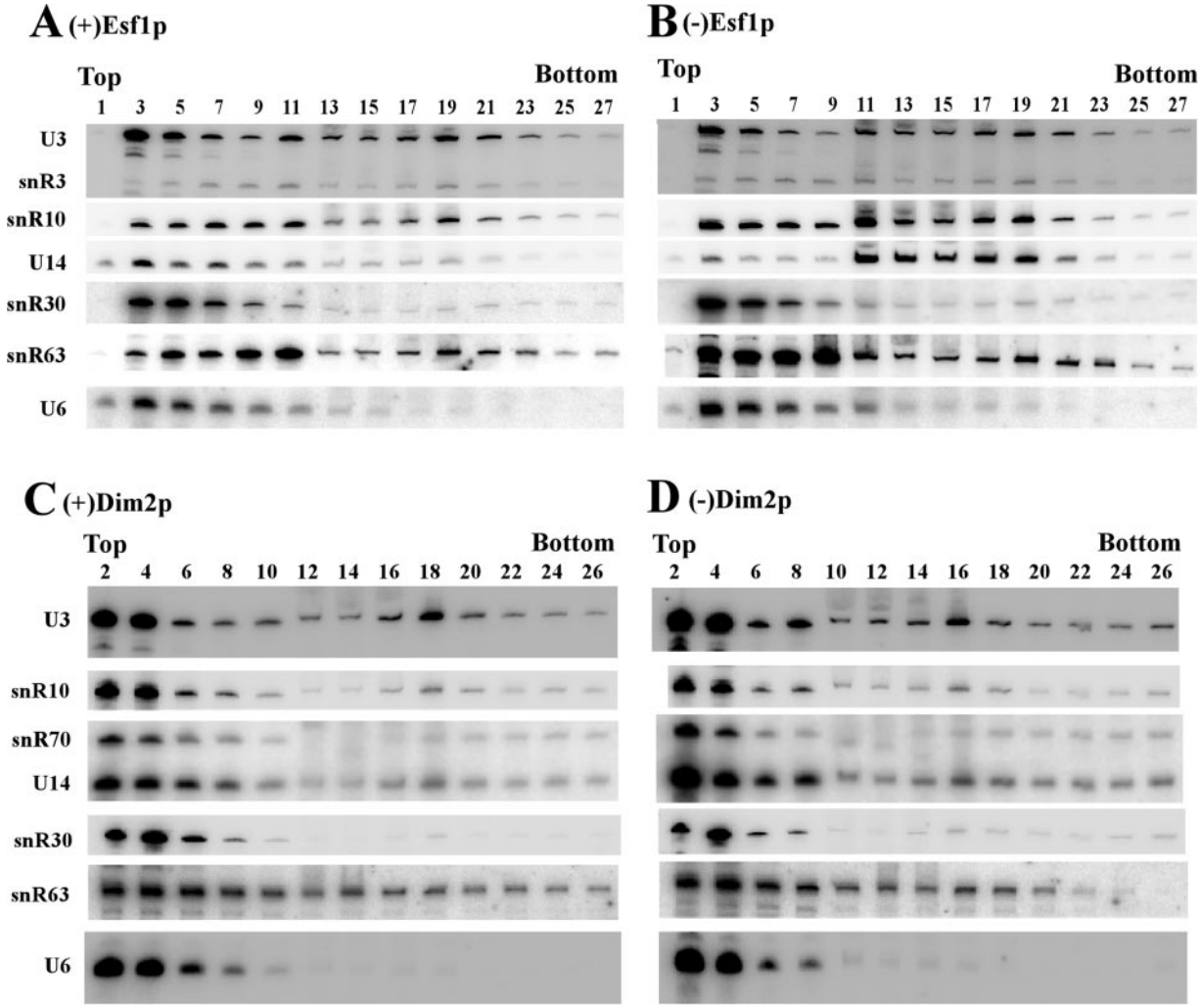

FIG. 5. Density gradient patterns of snoRNAs from cells depleted of Esf1p or Dim2p. Test cells were depleted of Esf1p or Dim2p, and extracts were analyzed as described for depletion of Has1p (Fig. 3). (A and B) Extracts containing and lacking Esf1p, respectively. (C and D) Extracts containing and lacking Dim2p, respectively.

the control of the tetO7-TATA promoter, and production is shut off by the addition of doxycycline (41). With the Dim2p test strain, transcription is under the control of a Gal promoter (51). As expected, the snoRNA patterns for cells expressing the two new test proteins are similar to those of wild-type cells and the Has1p-positive control (compare Fig. 5A and C with 3B). In the presence of these proteins, the U3 snoRNP fractionates as expected (Fig. 5A and C). However, the patterns obtained for the depletion conditions are different from those for loss of Has1p and also different from each other.

When Esf1p was depleted, the patterns of U14 and snR10 snoRNAs were similar to what was observed with Has1p depletion, with strong accumulation in the 60S-90S and 40S rRNP fractions (Fig. 5B) and a reduced level of the free U14 snoRNP (fractions 3 to 7). However, no significant accumulation of other snoRNAs, including U3, is apparent in the 60S-90S region relative to that of control cells. Slightly more extract was loaded for the Esf1p-depleted cells than for the wild type, as can be seen by the abundance of U6 snRNA (Fig. 5A and B). Interestingly, the level of free snR63, a snoRNA that directs methylation, increased after depletion of Esf1p. Thus, U3 and other snoRNP species accumulate with the large pre-rRNP complexes upon Has1p depletion but not upon depletion of Esf1p. The behaviors of the U14 and snR10 species differed, as these were enriched in the pre-rRNP fractions under both depletion conditions. To determine whether U14 and snR10 accumulation in the large complexes is a common defect with loss of proteins required for maturation of 18S rRNA, we analyzed the distribution of snoRNAs in cells depleted of Dim2p. No significant accumulation was observed for these and four other snoRNAs examined (Fig. 5C and D).

