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Identification of Genes That Function in the Biogenesis and Localization of Small Nucleolar RNAs in *Saccharomyces cerevisiae*▽

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Small nucleolar RNAs (snoRNAs) orchestrate the modification and cleavage of pre-rRNA and are essential for ribosome biogenesis. Recent data suggest that after nucleoplasmic synthesis, snoRNAs transiently localize to the Cajal body (in plant and animal cells) or the homologous nucleolar body (in budding yeast) for maturation and assembly into snoRNPs prior to accumulation in their primary functional site, the nucleolus. However, little is known about the *trans*-acting factors important for the intranuclear trafficking and nucleolar localization of snoRNAs. Here, we describe a large-scale genetic screen to identify proteins important for snoRNA transport in *Saccharomyces cerevisiae*. We performed fluorescence in situ hybridization analysis to visualize U3 snoRNA localization in a collection of temperature-sensitive yeast mutants. We have identified Nop4, Prp21, Tao3, Sec14, and Htl1 as proteins important for the proper localization of U3 snoRNA. Mutations in genes encoding these proteins lead to specific defects in the targeting or retention of the snoRNA to either the nucleolar body or the nucleolus. Additional characterization of the mutants revealed impairment in specific steps of U3 snoRNA processing, demonstrating that snoRNA maturation and trafficking are linked processes.

After synthesis in the nucleus, all RNA species undergo a series of maturation steps and transport from the site of synthesis to the site of action, and it is increasingly clear that RNA biogenesis and trafficking are closely linked (54). Whether they are destined to function in the cytoplasm (e.g., rRNA, tRNA, mRNA) or the nucleus (e.g., snRNAs or snoRNAs), all RNAs undergo intranuclear trafficking. It is well established that interactions between *trans*-acting factors and specific features of RNA substrates can govern which transport pathways will be utilized (38, 59, 73). However, little is known about the intranuclear trafficking factors and how they affect the molecular movement of RNAs within the nucleus.

Small nucleolar RNAs (snoRNAs) provide excellent models to study intranuclear RNA transport. snoRNAs remain within the nucleus, where they undergo biogenesis including covalent alterations (e.g., 5′ cap hypermethylation and 3′-end processing), as well as assembly with specific proteins into stable and functional RNP complexes (snoRNPs) (40, 54, 70, 84). Ultimately, snoRNAs are targeted to nucleoli, where they function in rRNA maturation.

The numerous snoRNA species fall into two structurally and functionally distinct classes called the box C/D and box H/ACA snoRNAs (40, 54, 70, 84). Species of both RNA classes interact with pre-rRNA (via base pairing), mainly to guide site-specific nucleotide modification. The majority of box C/D snoRNAs orchestrate 2′-*O*-methylation (12, 41, 65, 89), while the bulk of the box H/ACA RNA species guide pseudouridylation (21, 64). A few RNAs of both classes (e.g., box C/D snoRNA U3 and box H/ACA snoRNA U17 [vertebrates]/snR30 [yeast]) are critical for promoting specific endonucleolytic cleavages of pre-rRNA transcripts required to generate mature 18S rRNA for the ribosome (33, 57).

Box C/D and box H/ACA snoRNAs stably associate with two distinct sets of four proteins. All box C/D snoRNAs bind to the 2′-*O*-methyltransferase fibrillarin/Nop1 (18, 78, 86, 88), as well as Nop56 (22, 71), Nop58 (47, 53), and 15.5-kDa protein/Snu13 (83, 94), while box H/ACA snoRNAs bind to the pseudouridine synthase dyskerin/Cbf5 (46, 92) and Gar1 (26), Nop10 (28), and Nhp2 (27, 28, 92). The association of core C/D or H/ACA RNP proteins is essential for the stability and/or proper nucleolar targeting of the snoRNAs (28, 46, 91–93).

Several studies have highlighted the role of intranuclear structures called Cajal bodies in snoRNP biogenesis (62, 77, 90). Cajal bodies are spherical structures present in vertebrate, invertebrate, and plant nuclei (13, 19, 20). In addition to containing several Cajal body-specific markers such as coilin, survival of motor neuron (SMN) protein, small Cajal body RNAs, and U7 snRNA, Cajal bodies also house many nucleolar constituents, including snoRNAs, fibrillarin, and Nopp140, and are often localized adjacent to and sometimes within the nucleolus (13, 19, 20). Box C/D snoRNAs injected into *Xenopus*

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oocytes transiently associate with Cajal bodies prior to nucleoli, and mutant snoRNAs that fail to be targeted to nucleoli accumulate within Cajal bodies, indicating that Cajal bodies may serve as important sites for maturation and assembly of snoRNPs (62). Model box C/D snoRNAs ectopically expressed in mammalian cells localize to Cajal bodies (as well as nucleoli) (77). Precursors of U3 (bearing 5′ monomethyl caps and extended 3′ ends) localize to U3 gene transcription sites and Cajal bodies, but not to nucleoli, whereas mature forms of U3 (with 5′ trimethyl caps and properly processed 3′ ends) are detected primarily at nucleoli (90). Together with the finding that the enzyme responsible for cap hypermethylation (TGS1) is enriched in Cajal bodies (90), these observations suggest that U3 maturation (including 5′ cap hypermethylation and 3′-end processing) likely takes place in Cajal bodies.

An intranucleolar structure that appears to be functionally homologous to the Cajal body was recently described in budding yeast (91). The yeast "nucleolar body" shares several important features with Cajal bodies. First, overexpressed box C/D snoRNAs specifically accumulate within the nucleolar body (91). Second, when the Cajal body-specific human SMN protein is ectopically expressed in yeast, it specifically localizes to the nucleolar body (91). Third, U3 precursors accumulate within the nucleolar body while mature U3 is enriched in the nucleolus and excluded from the nucleolar body (90). Finally, the 5′ cap hypermethylase (Tgs1) accumulates in the nucleolar body (58, 90). Collectively, these data suggest that yeast nucleolar bodies function homologously to plant and animal cell Cajal bodies as sites for snoRNA maturation.

The *cis*-acting elements required for nucleolar localization of snoRNAs have been identified by different groups using several experimental systems, including *Xenopus* oocytes, yeast, and mammalian cells. The box C/D motif and box H/ACA motif (binding sites for the core snoRNP proteins) were each shown to be necessary and sufficient for the nucleolar localization of box C/D snoRNAs (48, 50, 62, 77) and box H/ACA snoRNAs (49, 61, 76), respectively. While multiple proteins have been implicated in the assembly and/or transport of snoRNPs, including SMN (35, 66, 96), nucleolin/Nsr1 (25, 91), Nopp140/Srp40 (91, 101), p55/Rvb1 (63, 93), p50/Rvb2 (39, 63, 93), BCD1 (30, 67), PHAX (9), CRM1 (9), Naf1, and Shq1 (4, 15, 17, 99, 100), no systematic analysis has been undertaken to identify the *trans*-acting factors crucial for snoRNA trafficking within the nucleus. In this study, we carried out a genetic screen to identify the gene products important in the subnuclear localization of U3 snoRNA in *Saccharomyces cerevisiae*. We identified five genes, *NOP4*, *PRP21*, *TAO3*, *SEC14*, and *HTL1*, which are implicated in the biogenesis and proper nucleolar transport of snoRNAs for the first time. Further analyses indicate that these *trans*-acting factors are important for the accumulation of snoRNAs in either the nucleolar body or nucleoli.

MATERIALS AND METHODS

Yeast strains and plasmids. The yeast temperature-sensitive mutant collection was generated by UV mutagenesis from parental strains FY23 (*MAT***a** *ura3-52 trp1Δ63 leu2Δ1*) and FY86 (*MATα ura3-52 his3Δ200 leu2Δ1*) in Charles Cole's laboratory (1). The *gsp1-2* temperature-sensitive mutant was described previously (60, 98). Two YCp50-based (*URA3 CEN4 ARS1*) yeast genomic libraries (45, 75) were used for complementing the temperature-sensitive mutants. The plasmid overexpressing an artificial box C/D snoRNA (pG14-U14/MS2X2, 2μ) was obtained from Edouard Bertrand (91).

