RNA: STRUCTURE METABOLISM AND CATALYSIS:
The Shq1p·Naf1p Complex Is Required for Box H/ACA Small Nucleolar Ribonucleoprotein Particle Biogenesis

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Small nucleolar ribonucleoprotein particles (snoRNPs) are essential cofactors in ribosomal RNA metabolism. Although snoRNP composition has been thoroughly characterized, the biogenesis process of these particles is poorly understood. We have identified two proteins from the yeast *Saccharomyces cerevisiae*, Yil104c/Shq1p and Ynl124w/Naf1p, which are essential and required for the stability of box H/ACA snoRNPs. Depletion of either Shq1p or Naf1p leads to a dramatic and specific decrease in box H/ACA snoRNA levels in *vivo*. A severe concomitant defect in ribosomal RNA processing is observed, consistent with the depletion of this family of snoRNAs. Shq1p and Naf1p show nuclear localization and interact with Nhp2p and Cbf5p, two core proteins of mature box H/ACA snoRNPs. Shq1p and Naf1p form a complex, but they are not strongly associated with box H/ACA snoRNPs. We propose that Shq1p and Naf1p are involved in the early biogenesis steps of box H/ACA snoRNP assembly.

Small nucleolar RNAs (snoRNAs) are essential cofactors in ribosomal RNA metabolism. Although a few snoRNAs are necessary for discrete steps of the processing of the 35S pre-ribosomal RNA precursor, most of them are required to target modifications of the bases or of the sugar phosphate backbone within the ribosomal RNA precursor (for review, see Refs. 1 and 2). snoRNAs can be divided into two major structural families (3, 4) with different functions. Box C/D snoRNAs have been shown to guide methylation of the 2'-hydroxyl groups of the ribose moiety of numerous nucleotides in the 35S rRNA precursor (5, 6). Box H/ACA snoRNAs guide the pseudouridylation of the rRNA precursor (7, 8). These small nucleolar RNAs do not function as isolated RNA molecules, but they assemble with protein complexes to form ribonucleoprotein particles (snoRNPs). The protein composition of the two major families of snoRNPs has been extensively characterized. The protein core of the box C/D snoRNP is composed of Nop1p, Nop58p, Nop56p, and Nop10p (9-10) with Nop1p as the candidate methyltransferase (2). The core of the box H/ACA snoRNP contains four proteins, Gar1p, Nhp2p, Nop1p, and Chb5p, which is presumably responsible for the pseudouridyl synthetase activity (4, 11-15). Although the RNA motifs required for the association of the snoRNP proteins with the box H/ACA snoRNAs have been investigated (16-17), little is known about the mechanisms by which the RNA and the protein components become assembled into mature snoRNPs. Some box H/ACA snoRNAs are generated from independent transcription units, whereas others are embedded within the introns of other genes and must be processed from the excised introns after splicing (1). Most independently transcribed box H/ACA snoRNAs are likely processed at their 3' end by a complex that includes the Nrd1p and Sen1p proteins, presumably associated with RNA polymerase II (18). A few independently transcribed box H/ACA snoRNAs also undergo 5' end processing by the double-stranded RNA endonuclease Rnt1p (19). The packaging of box H/ACA snoRNAs into snoRNPs is likely to occur at an early stage of biogenesis, since unprocessed snoRNA precursor accumulation requires the presence of the box H/ACA snoRNPs Nhp2p and Nop10p (13). After or during the course of the maturation of the snoRNAs, the snoRNPs are exported to the nucleolar compartment to function in ribosomal RNA processing and/or modification. Whether fully mature or partially assembled snoRNPs are exported to the nucleolus is a matter of debate. Recently a yeast putative RNA helicase was identified as an important factor for biogenesis of both box C/D and H/ACA snoRNPs (20). However the precise mechanisms by which the box C/D and box H/ACA snoRNPs assemble are likely to be different since both the RNA and the protein components differ. We present evidence that two novel essential yeast proteins, Yil104c and Ynl124w, are required specifically for accumulation of box H/ACA snoRNAs and interact with two components of the box H/ACA snoRNP, Nhp2p and Chb5p. Because these two proteins are nuclear but do not seem to exhibit strong nuclear localization, we propose that they participate in the early biogenesis of these snoRNPs.