Taken together, these results indicate that Has1p depletion specifically causes accumulation of all snoRNAs tested in the 35S pre-rRNA region. The strongest effects were observed for the U3 and U14 snoRNAs, but other species were also affected. Because the snoRNAs evaluated include five C/D snoRNAs (U3, U14, snR63, snR70, and snR77) and three H/ACA snoRNAs (snR3, snR10, and snR30), the accumulation defect for the large pre-rRNPs (60S-90S region) is not restricted to one family of snoRNAs. Under the Esf1p deple-

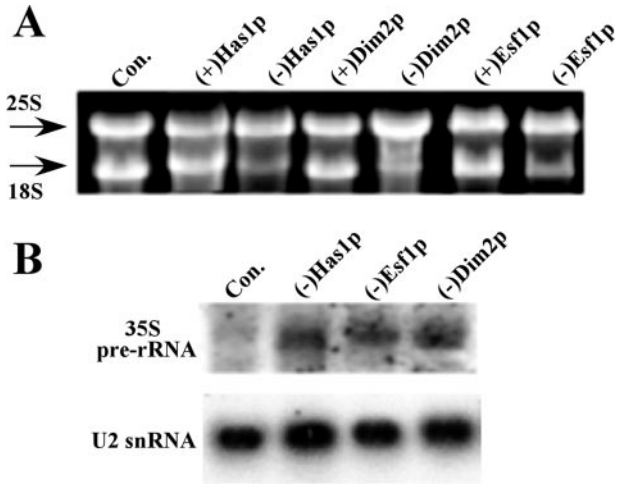

FIG. 6. Pattern of rRNA species in the protein-depleted strains. Total RNA was prepared from test cells depleted of Has1p, Esf1p, or Dim2p and fractionated on a 1.2% agarose gel. Visualization was by ethidium bromide staining (A) and Northern hybridization analysis (B). (A) 18S and 25S rRNAs. (B) 35S pre-rRNA. Pre-rRNA was detected with an oligonucleotide specific to the 5′ ETS region. U2 snRNA was used as an internal control. Con., total RNA from control cells; (+)Has1p, (+)Dim2p, and (+)Esf1p, nondepleted test cells; (−)Has1p, (−)Dim2, and (−)Esf1, depleted test cells.

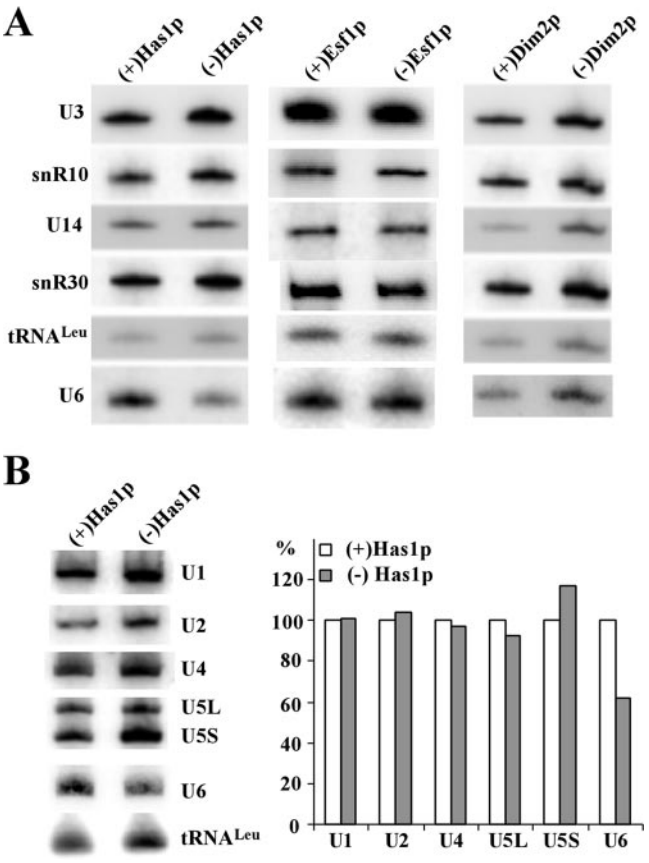

FIG. 7. Comparison of snoRNA and snRNA patterns in protein-depleted cells. Total RNA from control cells (+) or cells depleted (−) of Has1p, Esf1p, or Dim2p was fractionated by gel electrophoresis and subjected to Northern analysis. The oligonucleotide probes for the different snoRNAs (A) and snRNAs (B) are indicated. The relative abundances of the spliceosomal snRNAs were calculated based on the level of tRNA, using the program Image J (B, right).

tion condition, the U14 and snR10 snoRNAs were enriched in the 35S pre-rRNA fractions but not the other snoRNAs tested, including U3.

Depletion of the three proteins analyzed here is known to cause accumulation of 35S pre-rRNA (11, 41, 51). However, because of possible differences in the levels of transcription control in the different test strains, we wondered whether the greater accumulation of snoRNAs in the 60S-90S pre-rRNP complexes in the Has1p-depleted cells might stem from a substantially higher level of 35S pre-rRNA. To test this possibility, we examined the relative abundances of the mature 18S and 25S rRNAs and the level of 35S pre-rRNA in cells depleted of the various proteins. The staining patterns indicate that 18S rRNA is reduced at comparable levels in the depleted strains, with ~15% remaining relative to levels in wild-type or nonrepressed test cells (Fig. 6A). This situation suggests that disruption of 18S rRNA production is similar in each case.

The levels of 35S pre-rRNA were compared by hybridization probing of total RNA with an oligonucleotide specific for the 5′ ETS region. As is typical for such analyses, 35S pre-rRNA was barely detectable in wild-type control cells. However, it accumulated at higher levels in cells depleted of Has1p, Esf1p, or Dim2p, and importantly, the levels of U2 snRNA used as a loading control were comparable (Fig. 6B). The results show that snoRNP accumulation in the 60S-90S pre-rRNP complexes in Has1p-depleted cells is not due to higher accumulation of 35S pre-rRNA in the test strain. This interpretation is also supported by the fact that in Dim2p-depleted cells, no snoRNPs accumulated with 35S pre-rRNA at levels similar to those seen with Has1p depletion. Thus, based on the snoRNA distribution patterns, we conclude that Has1p is required for snoRNP release from pre-rRNA. The results further suggest that Esf1p is needed for the release of the U14 and snR10 snoRNPs.