FISH and immunofluorescence. Fluorescence in situ hybridization (FISH) analysis and immunofluorescence analysis in yeast were performed as previously described (60). The antisense deoxyoligonucleotide probes against yeast U3 snoRNA (5′ATTCAGTGGCTCTTTTGAAGAGTCAAAGAGTGACGATTCCTATAGAAATGA3′) and poly(A) mRNA [oligo(dT)50] were synthesized by Operon Technologies with a single Cy3 label at the 5′ end. The aminoallyl-modified snR30 probe (GGTT[AmC6~dT]TACCCAAATGA[AmC6~dT]CATGGACCAAC[AmC6~dT]AGGGTCATTTCA[AmC6~dT]AGTGTTTAGGAA[AmC6~dT]A) and the aminoallyl-modified U3 probe (T[AmC6~dT]GGATTCAGTGGC[AmC6~dT]CTTTTGAAGAG[AmC6~dT]CAAAGAGTGACGA[AmC6~dT]TCCTATAGAAA[AmC6~dT]G) were synthesized by Operon Technologies and labeled with Oregon green 488-X reactive dye according to the manufacturer's (Molecular Probes/Invitrogen) protocol. The Cy3-labeled U14 probes were described previously (29). The MS2 probe used to detect an ectopically expressed artificial C/D snoRNA that serves as a nucleolar body marker was described previously (91). Briefly, the strains were grown in yeast extract-peptone-dextrose medium at 25°C and shifted to 37°C for 4 h. The cells were fixed with formaldehyde and collected by centrifugation. The harvested cells were spheroplasted with 300 μg of Zymolyase 100T (U.S. Biological), resuspended in P solution (1.2 M sorbitol in 0.1 M potassium phosphate buffer, pH 6.5), and applied to 14-well Teflon-faced microscope slides (CellPoint Scientific, Inc.) precoated with 0.1% polylysine. After the cells were permeabilized with 0.5% IGEPAL, hybridization was performed with 150 ng of Cy3-labeled probe overnight at 37°C. In some experiments, indirect immunofluorescence was performed following the FISH analysis as described previously (98), with a 1:1,000 dilution of anti-Nop1p (A66) monoclonal antibody (3) or a 1:5,000 dilution of anti-Nsr1p monoclonal antibody (C21) and a 1:100 dilution of Cy2-conjugated anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories). Cells were stained with 1 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI), air dried, and mounted in 1 mg *p*-phenylenediamine/ml 90% glycerol in 1× phosphate-buffered saline. Cells were viewed with a Zeiss Axioskop 2 Mot Plus fluorescence microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were acquired at a magnification of ×100 (Plan Apochromat objective, numerical aperture 1.4) with a cooled charge-coupled device Retiga Exi Fast 1394 camera (Qimaging, Burnaby, British Columbia, Canada) and IPLab Spectrum software.

Mutant screen. Approximately 1,100 temperature-sensitive mutants were screened for U3 snoRNA localization by FISH. To screen the large collection of mutants, a pool of three mutants was grown to log phase at 25°C and shifted to 37°C for 4 h prior to FISH analysis. When a pool with phenotypes of U3 mislocalization was identified, individual strains in the pool were reexamined by FISH and the phenotype of each identified mutant was confirmed by an additional, independent experiment.

Tetrad analysis. In order to determine if a selected mutant contains a mutation in a single gene or mutations in more than one gene, tetrad analysis was performed. A selected mutant was crossed with a wild-type parental strain of the opposite mating type (either strain FY23 or FY86 [1] [described in the yeast strain and plasmid section]). The resulting diploid was sporulated, and temperature-sensitive growth and U3 localization at the nonpermissive temperature (37°C) were analyzed in each of the four haploid progeny. Only those mutants that showed 2:2 segregation of the temperature-sensitive phenotype linked to U3 mislocalization were selected for subsequent analysis.

Identification of genes. A yeast YCp50-based (*URA3 CEN4*) genomic library (45, 75) was introduced into the temperature-sensitive mutants, and transformants that grew at 37°C on plates lacking uracil were selected. Plasmids were extracted from the complemented transformants and transformed back into the corresponding temperature-sensitive mutant. The complementation of the temperature-sensitive growth and snoRNA mislocalization phenotype (by FISH) by the isolated plasmid was confirmed. The insert was identified by partial sequencing and an NCBI BLAST search.

Complementation of the U3 mislocalization phenotypes. Each temperature-sensitive mutant was transformed with a plasmid containing a wild-type copy of the relevant gene, and complementation of both the temperature-sensitive growth phenotype and the U3 snoRNA mislocalization phenotype was confirmed. The plasmids expressing Nop4 (pRS316NOP4), Tao3 (2μ URA3 TAO3), and Htl1 (pRS316HTL1) were described previously (16, 74, 81). The plasmid expressing Prp21 (pRS316-*PRP21*) was constructed in our laboratory and contains chromosome X, 52030 to 55454, which includes the complete *PRP21* gene and partial (presumably nonfunctional) sequences of the *RCY1* and *ECM25* genes, as well as a dubious gene, *YJL202C*, which is unlikely to encode a functional protein based on available experimental and comparative data (10, 37). The plasmid expressing Sec14 (pRS316-*SEC14*) was constructed in our laboratory and contains chromosome XIII, 422909 to 428713, which contains the complete *SEC14* gene and partial *CTF18* and *NAM7* gene fragments.

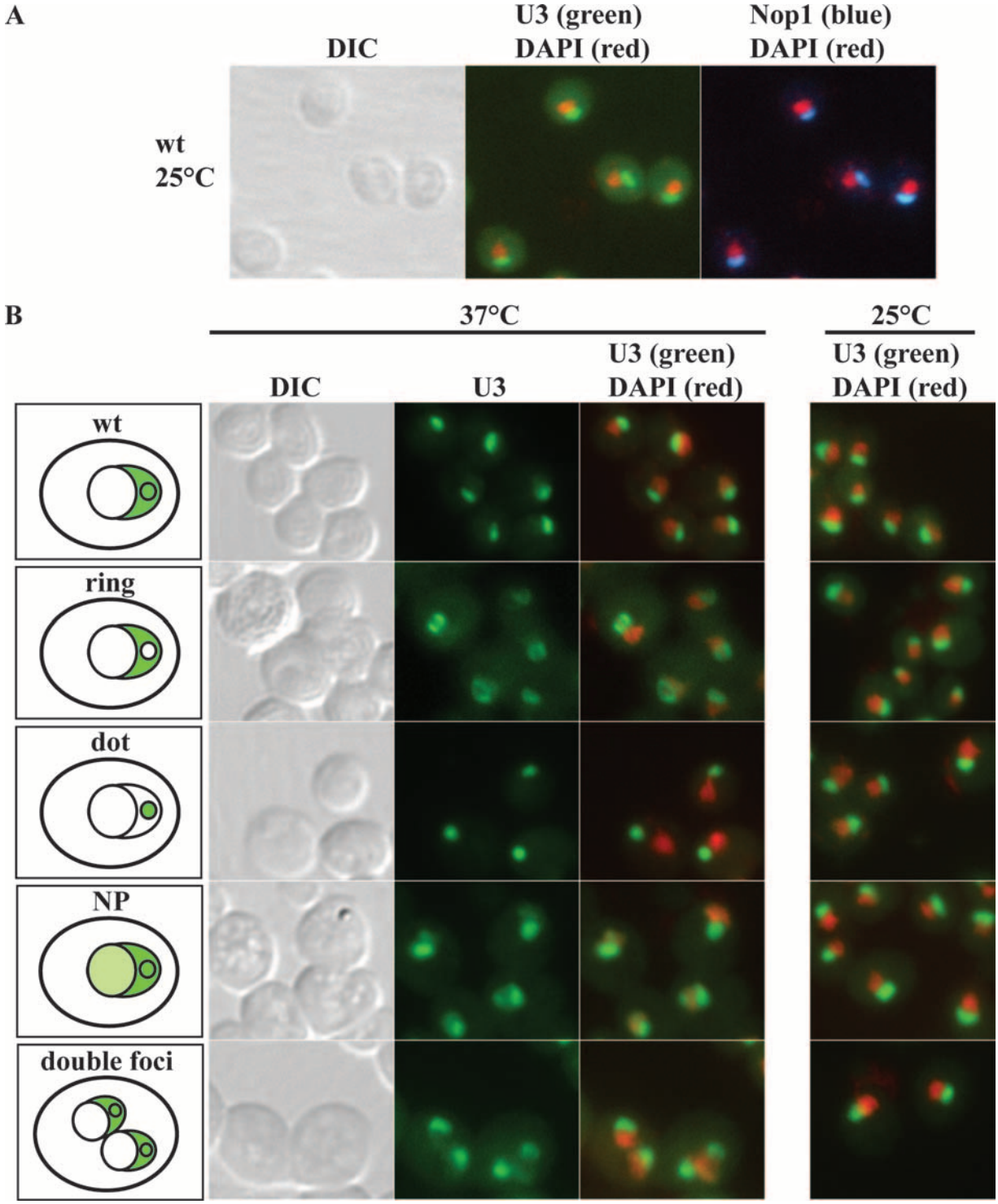

FIG. 1. Aberrant patterns of U3 snoRNA localization detected in temperature-sensitive mutants at the restrictive temperature. Yeast cells were fixed and hybridized with Cy3-labeled anti-U3 probe (green). The nucleolar protein Nop1 was detected by immunofluorescence with an anti-Nop1 antibody (blue). DAPI was used to stain DNA and indicate the location of the nucleoplasm (red). Corresponding DIC (differential interference contrast) images are shown. (A) U3 snoRNA is normally detected in the nucleoli of *S. cerevisiae* by FISH. (B) FISH analysis was performed on yeast temperature-sensitive mutants following a 4-h shift from 25°C to 37°C (37°C panels). Representative phenotypes are shown. The 25°C panels show the nucleolar localization of U3 in the yeast temperature-sensitive mutants at the permissive temperature (25°C). The illustrations on the left show the relationship of the patterns to the nucleolus (crescent) and nucleolar body (small circle) based on the subsequent analysis presented in this work. wt, wild type; NP, nucleoplasm.