**EXPERIMENTAL PROCEDURES**

The two-hybrid screen with Rnt1p as bait was performed as described (21). Replacement of the *YIL104C* and *YNL124W* promoters by a galactose promoter and an HA-epitope was performed using a PCR-based procedure as described (22). For *in vivo* depletion of Shq1p and Naf1p, after preculture in a medium containing galactose, strains were shifted in a rich medium containing 2% raffinose, 2% sucrose, and 2% galactose. RNA extraction and acrylamide Northern blot analysis were performed as in Chanfreau *et al.* (23). Agarose Northern blot was performed using standard protocols. The list of oligonucleotides used for snoRNA probes is indicated in Chanfreau *et al.* (19). The sequences of
the rRNA oligonucleotide probes are 5′-ETS, CCGAATTTGACCAGATATCGAG; 3′ETS, GCCGATGATGATTCAT; A1, rRNA, ACTAATCCTAAAAGAGAAGACAACAAGC; A1-A2, CCGTGTATATGCTGATA; A2-A3, ATGAAAATCCACAGTG; ITS2, GGGCAGAATTCCTCAAGC; A1-A2, CGGTTTTAATTGTCCTA; 5′-ETS, CCCGGGATCATAGAATTC; A0 rRNA, ACTATCGTGG; 3′ETS, CGGAATGGTACGTTTAGT. Native sedimentation coefficient was determined by ultracentrifugation (24), and immunoprecipitation of RNAs with IgG beads was performed as described (12). Glycerol gradient sedimentation analysis was performed as described (25) with the following modifications. For each strain, 1 liter of cells was harvested at A600 nm 1.0 and resuspended in 1.5 ml of Buffer GG (5 mM MgCl2, 20 mM Tris-HCl, pH 8.5, 150 mM NaCl, 0.2% Triton X-100, 1 mM dithiothreitol, protease inhibitors tablet from Roche Molecular Biochemicals). The cell paste was dropped in liquid N2 to form frozen droplets. Frozen droplets were then ground for 20 min in a mortar in the presence of liquid N2. Ground cells were thawed on ice, and the cell lysate was cleared by centrifugation for 30 min at 17,000 rpm in a Beckman JA25.50 rotor. Cleared lysates were thawed on ice, and the cell lysate was cleared by centrifugation for 30 min at 17,000 rpm in a Beckman SW-41 rotor. 500-μl fractions were collected of which 80 μl were precipitated with trichloroacetic acid for Western blot analysis, and the remainder was used for RNA extraction as described (19). Native sedimentation coefficient markers (Amersham Biosciences) were included in a gradient in run parallel and analyzed by silver staining.

In vitro translation of proteins was performed using a TNT-T7 kit (Promega) using PCR products as in vitro transcription-translation templates. For each open reading frame, the upstream primer contained a T7 promoter, the Kozak translation initiation sequence, and the beginning of the open reading frame, whereas the reverse primer contained the end of the open reading frame, the stop codon followed by a 20-nucleotide poly(T) tail at the 5′ end. PCR products were generated from genomic DNA using these two primers. Calmodulin-binding protein-tagged versions of Shq1p were obtained by PCR amplification of YIL104C using genomic DNA from the TAP-tagged Yil104c yeast strain with an upstream oligonucleotide containing a T7 promoter, the Kozak translation initiation sequence, and the beginning of the open reading frame, whereas the reverse primer containing the end of the open reading frame, the stop codon and a 20-nucleotide poly(T) tail at the 5′ end. PCR templates were incubated with reticulocyte lysate-T7 RNA polymerase mix from the Promega TNT kit and either unlabeled or [35S]methionine for 1 h. Calmodulin beads were washed 3 times for 5 min each with 1 ml of HEMN 200 buffer (40 mM Hepes, pH 7.5, 5 mM MgCl2, 200 mM KCl, 0.2 mM EDTA, 0.05% Nonidet P-40, 1 mM dithiothreitol with complete protease inhibitors from Roche Molecular Biochemicals) and blocked with 1% bovine serum albumin for 30 min. Translated products were incubated with 50 μl of prepared beads and 1 ml of HEMN 200 buffer at room temperature for 2 h with rotation. Beads were washed 4 times for 5 min with 1 ml of HEMN 200 buffer. Input and precipitates were boiled in SDS loading buffer and loaded on SDS gels. Indirect immunofluorescence was performed as described (26).