Has1p depletion affects U6 snRNA accumulation mainly at the level of free U6 snRNP. In evaluating the effects of Has1p loss, we noted that the level of U6 snRNA seemed reduced compared with that of another control, $tRNA^{Leu}$ (Fig. 3). To determine whether this was indeed the case, we examined the levels of U6 snRNA in the various protein-depleted strains by Northern analysis. Probes were included for several snoRNAs, and $tRNA^{Leu}$ was used as a control for RNA loading. The results indicate that the levels of the snoRNAs tested are not affected by depletion of the various proteins (Fig. 7), consistent with previous studies (11, 41, 51). However, the results confirm that the level of U6 is reduced with loss of Has1p but not with depletion of Esf1p or Dim2p (Fig. 7A). The fact that the decrease in U6 content occurs only with Has1p depletion indicates that its loss is not a secondary effect of ribosome deficiency, as depletion of the latter proteins also creates that condition. Since the U6 snRNA is required for pre-mRNA splicing together with four other snRNAs, we also analyzed the levels of the four other snRNAs. The results show that only U6

is significantly affected by the loss of Has1p, with a reduction of ~40% (Fig. 7B).

The U6 snRNA occurs in several types of snRNP complexes, including the free snRNP, a U4.U6 dimer, a U4.U6/U5 trimer, and the spliceosomal complex B that contains all five snRNPs (47, 55). To determine whether Has1p is involved in the formation of U6 complexes, sucrose density gradient fractions were probed for all five snRNAs. Depletion of Has1p does not significantly affect the patterns of the U1, U2, U4, and U5 RNAs relative to control cells (Fig. 8A and B). However, a striking change is apparent in the distribution of the U6 snRNP complexes, in particular the relative amount of free snRNP (Fig. 8B).

In control cells, U6 occurs in two regions of the gradient: fractions 6 to 10, which include both the free U6 snRNP and the U4/U6 dimer, and fractions 20 to 24, which correspond to the 25S trimer of U4.U6/U5 (12). Depletion of Has1p results in a dramatic reduction in the amount of free U6 RNP (Fig. 8B, fractions 6 to 8). No effect is apparent in the level of the U6-containing trimeric complex, suggesting that assembly of this complex is not affected by depletion of Has1p. Note that for the Has1p depletion patterns, more extract was loaded, as can be seen from the level of the $tRNA^{Leu}$ control. To test the possibility that the reduced level of free U6 RNP is linked to ribosome loss, we also analyzed the snRNP patterns for Esf1p-depleted cells in the same way (Fig. 8C). No differences were observed for U6 or any other snRNA relative to control cells. These last results argue that the reduction in free U6 snRNP content that occurs with Has1p depletion is not a general effect of impaired ribosome production.

Since Has1p depletion affects both the level of U6 snRNA and its distribution among U6-containing complexes, we addressed the possibility that Has1p might associate stably with the U6 snRNP. To this end, we immunoprecipitated HA-tagged Has1p and examined the precipitate for the presence of U6 snRNA. Negative data indicated that U6 was not specifically precipitated (Fig. 1C and data not shown), suggesting that Has1p is not stably associated with the U6 complexes. To determine whether the reduced level of U6 snRNPs caused by Has1p depletion impairs mRNA splicing, we analyzed the level of Tef4 mRNA (24) and its precursor by RT-PCR. No detectable effect was observed (data not shown). This was not surprising as the overall decrease in U6 was not large, estimated at ~40%, and the level of free U6 might be adequate for efficient assembly of the trimeric complex and spliceosome.

Point mutations in ATPase and helicase motifs of Has1p block release of U14 from pre-rRNA complexes. To determine whether the ATPase and helicase activities of Has1p are involved in snoRNA release from pre-rRNP complexes, we examined the effect of disrupting these activities with point mutations known to disrupt function: K92A (motif I) for ATPase activity and H375E (motif VI) for helicase activity. Both mutations are lethal (42). The test cells lack the chromosomal gene for Has1p and are complemented with a plasmid containing an HA-tagged wild-type Has1p allele under galactose control. A second plasmid expresses mutant or wild-type Has1p with a Myc tag (referred to below as normal protein) from the natural Has1p promoter. After cells were shifted to glucose medium to deplete the Gal-controlled Has1p, the constitutively expressed normal protein could support cell growth, but