TABLE 1. Genes identified from the U3 snoRNA mislocalization yeast mutants

Gene name	U3 snoRNA mislocalization pattern	Proposed function(s) of gene product	Reference(s)
NOP4	Ring	Nucleolar protein required for cleavage of 35S pre-rRNA (at A0, A1, and A2 sites) and for accumulation of 27S pre-rRNA	8, 81, 82
PRP21	Ring	Nuclear protein required for spliceosome assembly	2, 79, 95, 97
TAO3	Ring	Involved in cell morphogenesis	16
SEC14	Dot	Role in protein secretion from the Golgi body to the plasma membrane	6, 7, 87
HTL1	Dot	Nuclear protein component of RSC chromatin remodeling complex, which functions in transcription regulation and elongation	51, 74

Sequencing of the *nop4*, *prp21*, and *sec14* mutant alleles. The relevant genes in the *nop4*, *prp21*, and *sec14* mutants were amplified by PCR with oligonucleotides approximately 150 nucleotides upstream of the start codon and 50 nucleotides downstream of the stop codon. Genomic DNAs extracted from these mutant strains were used as templates for the PCRs. The genomic DNA extracted from wild-type parental strain FY23 was used as a control. Mutations were confirmed by at least two sequencing reactions with independent PCR products. The wild-type control PCR products were also sequenced and did not contain mutations found in the mutant genes.

Northern analysis. RNA was extracted as previously described (85), separated on denaturing 1.2% agarose-formaldehyde and acrylamide gels, transferred to nylon membranes, and analyzed by Northern blot hybridization. The oligonucleotide probes used were as follows. For U3 analysis (see Fig. 3), the probes used were probe A (CCAAGTTGGATTCAGTGGCTC), probe B (CAAAAGCTG CTGCAATGG), and a 5S rRNA probe (GTGGTTAACTTGTCA). For rRNA analysis (see Fig. 4), we hybridized panels I and VI with GGCCAGCAATTTC AAGTTA (probe e), panel II with TTGTTACCTCTGGGCCC (probe c), panel III with CGGTTTTAATTGTCCTA (probe b), panel IV with CTCCGCTTAT TGATATGC (probe f), panel V with CATGGCTTAATCTTTGAGAC (probe a), panel VII with GCGTTCTTGATCGATGC (probe d), and panel VIII with CTACTCGGTCAGGCTC (5S rRNA probe).

Electron microscopy (EM). Strains were fixed for 1 h at room temperature in 1.6% glutaraldehyde in 0.1 M Sörensen buffer (pH 6.5), processed by acetylation as previously described (91), and embedded in Epon. Ultrathin sections were mounted on nickel grids and stained with uranyl acetate and lead citrate. Samples were examined in a JEOL CX 100 II transmission electron microscope operated at 60 kV.

RESULTS

Screen for yeast temperature-sensitive mutants defective in U3 snoRNA localization. To identify genes involved in trafficking and assembly of snoRNPs, we carried out a large-scale genetic screen of *S. cerevisiae* seeking to identify mutants with altered patterns of U3 snoRNA localization. The localization of U3 snoRNA was detected by FISH with a U3-specific probe (60). In wild-type yeast, U3 snoRNA is predominantly localized in the nucleolus, which, depending on cell orientation, appears as an oblong, curved, or crescent-shaped subnuclear structure adjacent to the nucleoplasm (marked by general DNA staining with DAPI (Fig. 1A). The nucleolar localization of U3 snoRNA was confirmed by its colocalization with a nucleolar marker protein, Nop1 (Fig. 1A).

We analyzed a random collection of ~1,100 temperature-sensitive yeast mutants (1) by FISH in search of phenotypes of U3 mislocalization. As we were primarily interested in fast-acting mutations (and in avoiding secondary effects), mutants were analyzed following a 4-h shift from the permissive temperature (25°C) to the nonpermissive temperature (37°C). Initially, we identified 43 mutants that reproducibly exhibited abnormal patterns of U3 localization specifically at the nonpermissive temperature. We observed four distinct phenotypic classes. Representative U3 mislocalization patterns are shown in Fig. 1B. The classes included mutants where U3 snoRNA localized within the nucleus to (i) a ring-like structure (ring); (ii) a smaller, spherical structure (dot) which appears to be distinctly separated from the nucleoplasm; (iii) the nucleoplasm at levels higher than observed in the wild type (as well as the nucleolus) (NP); and (iv) double U3 foci within a single cell (suggestive of a cell division defect).

Further characterization was performed on nine mutants with either a ring- or a dot-like U3 localization pattern. Standard genetic analysis (see Materials and Methods) was carried out to confirm that each mutant phenotype was caused by a single recessive mutation that was linked to the U3 localization pattern. Seven of the nine mutants met these criteria and were selected for further analysis. To identify the mutated genes in each of the U3 mislocalization mutants, we transformed two different yeast genomic libraries (*URA3 CEN*) (45, 75) into the selected mutants. This approach led to the identification of the mutant gene in five of the seven mutants, including *NOP4*, *PRP21*, *TAO3*, *SEC14*, and *HTL1* (Table 1 contains a general description of each gene product). We found that the wild-type copy of the relevant genes complemented the U3 mislocalization phenotypes, as well as the temperature-sensitive growth phenotype, of the mutants (data not shown).

Identification of the mutations in the temperature-sensitive mutants. Mutations in the relevant genes in the temperature-sensitive mutants were identified by PCR amplification and sequencing. Our results showed that in the *nop4* mutant, there is a mutation (T to C) at position 471368 on chromosome XI causing an amino acid change from leucine to serine at position 478 of the Nop4 protein. This particular leucine lies within the fourth RRM (RNA recognition motif) of Nop4 and is conserved both within the RRM domain and within RBM28, a human orthologue candidate for Nop4. In the *prp21* mutant, a mutation (T to C) at position 53822 on chromosome X was identified that causes an amino acid change from leucine to serine at position 161 of the Prp21 protein. The leucine residue lies outside of any obvious identified functional domain but is conserved with human SF3a. In the *sec14* mutant, a mutation (T to C) at position 425262 on chromosome XIII was identified that causes an amino acid change from leucine to proline at position 40 of the Sec14 protein. The leucine lies within a CRAL-TRIO domain and is also conserved in human Sec14-like proteins. Because of technical problems, we did not manage to amplify the *htl1* or *tao3* mutant allele.

U3 mislocalization phenotypes are not the result of global changes in RNA localization. In order to determine whether

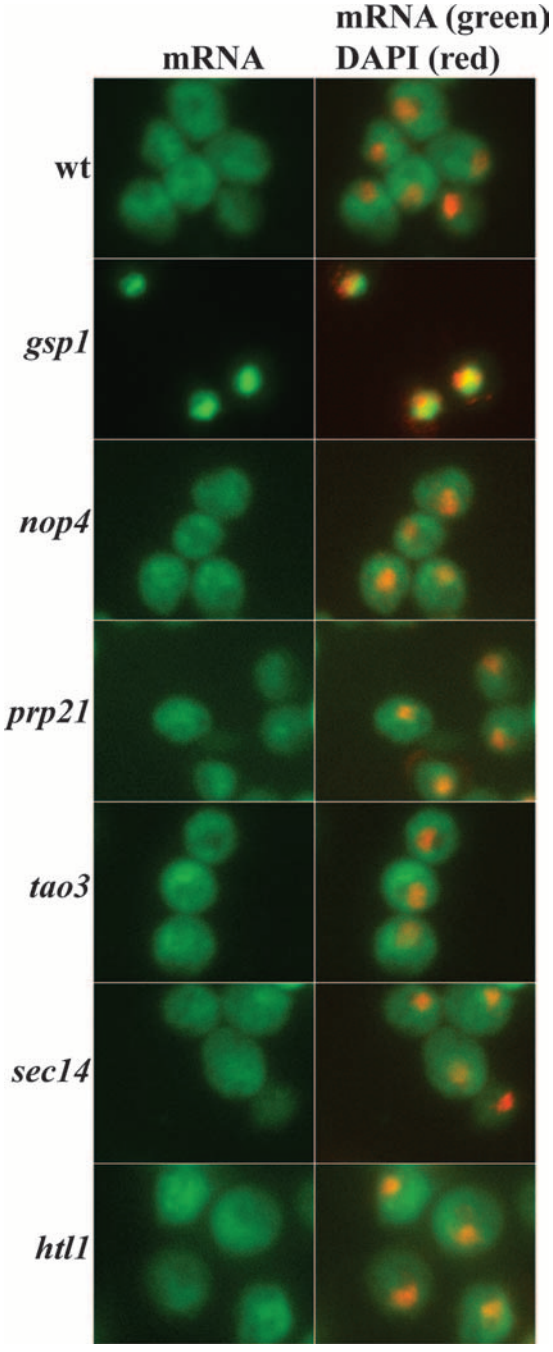

FIG. 2. mRNA export is not affected in temperature-sensitive mutants. FISH analysis was performed on temperature-sensitive mutants maintained at the permissive temperature (25°C) or following a 4-h shift to the nonpermissive temperature (37°C). Poly(A) RNA was detected with a Cy3-labeled oligo(dT) probe (green). Poly(A) RNA accumulates within the nuclei of Ran cycle mutant cells (*gsp1*) but not in wild-type (wt) control cells or any of the mutants identified in the U3 screen. DAPI was used to stain DNA and indicate the location of the nucleoplasm (red).