RESULTS

The Yil104c-Shq1p Protein Is Required for Box H/ACA snoRNA Accumulation—Yeast RNase III, Rnt1p, is required for processing of a large number of small nucleolar RNAs from both the box C/D and box H/ACA families (19, 23). To identify proteins that may influence RNase III cleavage in vivo, a two-hybrid screen was performed using Rnt1p as bait. Among the candidate clones, we found three clones corresponding to different fragments of the essential protein Yil104c (Fig. 1A). The junction of these fusion proteins was sequenced and was shown to correspond to positions 170769, 170925, and 170961 of chromosomal IX (Fig. 1B). The YIL104C open reading frame spans positions 169979–171502 of chromosome IX. Because each fusion was about 1-kilobase long, as estimated by agarose gel electrophoresis, the portions of Yil104c that interact with Rnt1p in the two-hybrid assay correspond to the C-terminal 244, 192, and 180 amino acids of Yil104c (Fig. 1B). We tried to confirm the interaction between Yil104c and Rnt1p by immunoprecipitation or in vitro pull-down experiments, but we failed to detect a specific interaction by these methods (data not shown). To characterize the function of Yil104c and its possible connection to yeast RNase III, we replaced the YIL104C endogenous promoter with a galactose-inducible promoter and an N-terminal HA epitope tag using homologous recombination. After preculture of the resulting strain in the presence of galactose, we shifted the yeast culture into a medium lacking galactose. The shift in culture conditions led to a rapid depletion of the protein, most of it being depleted after 3 h of growth in a medium lacking galactose (Fig. 2A). Because yeast RNase III is involved in the processing of small nuclear and small nucleolar RNAs (19, 27), we examined the level of these species after Yil104c depletion. We monitored the levels of various small RNAs from both the box C/D and H/ACA families and from the spliceosomal RNA family. Northern blot analysis showed that box C/D snoRNAs were rapidly depleted after Yil104c depletion (Fig. 2B). In contrast, box C/D snoRNAs and the RNA component of the RNase mitochondrial RNA processing ribonuclease complex were not affected by Yil104c depletion (Fig. 2B). The U5 spliceosomal small nuclear RNA was also unaffected (data not shown). The apparent increase of the level of the small RNAs of the box C/D family upon Yil104c depletion is due to a degradation of 25S and 18S rRNAs (data not shown).

We next investigated whether the depletion of box H/ACA snoRNAs in Shq1p-depleted cells resulted in a defect in the processing of the precursor of 5′SS. Such a processing defect would be expected, since depletion of the box H/ACA snoRNAs snR10 and snR30 is expected to block or to reduce the efficiency of small nucleolar RNAs from both the box C/D and box H/ACA families (19, 23). To identify proteins that may influence RNase III cleavage in vivo, a two-hybrid screen was performed using Rnt1p as bait. Among the candidate clones, we found three clones corresponding to different fragments of the essential protein Yil104c (Fig. 1A). The junction of these fusion proteins was sequenced and was shown to correspond to positions 170769, 170925, and 170961 of chromosomal IX (Fig. 1B). The YIL104C open reading frame spans positions 169979–171502 of chromosome IX. Because each fusion was about 1-kilobase long, as estimated by agarose gel electrophoresis, the portions of Yil104c that interact with Rnt1p in the two-hybrid assay correspond to the C-terminal 244, 192, and 180 amino acids of Yil104c (Fig. 1B). We tried to confirm the interaction between Yil104c and Rnt1p by immunoprecipitation or in vitro pull-down experiments, but we failed to detect a specific interaction by these methods (data not shown). To characterize the function of Yil104c and its possible connection to yeast RNase III, we replaced the YIL104C endogenous promoter with a galactose-inducible promoter and an N-terminal HA epitope tag using homologous recombination. After preculture of the resulting strain in the presence of galactose, we shifted the yeast culture into a medium lacking galactose. The shift in culture conditions led to a rapid depletion of the protein, most of it being depleted after 3 h of growth in a medium lacking galactose (Fig. 2A). Because yeast RNase III is involved in the processing of small nuclear and small nucleolar RNAs (19, 27), we examined the level of these species after Yil104c depletion. We monitored the levels of various small RNAs from both the box C/D and H/ACA families and from the spliceosomal RNA family. Northern blot analysis showed that box C/D snoRNAs were rapidly depleted after Yil104c depletion (Fig. 2B). In contrast, box C/D snoRNAs and the RNA component of the RNase mitochondrial RNA processing ribonuclease complex were not affected by Yil104c depletion (Fig. 2B). The U5 spliceosomal small nuclear RNA was also unaffected (data not shown). The apparent increase of the level of the small RNAs of the box C/D family upon Yil104c depletion is due to a degradation of 25S and 18S rRNAs (data not shown).