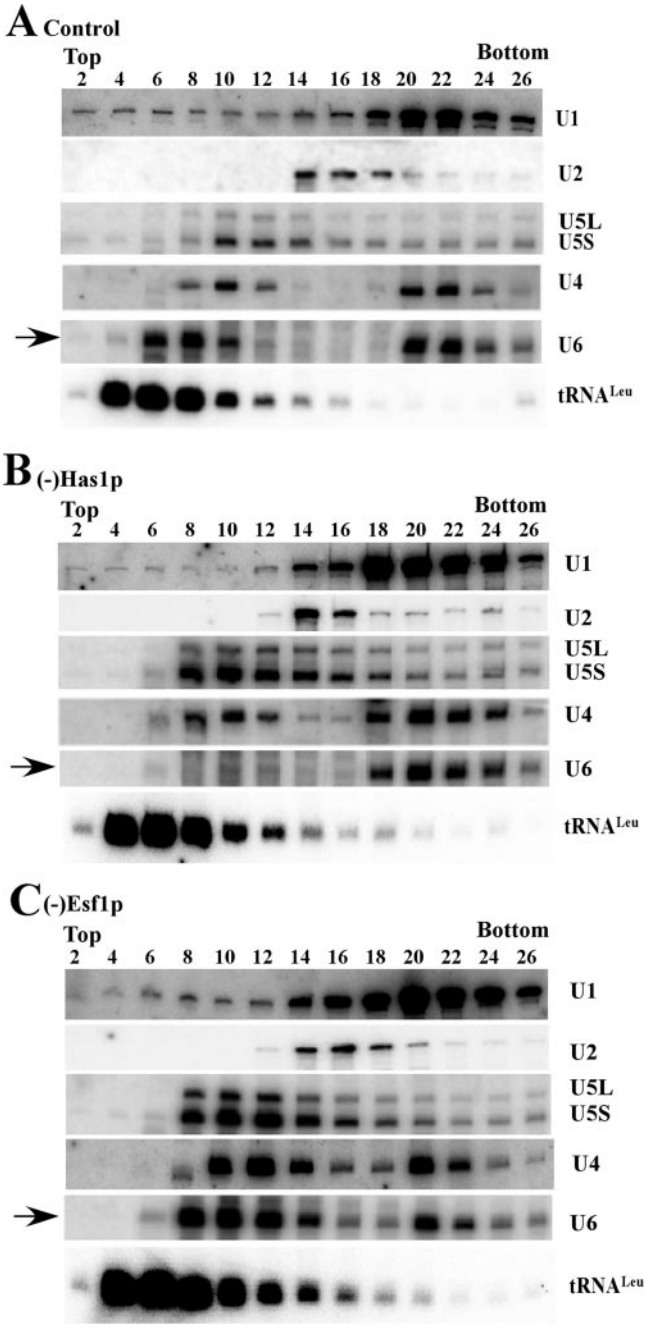

FIG. 8. Has1p depletion causes a loss of free U6 snRNPs. Extracts from control cells and cells depleted of Has1p or Esf1p were fractionated for 18 h by sucrose density gradient centrifugation. RNA prepared from gradient fractions was subjected to Northern analysis, using probes specific for the splicing snRNAs as indicated. As a control, the blots were also probed for $tRNA^{Leu}$.

the mutated Has1p variants could not (Fig. 9A). Cells expressing mutant Has1p grew normally in galactose medium, indicating that the mutations do not have a dominant-negative effect (Fig. 9A).

The density gradient patterns of snoRNA were then examined in the presence of normal or mutant Has1p. As expected, U14 snoRNA was mostly released from pre-rRNA with normal

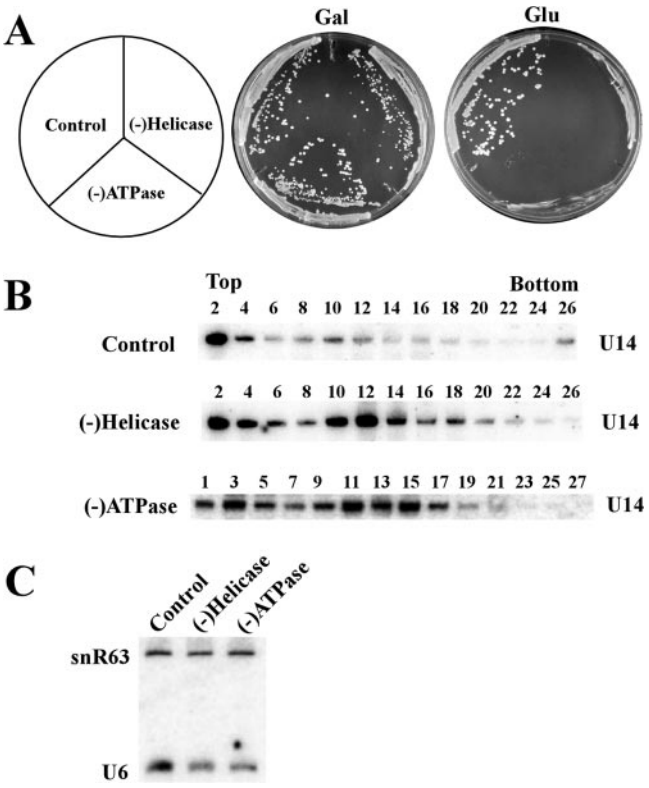

FIG. 9. Point mutations in the ATPase and helicase elements of Has1p block U14 release from pre-rRNA and affect U6 accumulation. (A) Growth of test cells with mutations in the ATPase (motif I) and helicase (motif VI) domains. All test strains express HA-tagged wild-type Has1p under galactose regulation and Myc-tagged mutant or wild-type Has1p regulated by its own promoter. (B) The U14 sedimentation patterns for strains expressing mutant or control Has1p were examined as described in the legend to Fig. 3. (C) Northern analysis of U6 snRNA in mutant and control strains.

protein (Fig. 9B, upper panel) but not with either defective protein (helicase mutation [middle panel] or ATPase mutation [lower panel]), indicating that these motifs are required for the function that Has1p plays in snoRNA release. Because the U3 snoRNA has a special function, we also analyzed the effect of the Has1p mutations on its release. No release was apparent with the normal Has1p (data not shown), precluding further analysis. The lack of complementation could reflect a deficiency in Has1p in this experiment, as the expression level of the plasmid-encoded normal protein is low (data not shown) and might not be sufficient for U3 release. Another possibility is that the N-terminal Myc tag sequence on the control Has1p may pose a special problem for U3 release.

Finally, U6 snRNA accumulation was also examined in the presence of the mutant Has1p variants. A wild-type level of U6 was observed for control cells expressing the Myc-tagged normal Has1p but not for cells dependent on the defective Has1p. Thus, the ATPase and helicase domains are also required for maintaining the level of U6 (Fig. 9C). Taken together, these data indicate that the ATPase and helicase activities of Has1p are required for its function in normal snoRNA release and U6 production.

DISCUSSION

Has1p is a nucleolar protein previously identified by affinity isolation in 90S and (mostly) pre-60S preribosomal RNP complexes (11, 13, 21). In one model, Has1p is proposed to associate with the 90S preribosomal particle, which contains both small- and large-subunit rRNAs and proteins and remains associated with early pre-60S particles derived from the larger complex (17, 39). Depletion of Has1p inhibits the processing cleavages at sites A_0, A_1, and A_2, leading to a decrease in 18S rRNA synthesis and, interestingly, a delay in maturation of 25S rRNA as well (11). Recently, Has1p was shown to be able to unwind an RNA/DNA helix in vitro in an ATP-dependent manner, demonstrating that it is an authentic helicase (42). Mutations in conserved motifs I and VI can disrupt both ATPase and helicase activities, respectively, and inactivation of these domains is lethal in vivo (42). These findings indicate that both ATPase and helicase activities are required for Has1p function in living cells.