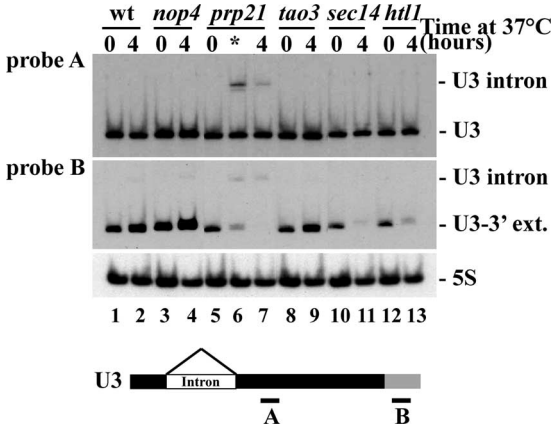

FIG. 3. Levels and processing of U3 snoRNA in temperature-sensitive mutants. Northern analysis was performed on wild-type (wt) and mutant cells maintained at the permissive temperature (25°C) (0-h lanes) or following a 4-h shift to the nonpermissive temperature (37°C) (4-h lanes). The *prp21* mutant showed a partial growth defect at the semipermissive temperature of 30°C and was further analyzed at this temperature (*, lane 6). Precursor forms of U3 containing an intron (U3 intron) or a 3′-extended species (U3-3′ ext.), as well as mature U3 snoRNA (U3), were detected with oligonucleotides A and B, which are specific to different regions of U3, as shown in the schematic (see Materials and Methods for details). 5S rRNA was probed as an internal RNA loading control.

the mutated genes globally affect the transport of cellular RNAs, export of polyadenylated mRNA was analyzed in each of the U3 mislocalization mutants. FISH analysis with an oligo(dT) probe to label poly(A) RNA was performed on the temperature-sensitive mutants after a 4-h temperature shift from 25°C to 37°C (Fig. 2). The wild type and a conditional *gsp1-2* mutant that is defective in mRNA export at 37°C (60, 98) were analyzed as controls. As expected, poly(A) mRNA localizes primarily to the cytoplasm in wild-type cells following a 4-h shift to 37°C. In the *gsp1* mutant at the nonpermissive temperature (37°C), export is blocked and thus poly(A) mRNA is predominantly localized to the nucleus. For each of the mutants that we identified, poly(A) mRNA is predominantly localized in the cytoplasm, even following a 4-h shift to the nonpermissive temperature. We conclude that mRNA export is not affected in these mutants.

Defects in U3 processing are observed in some mutants. In order to determine if the accumulation and processing of U3 snoRNA are affected by the mutated genes, we performed Northern analysis with two different probes, one specific for a region of the mature U3 protein and one that recognizes only 3′-extended U3 precursors (Fig. 3). No significant change in the amount of the mature U3 was detected in any of the mutants (U3 band, probe A panel). Moreover, the processing of U3 is similar to that in the wild type in the *nop4* and *tao3* mutants (U3-3′ ext., probe B panel, and U3 intron, probe A and B panels). In the *prp21* mutant, the level of U3 precursor containing the intron (U3 intron) increased and the level of 3′-extended precursors (U3-3′ extension) decreased significantly. A significant decrease in the levels of the 3′-extended pre-U3 forms with no change in the precursor with the intron was observed for both the *sec14* and *htl1* mutants. Thus, some of the mutants display defects in U3 snoRNA maturation/processing, as well as localization.

Pre-rRNA processing is affected in the mutants. Given that U3 and other snoRNAs function in rRNA biogenesis, we tested whether the mutated genes resulted in defects in the processing of precursor rRNAs by performing Northern blot assays with RNAs isolated from the strains grown at the per-

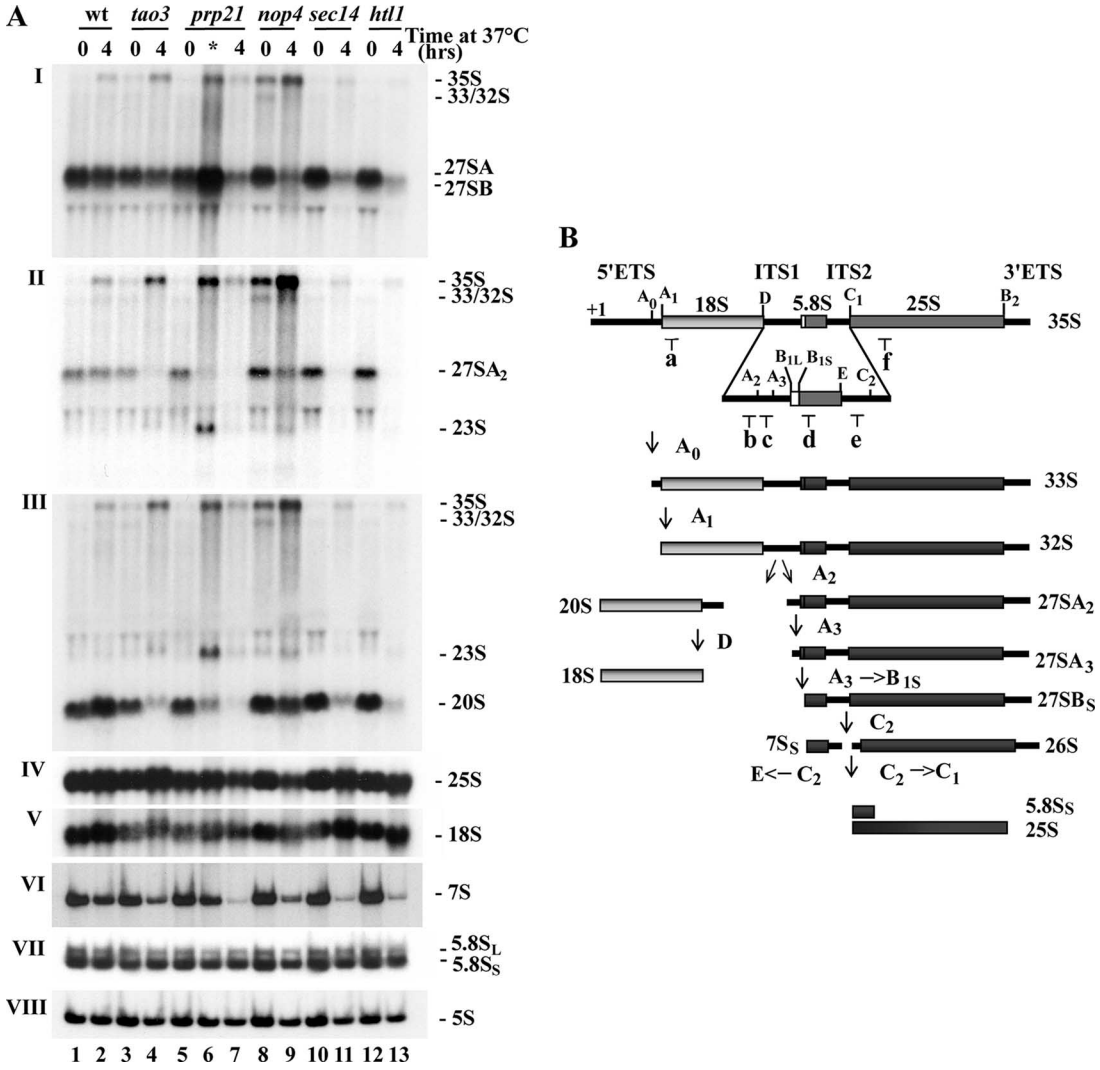

FIG. 4. rRNA levels and processing in temperature-sensitive mutants. (A) Northern analysis was performed with total RNA isolated from the temperature-sensitive mutants and the wild-type (wt) strain at the permissive temperature (25°C) or after a 4-h shift to the nonpermissive temperature (37°C) (0-h and 4-h lanes, respectively). Lane 6 shows analysis of the *prp21* mutant at the semipermissive temperature of 30°C (*). The relative positions of the oligonucleotide probes (a to f) used for each panel are diagrammed in panel B, and the sequences are given in Materials and Methods. We hybridized panels I and VI with probe e, panel II with probe c, panel III with probe b, panel IV with probe f, panel V with probe a, panel VII with probe d, and panel VIII with a probe against the 5S rRNA. (B) Primary Pol I transcript (35S) and simplified pre-rRNA processing pathway. The sequences of the mature 18S, 5.8S, and 25S rRNAs are embedded within external (5′ and 3′-ETS) and internal (ITS1 and ITS2) transcribed spacers. Cleavage sites A_0 to E are indicated.