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of several steps of the 35S rRNA precursor required for the production of the 18 S rRNA. This phenotype is likely due to the rapid depletion of snR10 and snR30, which are required for these steps (28, 29).

The Ynl124w/Naf1p Protein Is Required for Box HACA snoRNA Accumulation—Exhaustive two-hybrid screen analyses indicated that Shq1p interacts with an essential protein of the yeast genome, Ynl124w (30). Ynl124w also interacted with the box HACA snoRNP proteins Cbf5p and Nhp2p in the same study (30). Furthermore, mass spectrometry analysis of proteins copurifying with an overexpressed FLAG-tagged version of Cbf5p identified Ynl124w and Shq1p (31). Naf1p also shows sequence similarities with the box HACA snoRNP protein Gar1p and the RNA-binding protein Nrd1p in various regions of the protein sequence (Fig. 4A). These observations and our results showing that Shq1p is required for box HACA snoRNA accumulation suggested a role for Ynl124w in box HACA snoRNA metabolism. We therefore analyzed the function of the Ynl124w protein in vivo using the same procedure described for Shq1p, i.e. conditional depletion of Ynl124w after the replacement of the YNL124W endogenous promoter with a galactose-inducible promoter and an N-terminal HA epitope tag.

Because this study was in progress, the YNL124W open reading frame became labeled in the Saccharomyces genome data base as NAF1 (nuclear association factor 1). We will therefore use the name Naf1p to describe the protein product of YNL124W. Upon depletion of Naf1p, we observed a rapid and specific depletion of box HACA snoRNAs, whereas the level of other small RNA species remained unchanged (Fig. 4B). The level of Nhp2p was also unaffected after Naf1p depletion (data not shown), as observed during Shq1p depletion (Fig. 2A). We conclude that Shq1p and Naf1p are similarly required for box HACA snoRNA accumulation. Not surprisingly, depletion of Naf1p resulted in ribosomal RNA processing defects (Fig. 4C) that were similar to those observed during Shq1p depletion (Fig. 3B). Because of the similarities of the phenotypes observed during Naf1p and Shq1p depletion, we checked whether the stability of Naf1p requires the presence of Shq1p. We constructed a yeast double-tagged strain (DT) where the GAL:HA-SHQ1 strain also carries a TAP (32)-tagged version of Naf1p. Upon depletion of Shq1p, the level of Naf1p remained constant (data not shown), suggesting that the stability of Naf1p does not require wild-type levels of Shq1p.

**Shq1p and Naf1p Are Not Strongly Associated with Box HACA snoRNPs**—A straightforward explanation for the rapid depletion of box HACA snoRNAs upon Shq1p and Naf1p depletion would be that Shq1p and Naf1p are integral components of box HACA snoRNPs but that they had not been identified in previous work studying the protein composition of box HACA snoRNPs. If this were the case, we would expect Shq1p and Naf1p to associate strongly and specifically with box HACA snoRNAs, as described previously for core proteins of box HACA snoRNPs (11–14). To test this hypothesis, we generated chromosomal TAP-tagged versions of Shq1p and Naf1p. The TAP-tagged versions of these two proteins were functional, since introduction of the TAP tag at the C terminus of these proteins did not induce a significant growth defect (data not shown). Extracts were first made from these two strains followed by incubation with IgG beads to bind the protein A portion of the TAP tag. After extensive washing, RNAs were extracted from the precipitates and detected by Northern blots. As a control for a weak association with box HACA snoRNAs, we used a TAP-tagged Nop1p strain, since it was shown previously that a protein A-tagged version of Nop1p is weakly associated with box HACA snoRNAs at low salt concentrations (12). Northern blot analysis showed that a small amount of box...
H/ACA snoRNAs were associated with Shq1p and Naf1p but not significantly higher than with an untagged control (Fig. 5A). In contrast, a weak but significant association could be detected with Nop1p. In addition, Nop1p was strongly associated with box C/D snoRNAs at both salt concentrations, whereas no significant association was found for Shq1p and Naf1p. The lack of strong association of both Shq1p and Naf1p with box H/ACA snoRNAs in whole-cell extracts suggests that Shq1p and Naf1p are not integral components of box H/ACA snoRNPs.