In this study, we found that the Has1p helicase is required for the release of all snoRNAs tested from pre-rRNA; the strongest effects were for the U3 and U14 processing snoRNAs. In addition, Has1p was shown to be important for U6 snRNA accumulation. In a search for proteins specific to individual snoRNPs, we initially found Has1p associated with the snR30 complex. Further study showed that it is not specific to snR30, as it was also coisolated with a second H/ACA snoRNP (snR3) and with two C/D snoRNP complexes (data not shown). In addition, each of six snoRNAs examined was found to coimmunoprecipitate with an HA-tagged variant of Has1p, suggesting that snoRNP association is widespread for Has1p. Pre-rRNA was also detected in the snR30 snoRNP preparation and in immunoprecipitated Has1p. This situation could stem from the association of Has1p with pre-rRNA complexes, which has been documented (11, 17, 39), and that association could reflect the specificity of Has1p for pre-rRNA or snoRNPs bound to pre-rRNA. Thus, the interaction of Has1p with snoRNPs might be indirect; however, it is also possible that Has1p directly contacts snoRNPs and also pre-rRNA to participate in snoRNP function.

Has1p is not required for snoRNA formation or stability (11); however, we find that its depletion causes all snoRNAs tested to accumulate in pre-rRNA complexes. Importantly, the accumulated snoRNAs, such as U3 and U14, remain associated with 23S and 35S pre-rRNAs in the absence of Has1p, even after deproteinization (Fig. 4), indicating that Has1p could function in a snoRNP release mechanism(s). This is further supported by the fact that Has1p with defective ATPase or helicase function cannot release the U14 snoRNP from pre-rRNA (Fig. 9).

Notably, depletion of Has1p leads to enrichment of both C/D and H/ACA snoRNAs with pre-rRNA (Fig. 3). The enriched snoRNAs, which function as snoRNPs, bind to either 18S or 25S rRNA, indicating that the snoRNAs affected by Has1p depletion are not restricted to a single family or functional type or to a particular pre-rRNA region. The results suggest that Has1p depletion affects the release of many, if not all, snoRNAs from pre-rRNA. Since Has1p is involved in the release of snoRNAs from the 25S rRNA region of pre-rRNA,

this may be part of the reason that loss of Has1p delays the production of 25S rRNA (11).

The defect in snoRNA release with Has1p depletion is not a secondary effect of a deficiency in rRNA. Depletion of Esf1p, which is also required for 18S rRNA production (41), has a pre-rRNA processing defect similar to that of Has1p. However, depletion of Esf1p causes the U14 and snR10 snoRNAs to accumulate in the 60S-90S pre-rRNA region, but not the other snoRNAs examined, including U3. Depletion of Dim2p, which is also involved in 18S rRNA maturation (51), has no effect on snoRNA accumulation in pre-rRNP complexes (Fig. 5). These data indicate that the Has1p depletion defect on snoRNA association with pre-rRNA is specific to that protein. The snoRNA accumulation defect is not due simply to accumulation of 35S pre-rRNA itself. Depletion of Esf1p and Dim2p causes similar enhancements of 35S pre-rRNA accumulation but not the same pattern or similar patterns of snoRNA accumulation (Fig. 6). Taken together, these data argue that Has1p is required for snoRNA release. However, we cannot rule out the possibility that this defect results from an indirect effect of Has1p deficiency. For example, interference with an upstream process, such as pre-rRNP remodeling, is a possibility.

Recently, another RNA helicase-like protein, Dbp4p, was shown to be involved in snoRNA release from large pre-rRNA complexes (27). This protein was identified earlier as a suppressor of a lethal mutation in U14 snoRNA and shown to be required for production of 18S rRNA (32). Depletion of Dbp4p results in accumulation of U14 in preribosome complexes. In addition, a second C/D snoRNA (snR41) that targets methylation to 18S rRNA also accumulated in similar complexes. However, depletion of other RNA helicases, including Dbp8p, Dhr1p, Dhr2p, and Rok1p, which are all involved in 18S rRNA processing, did not cause accumulation of any snoRNA tested in the large pre-rRNA complex (27). The results from that study provide additional strong support that the defects observed here for Has1p are specific and that at least two helicases are involved in snoRNP release from pre-rRNP complexes.

The release of snoRNPs is likely an intricate process because of the complexity and dynamic nature of the evolving substrates and interfacing of the processes of rRNA transcription, maturation, and ribosome assembly. As demonstrated in this study, Has1p is involved in the release of all eight snoRNAs tested from pre-rRNA, with the strongest effect on release of the U3 and U14 RNAs. However, depletion of Esf1p also causes strong accumulation of U14 and snR10 in the 60S-90S pre-rRNP fractions but not the other snoRNAs examined. Previous work showed that depletion of yet another protein required for 18S rRNA processing, Esf2p, leads to accumulation of U3, snR3, and, to a lesser extent, U14 in 60S-90S complexes but not in the 40S pre-rRNA region (22). This pattern differs from those of Has1p and Esf1p depletion and suggests that Esf2p may function before cleavage of 35S rRNA into small- and large-subunit pre-rRNAs. As noted, depletion of the Dbp4p helicase causes the U14 and snR41 C/D snoRNAs to accumulate with pre-rRNA (27). Together, these findings indicate that snoRNA release is indeed complicated.

Depletion of proteins that influence snoRNA accumulation with pre-rRNA can result in the enrichment of different snoRNA species. One interpretation for the different patterns is that the individual snoRNAs are released from pre-rRNA in multiple steps, by the same or different processes, and these are affected in different ways. In this context, U14 seems associated with pre-rRNP for a longer period of time than other snoRNAs. This could be due to involvement with multiple pre-rRNP complexes in the processing pathway. Indeed, we found that U14 remains associated with both 23S and 35S pre-rRNA complexes after depletion of Has1p (Fig. 3 and 4), and earlier results showed cosedimentation of U14 with 27S pre-rRNA in normal cells (56). Because the U14 snoRNP functions in both processing and methylation, it may be released by more than one process.