missive and nonpermissive temperatures (Fig. 4). Our probes detected the primary transcript (35S rRNA), processing intermediates (e.g., 32S, 20S, 27S, and 7S rRNAs), or mature rRNAs (18S, 5.8S, and 25S) (Fig. 4A; panel B contains a schematic of the rRNA processing pathway). Compared to wild-type cells, the level of the mature rRNAs (18S, 5.8S, and 25S) did not change significantly after the 4-h temperature shift. However, alterations in the levels of the primary transcripts and specific processing intermediates were observed in each of the mutants, indicating that the mutations do negatively impact pre-rRNA processing or stability. For example, in the *nop4*, *prp21*, and *tao3* mutants, the relative levels of the 35S pre-rRNA increased while the levels of the processing intermediates, including 27S (pre-25S), 20S (pre-18S), and 7S (pre-5.8S), decreased, indicating that the early processing steps were inhibited. In the *sec14* and *htl1* mutants, the level of 35S pre-rRNA appeared not to change while amounts of the 27S, 20S, and 7S pre-rRNAs decreased considerably, suggesting a possible destabilization of the processing intermediates.

Localization of an H/ACA snoRNA is affected in dot mutants but not ring mutants. We screened for mutations that affected the localization of U3, a member of the box C/D family of snoRNAs. To address whether the mutants affect the localization of both major families of snoRNAs, we analyzed the localization of an H/ACA snoRNA, snR30 (5, 57). snR30 and U3 snoRNAs were detected simultaneously in the same yeast cells at the permissive and nonpermissive temperatures (Fig. 5). In wild-type cells, snR30 snoRNA colocalizes with U3 in

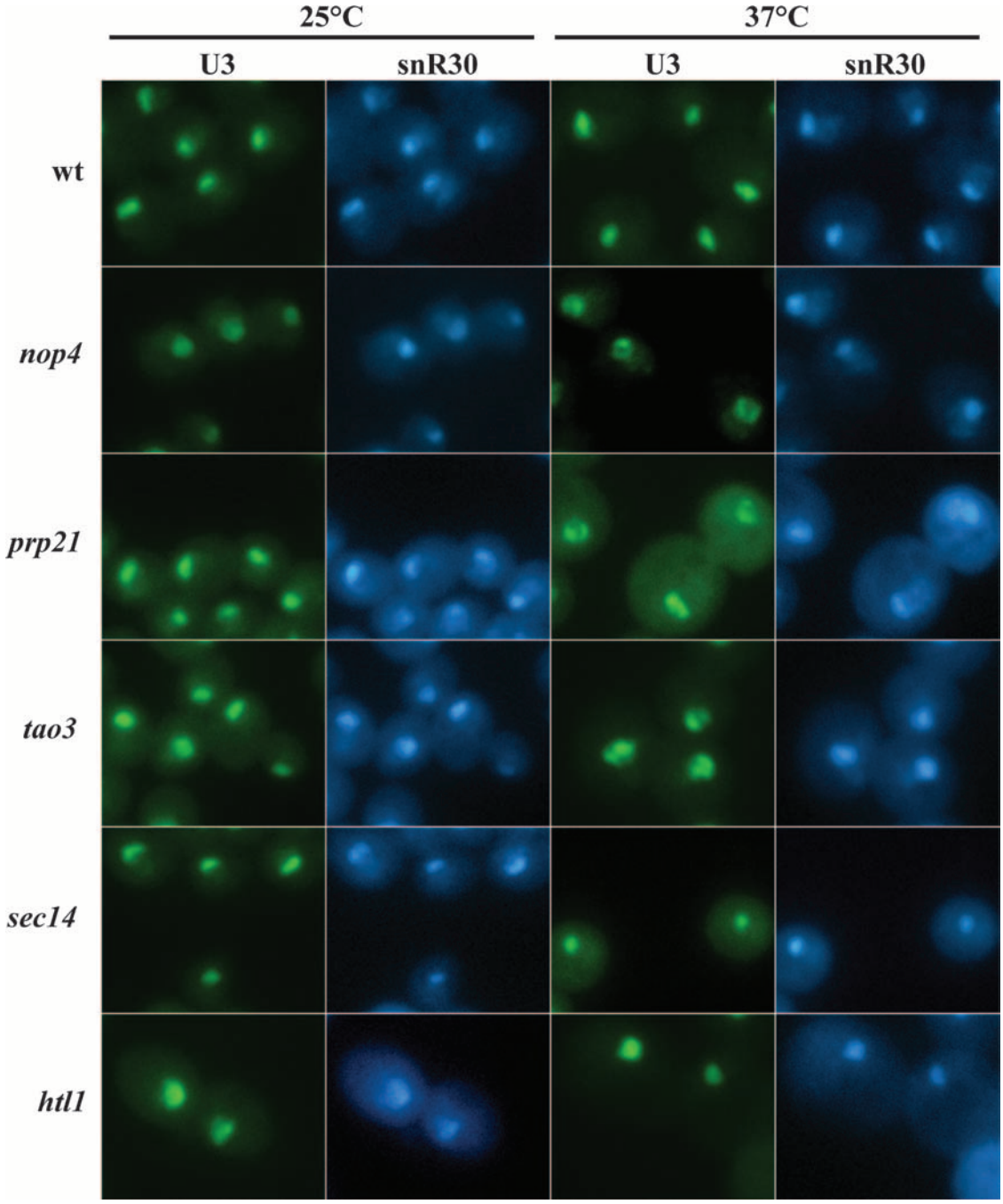

FIG. 5. Localization of a box H/ACA snoRNA (snR30) in temperature-sensitive mutants. FISH analysis was performed on wild-type (wt) and mutant strains at the permissive temperature (25°C) or after a 4-h shift to the nonpermissive temperature (37°C). U3 snoRNA and snR30 snoRNA were detected simultaneously with a Cy3-labeled U3-specific probe (green) and an Oregon green-labeled snR30-specific probe (blue).

the nucleolus at both temperatures. However, in mutants where U3 localizes to a ring (*nop4*, *prp21*, and *tao3*), snR30 occupied the whole area of the nucleolus, indicating that the relevant gene products in these ring mutants are not common factors required for proper nucleolar localization of both C/D and H/ACA snoRNAs. The gene product affected in the *tao3* mutant likely functions in a pathway common to the C/D RNAs, as we found that another C/D snoRNA, U14, was also observed in a ring in this mutant (data not shown). However, U14 exhibited normal nucleolar localization in both the *nop4* and *prp21* mutants (data not shown), indicating potential U3-specific effects of these two mutants (see Discussion). In the mutants where U3 is localized in a dot (*sec14* and *htl1*), snR30 colocalized with U3, which might reflect defects common to both C/D and H/ACA snoRNAs. Consistent with this possibility, U14 also localized in a dot in the *sec14* and *htl1* mutants (data not shown).

Localization of snoRNP-related nucleolar proteins. In order to determine whether the mutants also mislocalize nucleolar pro-

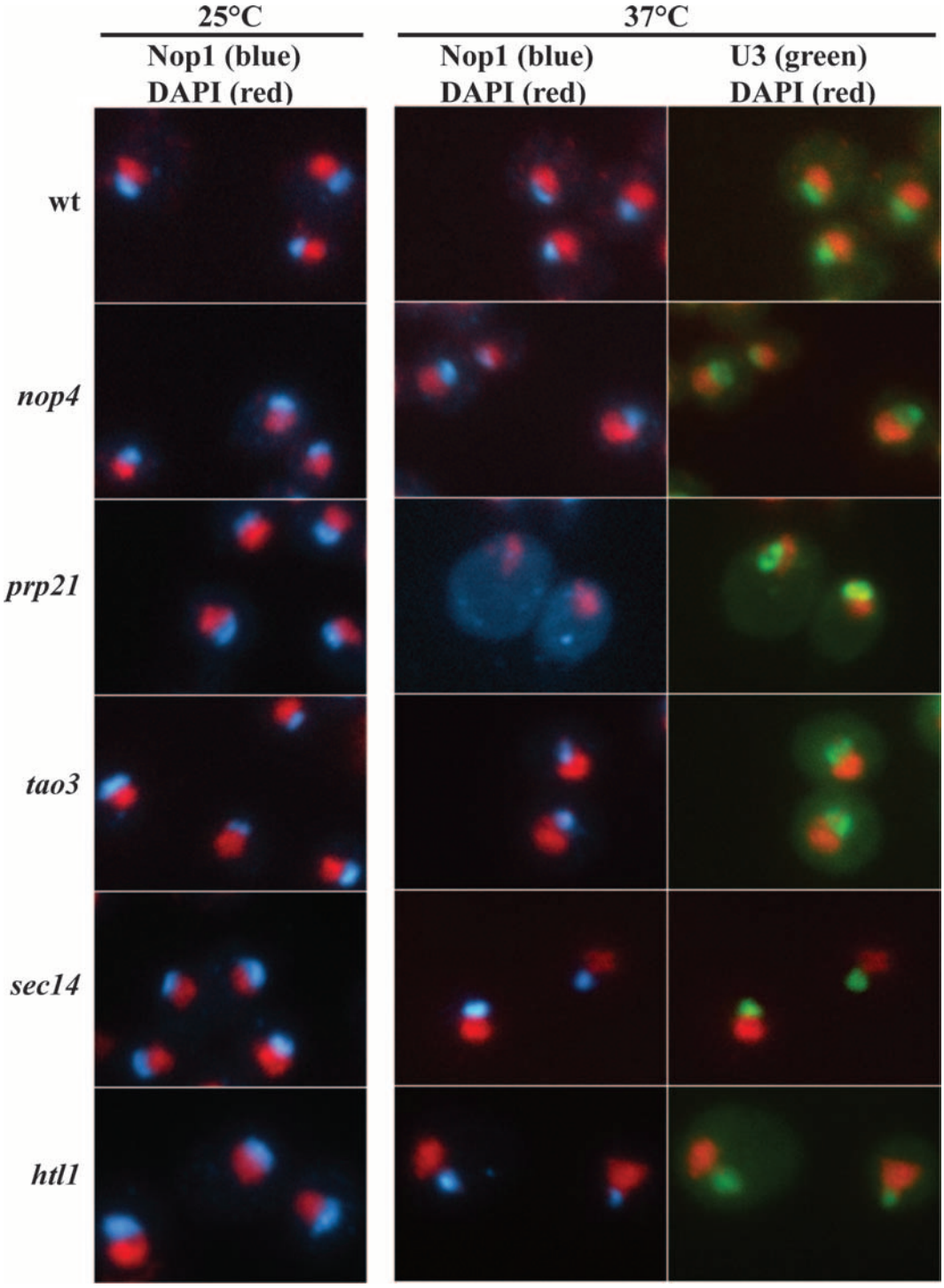

FIG. 6. Localization of nucleolar protein Nop1 in temperature-sensitive mutants. FISH analysis was performed on the wild-type (wt) and mutant strains at the permissive temperature (25°C) or after a 4-h shift to the nonpermissive temperature (37°C). U3 snoRNA (green) was detected by FISH with a Cy3-labeled U3-specific probe (60). The subcellular localization of Nop1 (blue) was detected in the same cells by immunofluorescence with antibodies specific for Nop1. DAPI was used to stain DNA (red).

teins, the subcellular distribution of both U3 and either Nop1 (Fig. 6) or Nsr1 (data not shown) was analyzed in the same set of cells by a combined FISH-immunofluorescence procedure. Nop1 is a core component of C/D snoRNPs (78, 86), and Nsr1 is a nucleolar protein implicated in the snoRNP assembly and transport pathway (52, 91). The three ring mutants showed distinctive phenotypes. For the *nop4* mutant, both Nop1 and Nsr1 showed a normal nucleolar localization pattern. For the *prp21* mutant, both the Nop1 and Nsr1 proteins were dispersed throughout the cells and did not show accumulation in any specific locus. For the *tao3*

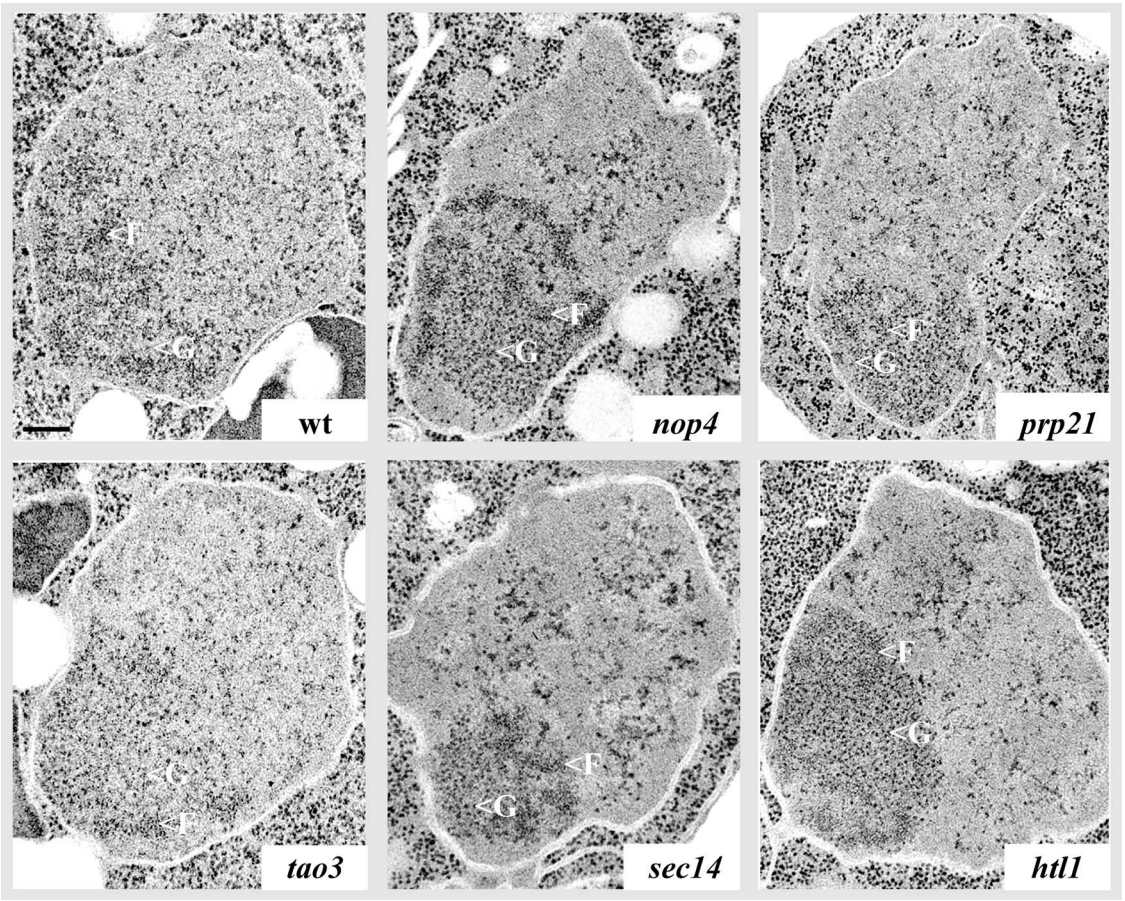

FIG. 7. Temperature-sensitive mutants do not exhibit major alterations in nucleolar structure. Cells grown for 4 h at the nonpermissive temperature (37°C) were collected, processed by acetylation, and inspected by EM. Representative nuclei are presented. wt, wild type; F, fibrillar strands; G, granular areas. Scale bar is 0.2 μm.

mutant, Nop1 was enriched in the area where U3 is absent (i.e., in the hole of the ring) while Nsr1 showed a normal nucleolar distribution. Finally, both the Nop1 and Nsr1 proteins colocalized with U3 in the dot-like structure of the *sec14* and *htl1* mutants. Thus, several distinct profiles were observed in the mutants with regard to effects on the localization of nucleolar proteins.

The structure of nucleoli is not significantly altered in the mutants. We naturally considered the possibility that the U3 localization (ring and dot) patterns related to an underlying alteration in nucleolar structure. While some of the data presented earlier (e.g., normal patterns of localization of snR30 [Fig. 5] or nucleolar proteins [Fig. 6]) indicated that this was not likely to be the case, we examined nucleolar structure in each of the mutants at the ultrastructural level by EM (Fig. 7). EM analysis revealed that all temperature-sensitive mutants maintained a largely normal nucleolar structure; no major changes were observed in any of the mutants. The *prp21*, *sec14*, and *htl1* mutants did display a slightly more compact but otherwise normal nucleolus. Collectively, these results argue that the U3 ring and dot patterns are not likely to arise because of a grossly altered nucleolar structure.