The findings that loss of nonhelicase proteins Esf1p and Esf2p also results in accumulation of snoRNAs in pre-rRNP complexes indicate that the release of snoRNAs from pre-rRNA is not based solely on RNA helicase activity. From this study and those described above, it seems likely that snoRNA release from pre-rRNA requires multiple factors that function in a coordinated way. These factors could include both RNA helicases, such as Has1p and Dbp4p, and nonhelicase proteins, such as Esf1p and Esf2p. Affinity selection has demonstrated that Nop1/fibrillarin and Cbf5p can be affinity selected using Esf1p as bait, suggesting that Esf1p interacts directly or indirectly with snoRNPs (28, 41). Based on these early studies, more proteins involved in snoRNA release are likely to be identified. Thus far, Has1p, Dbp4p, and Esf1p have all been shown by affinity isolation to interact with the protein Esf2p (22), and all four proteins are required for normal snoRNA release. Several other proteins associated with Esf2p have also been identified by affinity selection (22). These include Lcp5 and Rrp5p, both of which are required for rRNA processing (52, 54), Enp2 and Bfr2, which are not well characterized, and six U3-specific proteins, suggesting that a network of proteins is required for snoRNP release. It will be important to test these proteins for roles in this process and to continue to search for other participating components.

The partial reduction in U6 snRNA level that accompanies Has1p depletion or mutation suggests that Has1p has multiple functions. The defect in U6 snRNA stability is not a secondary effect of an rRNA or ribosome deficiency, since depletion of Esf1p and Dim2p also affects ribosome maturation in similar ways but does not affect U6 stability. Based on immunoprecipitation screening, Has1p is not specifically associated with U6. Notably, loss of Has1p affects the level of free U6 snRNP but not the formation of the U4.U6/U5 trimeric complex (Fig. 8). Has1p does not appear to be involved in U6 function or recycling, as no defect in pre-mRNA splicing was detected under the depletion condition and no difference was observed in the fractionation patterns for the other splicing snRNAs. Thus, Has1p is most probably involved in some aspect of U6 snRNP biogenesis, directly or indirectly. The U6 snRNA is distinct from other splicing snRNAs in several important ways, and these differences may explain why Has1p loss affects accumulation of U6 but not the other snRNAs. Notably, U6 RNA is transcribed by RNA polymerase III (6) whereas the other snRNAs are expressed by RNA polymerase II. In addition, during its assembly into an snRNP, U6 snRNA associates with a set of Sm-like (Lsm) proteins but not the Sm core proteins that are common for the other four snRNAs (45).

Our observation that Has1p influences the accumulation of U6 snRNA in addition to the maturation of rRNA suggests that its substrate specificity extends beyond pre-rRNA and snoRNP complexes. This finding also raises the possibility that ribosome and mRNA syntheses are linked at some level. A similar situation exists for the DEAH box helicase Prp43p, which has been shown to be involved in both mRNA splicing and maturation of rRNA (1, 8, 29, 30, 35). Other RNA helicases involved in rRNA synthesis may prove to have similarly interesting effects.

Defining the events that bring about the release of snoRNPs from rRNA and other substrates is an especially interesting problem. The findings from this study, together with those from two other recent reports (22, 27), suggest that snoRNP release (and perhaps binding) involves both helicase and nonhelicase proteins that function in a complex network. Elucidating the mechanisms by which snoRNP association and dissociation occur is an important challenge for the future.

ACKNOWLEDGMENTS

We are grateful for test strains kindly provided by Patrick Linder (Has1p), Timothy R. Hughes (Esf1p), and Denis L. J. Lafontaine (Dim2p). We thank Ben Liu, formerly in our laboratory, for sharing his snoRNA affinity tagging strategy prior to publication and Dorota Piekna-Przybylska for sharing her experience with this technology. We thank Qing Liu for helping with some experiments and for making the Has1p mutant strains and all other laboratory members for stimulating discussions.

This study was supported by a grant from the NIH (GM19351) to M.J.F.