Relationship between the U3 mislocalization phenotypes and the nucleolar body. As described earlier, the nucleolar body, a spherical structure housed within the nucleolus, appears to be involved in snoRNP biogenesis or trafficking (90, 91). The U3 patterns in the ring and dot mutants suggested to us that they may relate to the nucleolar body. To directly test this idea, we simultaneously examined the localization of nucleolar bodies (with an established nucleolar body marker [91]) and U3 snoRNA in each mutant (Fig. 8). The nucleolar body is found within the central hole of the ring-like U3 pattern in the *nop4*, *prp21*, and *tao3* ring mutants (Fig. 8). In the *sec14* and *htl1* dot mutants, U3 colocalized with nucleolar body (Fig. 8). These results indicate that the ring- and dot-like U3 mislocalization phenotypes physically relate to the nucleolar body and suggest defects in U3 snoRNA transport into or retention within this structure.

DISCUSSION

We screened a large bank of temperature-sensitive yeast mutants for defects in the nucleolar localization of U3 snoRNA. From this screen, we identified five gene products—Nop4, Prp21, Tao3, Sec14, and Htl1—from mutants with two U3 mislocalization patterns—ring-like and dot-like—that are intimately related to the yeast nucleolar body. The gene products identified from the ring mutants (Nop4, Prp21, and Tao3) are apparently important for the accumulation of snoRNAs within the nucleolar body (e.g., reduced delivery or accelerated

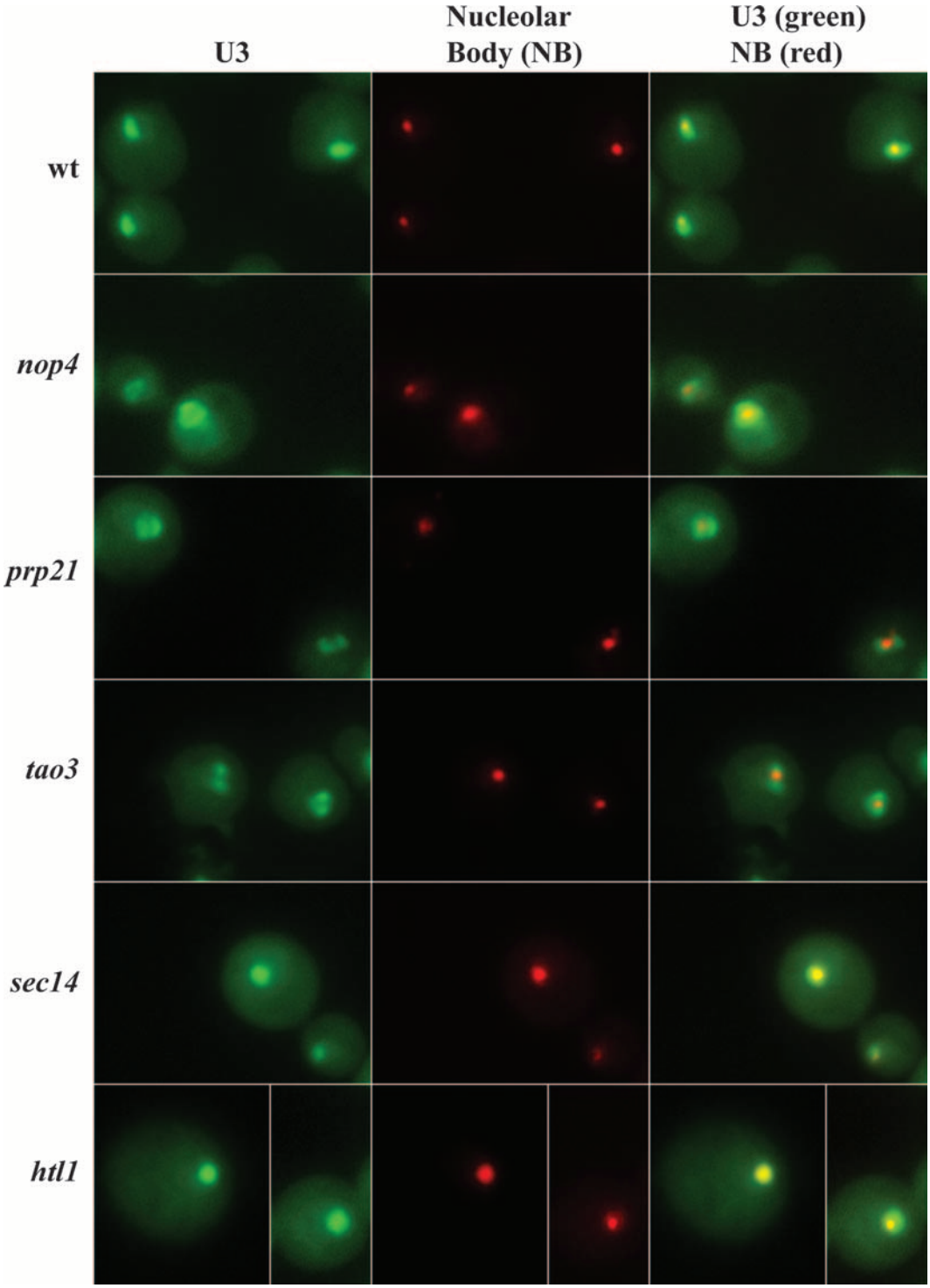

FIG. 8. Spatial relationship of the nucleolar body to U3 in temperature-sensitive mutants. Temperature-sensitive mutants were transformed with a plasmid overexpressing a box C/D snoRNA variant (pG14-U14/MS2X2) that is used as a marker for the nucleolar body (91). FISH analysis of endogenous U3 snoRNA (green) and the ectopically expressed variant snoRNA (red) was performed following a 4-h shift to the nonpermissive temperature (37°C). wt, wild type.

release), while those from the dot mutants (Sec14 and Htl1) appear to be important for releasing snoRNAs from the nucleolar body to the nucleolus (Fig. 9). This is the first time that these gene products have been implicated in the snoRNA maturation pathway.

Spatial organization of the snoRNA maturation pathway. The pathway of U3 snoRNA maturation is temporally and spatially organized (43, 90, 93). In yeast, newly synthesized U3 transcripts contain a 5′ monomethyl cap (m^7G), an intron, and extended nucleotides at the 3′ end. Conversion of pre-U3 to

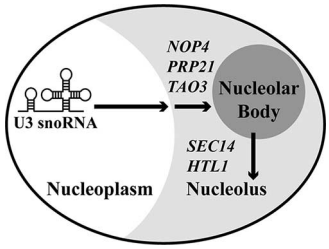

FIG. 9. Proposed roles of the newly identified *trans*-acting factors in the snoRNA trafficking pathway. After synthesis, intron removal, and core protein assembly in the nucleoplasm, yeast U3 snoRNA accumulates within the nucleolar body for cap hypermethylation, 3′-end trimming, and final assembly and then localizes to the nucleolus for function in rRNA biogenesis. The novel *trans*-acting factors identified in our study that are important for nucleolar body accumulation (NOP4, PRP21, and TAO3) and nucleolar targeting (SEC14 and HTL1) of U3 snoRNA are indicated.

functionally mature U3 involves multiple posttranscriptional events that include (i) hypermethylation of the 5′ monomethyl cap (m^7G) structure to a trimethyl cap ($m^{2,2,7}G$) by the enzyme trimethyl guanine synthase (Tgs1) (58); (ii) precise removal of the intron by the pre-mRNA spliceosome; (iii) trimming of nucleotides at the 3′ end by the exosome (43); (iv) assembly with the core box C/D snoRNP proteins, as well as U3-specific binding proteins (93); and (v) intranuclear trafficking to nucleolar compartments where substrate (pre-rRNA) is found. Early U3 maturation steps such as intron removal and assembly with core snoRNP proteins appear to take place in the nucleoplasm (93). Indeed, a growing body of evidence indicates that, in general, the early steps of snoRNP biogenesis are tightly coupled to transcriptional events, occurring at snoRNA gene loci during transcriptional elongation (4, 56, 72, 99). In contrast, later steps of U3 maturation, including 5′ cap hypermethylation and 3′-end trimming, appear to take place in the nucleolar body since the pre-U3 RNA, but not mature U3, is located in the nucleolar body and enzymes like Tgs1 that modify U3 also accumulate in nucleolar bodies (90). The mature U3 snoRNP moves from the nucleolar body to its functional destination, the nucleolus (90).

The U3 mislocalization patterns revealed in our mutant analysis likely reflect defects in stages of the U3 snoRNP biogenesis pathway (Fig. 9). The ring phenotype appears to represent an underaccumulation of U3 snoRNA in the nucleolar body. The mutated gene products in these mutants may be protein factors that either assist snoRNA trafficking to the nucleolar body or retain the snoRNA within the nucleolar body. The dot phenotype appears to reflect overaccumulation of U3 snoRNA in the nucleolar body and underaccumulation at other regions of the nucleolus. Accordingly, the mutated gene products in these mutants may be factors important for assisting snoRNA movement either into the nucleolar body (from the nucleoplasm or nucleolus) or from the nucleolar body to the nucleolus.

Similar roles in trafficking to and from the nucleolar body have been proposed previously for Srp40 and Nsr1 (91). Specifically, null mutants of *SRP40* (which encodes a yeast homolog of mammalian Nopp140) result in underaccumulation of a model box C/D snoRNA in the nucleolar bodies (analogous to our ring phenotype) and null mutants of *NSR1* (which encodes a yeast homolog of the mammalian nucleolin) result in overaccumulation of this snoRNA in the nucleolar body and reduced flow to other nucleolar regions (analogous to our dot phenotype).

Potential roles of ring genes *NOP4*, *PRP21*, and *TAO3* in snoRNA accumulation within the nucleolar body. The genes identified from the three ring mutants are *NOP4*, *PRP21*, and *TAO3*, which each encode an essential protein. Nop4 is a nucleolar protein critical for proper processing of pre-rRNA and production of functional ribosomes (8, 81). Depletion of Nop4 results in inhibition of cleavage at A_0, A_1, and A_2 sites of 35S pre-rRNA (8), which are each sites known to require U3 snoRNP activity (33). Nop4 depletion also leads to underaccumulation of 27SA and its processing products (8, 81). The effects of Nop4 depletion on pre-rRNA processing observed by others are quite similar to those that we observed with the temperature-sensitive allele of *NOP4* (Fig. 4). The molecular mechanism of Nop4 function is not understood. Our results raise the possibility that Nop4 functions in U3 snoRNA trafficking to (or retention within) nucleolar bodies. Defects in the ability of U3 to accumulate in nucleolar bodies are predicted to impair U3 snoRNA biogenesis and to affect ribosome biogenesis, consistent with the previously observed phenotypes.

Nop4 contains four RRMs, each of which is essential for Nop4 function in ribosome biogenesis (81, 82), suggesting that Nop4 may directly bind RNAs such as snoRNAs or rRNAs. However, the precise RNA substrates for Nop4 have not been identified. No stable association between Nop4 and the U3, U14, snR10, and snR30 snoRNAs was identified by coimmunoprecipitation (8, 81), suggesting that if there is any association between Nop4 and snoRNAs, it is transient. Following UV cross-linking, snR190, a box C/D snoRNA (41, 68), was coimmunoprecipitated with the Nop4 antibody (81), consistent with transient snoRNA-binding activity. In our study, we found that site-directed mutation of any of the four RRMs of Nop4 (81, 82) resulted in mislocalization of U3 snoRNA to the same ring-like pattern that was observed in the Nop4 mutant identified in our screen (unpublished data). Additional evidence that Nop4 may interact (at least transiently) with U3 and perhaps other snoRNAs comes from numerous genetic or physical associations that have been observed between Nop4 and snoRNP proteins. For example, a *nop4* allele was identified in a screen for synthetic lethality with a *nop1* allele, which suggests that Nop4 functionally interacts with Nop1, a core protein component of U3 and other box C/D snoRNPs (8). Moreover, proteomic studies using large-scale tandem affinity purification and mass spectrometry revealed that Nop4 associates with the C/D snoRNP core proteins Nop1 and Nop56, H/ACA snoRNP core proteins Cbf5 and Gar1, and U3-specific proteins Utp10 and Utp20 (23, 24, 42).

Like Nop4, Prp21 was identified in our screen as a protein that, when mutated, gave rise to a U3 ring mislocalization phenotype. Prp21 is an essential nuclear protein involved in the assembly of the prespliceosome and is required for intron splicing (2, 24, 79, 95, 97). Consistent with the known role of this protein in splicing, the intron-containing precursor forms of U3 snoRNA selectively accumulated in the *prp21* mutant, revealing that intron removal was impaired (Fig. 3). Given that this defect in U3 processing is associated with U3 mislocaliza-

tion to a ring pattern, this finding indicates that the splicing of the U3 intron is important for localization of the RNA in the nucleolar body. Precursors of U3 RNA in wild-type cells are normally not observed in nucleolar bodies, and there is evidence that intron removal of U3 may normally occur at the site of transcription (43, 90). In addition, our finding that the snoRNP-related proteins Nop1 and Nsr1 also become dramatically mislocalized in *prp21* mutant cells (Fig. 6A and B) suggests a likelihood of defects in snoRNP assembly as well. Other recent data also indicate that splicing and snoRNP assembly are coupled processes (31, 32, 72).

The third protein in this ring phenotypic class, Tao3, is an essential and conserved protein with unknown molecular functions. The only previous study of *TAO3* showed that a temperature-sensitive *tao3* mutant displays defects in cell morphogenesis (16). GFP-tagged Tao3 localized in the cytoplasm, as well as the polarized cell periphery (16). On the other hand, proteomic studies have revealed interactions of Tao3 with nuclear proteins and components with potential connections to snoRNPs. For example, Tao3 was identified in complex with Sbp1 (23), a nucleolar, single-strand nucleic acid binding protein that associates with snoRNAs (14). Tao3 was also found to associate with Yra1 (36), a member of the RNA and export factor binding protein family. RNA and export factor binding proteins are nuclear proteins that interact with RNA, and at least some family members are required for mRNA export from the nucleus (69, 80, 102). It is possible that Yra1, together with Tao3, interacts with snoRNAs and plays a role in their intranuclear trafficking. Moreover, Tao3 was identified in complex with Sup45 (24), which (as determined by yeast two-hybrid analysis) physically associates with Nop4 (34) (recall that mutant Nop4 also leads to U3 ring patterns). Our study showed that U3 processing is not affected in the *tao3* or *nop4* mutant (Fig. 3).

Potential roles of dot genes *SEC14* and *HTL1* in the release of snoRNAs from the nucleolar body. The two genes identified from the dot mutants are *SEC14* and *HTL1*. U3 processing was similarly affected in both *sec14* and *htl1* mutants (Fig. 3). Specifically, the 3′-extended U3 precursors were greatly reduced in both mutants after the temperature shift to 37°C without any detectable accumulation of the intron-containing precursor form of U3. The 3′-extended forms of pre-U3 are stabilized by binding of the Lhp1 (La homolog) protein (43), and the Lsm2-8 (like-Sm) complex acts as a chaperone with Lhp1 to stabilize pre-U3 species (44). The stabilization of the 3′-extended precursors is required for the final maturation of U3 (43, 44). It is possible that Sec14 and Htl1 play roles in the stabilization of U3 precursors by functional interaction with Lhp1 and/or the Lsm2-8 complex. Sec14 is essential for viability and was identified as a cytosolic phospholipid transfer protein required for protein secretion from the Golgi body to the plasma membrane (7, 87). However, continuous protein secretion is essential for ribosome synthesis (55), indicating indirect links of Sec14 to possible functions within the nucleus. Htl1 is not an essential protein but is important for yeast growth at 37°C (51). Htl1 is a nuclear protein (74) and is a component of the RSC chromatin remodeling complex, which functions in transcription regulation and elongation from the RNA polymerase II promoter (11, 74). Previous studies indicate that snoRNP assembly initiates at the transcription site, most likely during transcription elongation (4, 72, 99). It is possible that defects in transcription elongation in the *htl1* mutant lead to defects in snoRNA 3′-end processing and/or RNP assembly (and ultimately proper nucleolar targeting).

Summary. The findings presented here have increased our understanding of the key factors in snoRNA maturation and transport. We have identified factors required for the accumulation of a snoRNA in the nucleolar body (Nop4, Prp21, and Tao3) and for the trafficking of a snoRNA from the nucleolar body to the nucleolus (Sec14 and Htl1). Some of these proteins affect the localization of members of both major families of snoRNAs (e.g., Sec14 and Htl1 affect the localization of U3, U14, and snR30), and others may be specific for the C/D snoRNAs (e.g., Tao3 affects the localization of U3 and U14) or just U3 snoRNA (e.g., Nop4 and Prp21). A plausible explanation for the specific link between U3 RNA and Prp21 is the unique presence of an intron in this snoRNA. Our results refine the potential function of Nop4 to a role in snoRNA trafficking to or within the nucleolar body. This role would explain the pre-rRNA processing phenotypes previously observed with alterations in this protein (8, 81). The observation that Prp21, Sec14, and Htl1 are required for both snoRNA processing and nucleolar localization indicates a strong link between the maturation and transport pathways. Specifically, based on the *prp21* phenotype, accumulation of the snoRNA in the nucleolar body appears to depend on intron removal (which is thought to take place in the nucleoplasm [90]). The phenotypes produced by *sec14* and *htl1* suggest that exiting the nucleolar body is linked to later snoRNA maturation steps. More detailed molecular characterization of the identified proteins will now allow further delineation of the mechanisms by which these proteins influence the intranuclear trafficking of snoRNAs.

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