REFERENCES

1. **Arenas, J. E., and J. N. Abelson.** 1997. Prp43: an RNA helicase-like factor involved in spliceosome disassembly. Proc. Natl. Acad. Sci. USA **94:**11798–11802.
2. **Bachellerie, J. P., J. Cavaillé, and A. Hüttenhofer.** 2002. The expanding snoRNA world. Biochimie **84:**775–790.
3. **Bachler, M., R. Schroeder, and U. von Ahsen.** 1999. StreptoTag: a novel method for the isolation of RNA-binding proteins. RNA **5:**1509–1516.
4. **Bertrand, E., and M. J. Fournier.** 2004. The snoRNPs and related machines: ancient devices that mediate maturation of rRNA and other RNAs, p. 225–261. *In* M. O. J. Olson (ed.), The nucleolus. Landes Bioscience Publishing, Austin, Tex.
5. **Billy, E., T. Wegierski, F. Nasr, and W. Filipowicz.** 2000. Rcl1p, the yeast protein similar to the RNA 3′-phosphate cyclase, associates with U3 snoRNP and is required for 18S rRNA biogenesis. EMBO J. **19:**2115–2126.
6. **Brow, D. A., and C. Guthrie.** 1990. Transcription of a yeast U6 snRNA gene requires a polymerase III promoter element in a novel position. Genes Dev. **4:**1345–1356.
7. **Cavaillé, J., M. Nicoloso, and J. P. Bachellerie.** 1996. Targeted ribose methylation of RNA in vivo directed by tailored antisense RNA guides. Nature **383:**732–735.
8. **Combs, D. J., R. J. Nagel, M. Ares, Jr., and S. W. Stevens.** 2006. Prp43p is a DEAH-box spliceosome disassembly factor essential for ribosome biogenesis. Mol. Cell. Biol. **26:**523–534.
9. **Decatur, W. A., and M. J. Fournier.** 2003. RNA-guided nucleotide modification of ribosomal and other RNAs. J. Biol. Chem. **278:**695–698.
10. **Dragon, F., J. E. Gallagher, P. A. Compagnone-Post, B. M. Mitchell, K. A. Porwancher, K. A. Wehner, S. Wormsley, R. E. Settlage, J. Shabanowitz, Y. Osheim, A. L. Beyer, D. F. Hunt, and S. J. Baserga.** 2002. A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. Nature **417:**967–970.
11. **Emery, B., J. de la Cruz, S. Rocak, O. Deloche, and P. Linder.** 2004. Has1p, a member of the DEAD-box family, is required for 40S ribosomal subunit biogenesis in *Saccharomyces cerevisiae*. Mol. Microbiol. **52:**141–158.
12. **Fabrizio, P., S. Esser, B. Kastner, and R. Lührmann.** 1994. Isolation of *S. cerevisiae* snRNPs: comparison of U1 and U4/U6.U5 to their human counterparts. Science **264:**261–265.
13. **Fatica, A., and D. Tollervey.** 2002. Making ribosomes. Curr. Opin. Cell Biol. **14:**313–318.
14. **Filipowicz, W., and V. Pogacic.** 2002. Biogenesis of small nucleolar ribonucleoproteins. Curr. Opin. Cell Biol. **14:**319–327.
15. **Ganot, P., M. L. Bortolin, and T. Kiss.** 1997. Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. Cell **89:**799–809.
16. **Gavin, A. C., M. Bösche, R. Krause, P. Grandi, M. Marzioch, A. Bauer, J. Schultz, J. M. Rick, A. M. Michon, C. M. Cruciat, M. Remor, C. Höfert, M. Schelder, M. Brajenovic, H. Ruffner, A. Merino, K. Klein, M. Hudak, D. Dickson, T. Rudi, V. Gnau, A. Bauch, S. Bastuck, B. Huhse, C. Leutwein, M. A. Heurtier, R. R. Copley, A. Edelmann, E. Querfurth, V. Rybin, G. Drewes, M. Raida, T. Bouwmeester, P. Bork, B. Seraphin, B. Kuster, G. Neubauer, and G. Superti-Furga.** 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature **415:**141–147.
17. **Grandi, P., V. Rybin, J. Bassler, E. Petfalski, D. Strauss, M. Marzioch, T. Schäfer, B. Kuster, H. Tschochner, D. Tollervey, A. C. Gavin, and E. Hurt.** 2002. 90S pre-ribosomes include the 35S pre-rRNA, the U3 snoRNP, and 40S subunit processing factors but predominantly lack 60S synthesis factors. Mol. Cell **10:**105–115.
18. **Granneman, S., and S. J. Baserga.** 2005. Crosstalk in gene expression: coupling and regulation of rDNA transcription, pre-ribosome assembly and pre-rRNA processing. Curr. Opin. Cell Biol. **17:**281–286.
19. **Granneman, S., J. E. Gallagher, J. Vogelzangs, W. Horstman, W. J. van Venrooij, S. J. Baserga, and G. J. Pruijn.** 2003. The human Imp3 and Imp4 proteins form a ternary complex with hMpp10, which only interacts with the U3 snoRNA in 60-80S ribonucleoprotein complexes. Nucleic Acids Res. **31:**1877–1887.
20. **Hall, K. B., and W. T. Stump.** 1992. Interaction of N-terminal domain of U1A protein with an RNA stem/loop. Nucleic Acids Res. **20:**4283–4290.
21. **Harnpicharnchai, P., J. Jakovljevic, E. Horsey, T. Miles, J. Roman, M. Rout, D. Meagher, B. Imai, Y. Guo, C. J. Brame, J. Shabanowitz, D. F. Hunt, and J. L. Woolford, Jr.** 2001. Composition and functional characterization of yeast 66S ribosome assembly intermediates. Mol. Cell **8:**505–515.
22. **Hoang, T., W.-T. Peng, E. Vanrobays, N. Krogan, S. Hiley, A. L. Beyer, Y. N. Osheim, J. Greenblatt, T. R. Hughes, and D. L. J. Lafontaine.** 2005. Esf2p, a U3-associated factor required for small-subunit processome assembly and compaction. Mol. Cell. Biol. **25:**5523–5534.
23. **Hughes, J. M., and M. Ares, Jr.** 1991. Depletion of U3 small nucleolar RNA inhibits cleavage in the 5′ external transcribed spacer of yeast pre-ribosomal RNA and impairs formation of 18S ribosomal RNA. EMBO J. **10:**4231–4239.
24. **Kinzy, T. G., T. L. Ripmaster, and J. L. Woolford, Jr.** 1994. Multiple genes encode the translation elongation factor EF-1 gamma in *Saccharomyces cerevisiae*. Nucleic Acids Res. **22:**2703–2707.
25. **Kiss, T.** 2002. Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. Cell **109:**145–148.
26. **Kiss-László, Z., Y. Henry, J. P. Bachellerie, M. Caizergues-Ferrer, and T. Kiss.** 1996. Site-specific ribose methylation of preribosomal RNA: a novel function for small nucleolar RNAs. Cell **85:**1077–1088.
27. **Kos, M., and D. Tollervey.** 2005. The putative RNA helicase Dbp4p is required for release of the U14 snoRNA from preribosomes in *Saccharomyces cerevisiae*. Mol. Cell **20:**53–64.
28. **Krogan, N. J., G. Cagney, H. Yu, G. Zhong, X. Guo, A. Ignatchenko, J. Li, S. Pu, N. Datta, A. P. Tikuisis, T. Punna, J. M. Peregrín-Alvarez, M. Shales, X. Zhang, M. Davey, M. D. Robinson, A. Paccanaro, J. E. Bray, A. Sheung, B. Beattie, D. P. Richards, V. Canadien, A. Lalev, F. Mena, P. Wong, A. Starostine, M. M. Canete, J. Vlasblom, S. Wu, C. Orsi, S. R. Collins, S. Chandran, R. Haw, J. J. Rilstone, K. Gandi, N. J. Thompson, G. Musso, P. St. Onge, S. Ghanny, M. H. Lam, G. Butland, A. M. Altaf-Ul, S. Kanaya, A. Shilatifard, E. O'Shea, J. S. Weissman, C. J. Ingles, T. R. Hughes, J. Parkinson, M. Gerstein, S. J. Wodak, A. Emili, and J. F. Greenblatt.** 2006. Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. Nature **440:**637–643.
29. **Lebaron, S., C. Froment, M. Fromont-Racine, J.-C. Rain, B. Monsarrat, M. Caizergues-Ferrer, and Y. Henry.** 2005. The splicing ATPase Prp43p is a component of multiple preribosomal particles. Mol. Cell. Biol. **25:**9269–9282.
30. **Leeds, N. B., E. C. Small, S. L. Hiley, T. R. Hughes, and J. P. Staley.** 2006. The splicing factor Prp43p, a DEAH box ATPase, functions in ribosome biogenesis. Mol. Cell. Biol. **26:**513–522.
31. **Li, H. D., J. Zagorski, and M. J. Fournier.** 1990. Depletion of U14 small nuclear RNA (snR128) disrupts production of 18S rRNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**1145–1152.
32. **Liang, W.-Q., J. A. Clark, and M. J. Fournier.** 1997. The rRNA-processing function of the yeast U14 small nucleolar RNA can be rescued by a conserved RNA helicase-like protein. Mol. Cell. Biol. **17:**4124–4132.
33. **Liang, W.-Q., and M. J. Fournier.** 1995. U14 base-pairs with 18S rRNA: a novel snoRNA interaction required for rRNA processing. Genes Dev. **9:**2433–2443.
34. **Lygerou, Z., C. Allmang, D. Tollervey, and B. Seraphin.** 1996. Accurate processing of a eukaryotic precursor ribosomal RNA by ribonuclease MRP in vitro. Science **272:**268–270.
35. **Martin, A., S. Schneider, and B. Schwer.** 2002. Prp43 is an essential RNA-dependent ATPase required for release of lariat-intron from the spliceosome. J. Biol. Chem. **277:**17743–17750.

36. **Morrissey, J. P., and D. Tollervey.** 1993. Yeast snR30 is a small nucleolar RNA required for 18S rRNA synthesis. Mol. Cell. Biol. **13:**2469–2477.
37. **Nazar, R. N.** 2004. Ribosomal RNA processing and ribosome biogenesis in eukaryotes. IUBMB Life **56:**457–465.
38. **Ni, J., A. L. Tien, and M. J. Fournier.** 1997. Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA. Cell **89:**565–573.
39. **Nissan, T. A., J. Bassler, E. Petfalski, D. Tollervey, and E. Hurt.** 2002. 60S pre-ribosome formation viewed from assembly in the nucleolus until export to the cytoplasm. EMBO J. **21:**5539–5547.
40. **Peculis, B. A., and J. A. Steitz.** 1993. Disruption of U8 nucleolar snRNA inhibits 5.8S and 28S rRNA processing in the Xenopus oocyte. Cell **73:**1233–1245.
41. **Peng, W. T., N. J. Krogan, D. P. Richards, J. F. Greenblatt, and T. R. Hughes.** 2004. ESF1 is required for 18S rRNA synthesis in *Saccharomyces cerevisiae*. Nucleic Acids Res. **32:**1993–1999.
42. **Rocak, S., B. Emery, N. K. Tanner, and P. Linder.** 2005. Characterization of the ATPase and unwinding activities of the yeast DEAD-box protein Has1p and the analysis of the roles of the conserved motifs. Nucleic Acids Res. **33:**999–1009.
43. **Schattner, P., W. A. Decatur, C. A. Davis, M. Ares, Jr., M. J. Fournier, and T. M. Lowe.** 2004. Genome-wide searching for pseudouridylation guide snoRNAs: analysis of the *Saccharomyces cerevisiae* genome. Nucleic Acids Res. **32:**4281–4296.
44. **Schmitt, M. E., and D. A. Clayton.** 1993. Nuclear RNase MRP is required for correct processing of pre-5.8S rRNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**7935–7941.
45. **Seraphin, B.** 1995. Sm and Sm-like proteins belong to a large family: identification of proteins of the U6 as well as the U1, U2, U4 and U5 snRNPs. EMBO J. **14:**2089–2098.
46. **Sharma, K., and D. Tollervey.** 1999. Base pairing between U3 small nucleolar RNA and the 5′ end of 18S rRNA is required for pre-rRNA processing. Mol. Cell. Biol. **19:**6012–6019.
47. **Sharp, P. A.** 1994. Split genes and RNA splicing. Cell **77:**805–815.
48. **Tollervey, D.** 1987. A yeast small nuclear RNA is required for normal processing of pre-ribosomal RNA. EMBO J. **6:**4169–4175.
49. **Tycowski, K. T., M. D. Shu, and J. A. Steitz.** 1994. Requirement for intron-encoded U22 small nucleolar RNA in 18S ribosomal RNA maturation. Science **266:**1558–1561.
50. **Tycowski, K. T., C. M. Smith, M. D. Shu, and J. A. Steitz.** 1996. A small nucleolar RNA requirement for site-specific ribose methylation of rRNA in *Xenopus*. Proc. Natl. Acad. Sci. USA **93:**14480–14485.
51. **Vanrobays, E., J. P. Gélugne, M. Caizergues-Ferrer, and D. L. Lafontaine.** 2004. Dim2p, a KH-domain protein required for small ribosomal subunit synthesis. RNA **10:**645–656.
52. **Venema, J., and D. Tollervey.** 1996. RRP5 is required for formation of both 18S and 5.8S rRNA in yeast. EMBO J. **15:**5701–5714.
53. **Wegierski, T., E. Billy, F. Nasr, and W. Filipowicz.** 2001. Bms1p, a G-domain-containing protein, associates with Rcl1p and is required for 18S rRNA biogenesis in yeast. RNA **7:**1254–1267.
54. **Wiederkehr, T., R. F. Prétôt, and L. Minvielle-Sebastia.** 1998. Synthetic lethal interactions with conditional poly(A) polymerase alleles identify LCP5, a gene involved in 18S rRNA maturation. RNA **4:**1357–1372.
55. **Will, C. L., and R. Lührmann.** 2001. Spliceosomal UsnRNP biogenesis, structure and function. Curr. Opin. Cell Biol. **13:**290–301.
56. **Zagorski, J., D. Tollervey, and M. J. Fournier.** 1988. Characterization of an SNR gene locus in *Saccharomyces cerevisiae* that specifies both dispensible and essential small nuclear RNAs. Mol. Cell. Biol. **8:**3282–3290.