To test whether Shq1p would associate specifically with the precursors of box H/ACA snoRNAs rather than with the mature species, we constructed a yeast strain carrying a deletion of RNT1 in addition to the TAP-tagged Shq1p. The RNT1 deletion results in accumulation of some box H/ACA snoRNA precursors (19). Northern blot analysis of Shq1p-TAP precipitates in a rnt1 deletion background failed to reveal a significant association with the box H/ACA snoRNA precursors tested (snR36, snR43, and snR46, data not shown).

Overall, these results suggest that Shq1p and Naf1p are not strongly associated with either precursors or mature box H/ACA snoRNAs. To investigate further the relationships between Shq1p, Naf1p, and box H/ACA snoRNPs, we prepared extracts from Shq1p-TAP- and Naf1-TAP-tagged strains as

**Fig. 3.** Shq1p is required for ribosomal RNA processing. **A**, a schematic view of the ribosomal RNA-processing pathway. The black rectangles indicate the positions of the oligonucleotide probes used in **B**. **B**, ribosomal RNA processing defect in Shq1p-depleted cells. RNAs extracted from wild-type (WT) and GAL::SHQ1 yeast strains grown in medium containing (Gal) or lacking (Glu) galactose were run on denaturing formaldehyde-agarose gels, transferred to Nylon membranes, and hybridized with oligonucleotide probes shown in **A**. Shown are regions of the blots identifying precursors, intermediates, and products that showed a processing defect.
Fig. 4. Naf1p is required for box H/ACA snoRNAs accumulation and for rRNA processing. A, domain organization of the Naf1p protein. Dark boxes represent the regions of sequence similarities with Nrd1p. The gray box represents a region rich in glutamine (Q Rich). B, Naflp is required for box H/ACA snoRNAs accumulation. Legends are as in Fig. 2, except that a GAL::NAF1 strain was used. C, ribosomal RNA-processing defect in Naf1p-depleted cells. Legends are as in Fig. 3, except that a GAL::NAF1 strain was used.

Well as from the double-tagged strain (GAL::HA-SHQ1P, NAF1-TAP), and we fractionated them by centrifugation on glycerol gradients. We followed the sedimentation profiles of TAP-tagged Naflp and Shq1p by Western blot and compared them with the sedimentation profile of the snR42 box H/ACA snoRNA and of the box H/ACA snoRNP protein Nhp2p. The peak of Shq1p-TAP was found in fraction 18. Two peaks of Naflp sedimentation were observed, one of which was in the heavier fractions that may correspond to the rRNA complexes, since rRNAs were also observed in these fractions. The second peak corresponded to fraction 18, where the bulk of Naflp sediments and the major peak of Shq1p were also observed. Box H/ACA snoRNP components (snr42 and Nhp2p) were found throughout several fractions of the gradient, but no enrichment of these components was found in fraction 18.

To confirm the cosedimentation of Shq1p and Naflp in fraction 18, we used the double-tagged strain where Shq1p is HA-tagged and Naflp is TAP-tagged. This strain allowed the detection of both proteins in the same gradient fractions. Western blot analysis of sedimentation profiles in this double-tagged strain confirmed the cosedimentation of these two proteins in fraction 18. Strikingly, we found a third peak of sedimentation of Naflp-TAP in the glycerol gradient fractions made from this double-tagged strain. This third peak in fractions 6–7 overlapped with the major position of box H/ACA snoRNPs. We also found that there was much less Naflp present in fraction 18 than in the single-tagged Naflp-TAP strain. In contrast to the Naflp-TAP strain, where most of the Naflp protein was present in fraction 18, the bulk of the Naflp protein in the double-tagged strain was located in higher sedimentation coefficient fractions and much less in fraction 18. This result is possibly due to a deleterious effect of the double tag, since this double-tagged strain showed a significant growth defect compared with a wild-type strain or a single-tagged strain (data not shown). This negative effect of the double tag might result in a stabilization of transient interactions with box H/ACA snoRNPs that are otherwise not revealed in a gradient run in parallel on which native molecular markers were loaded. The profiles shown for the snR42 snoRNA are from fraction samples of the Naflp-TAP gradient, whereas the profiles shown for the Nhp2p and U3 are from fraction samples of the Shq1p-TAP gradient, but similar profiles were obtained in both gradients.

Fig. 5. Shq1p and Naflp are not strongly associated with box H/ACA snoRNAs. A, immunoprecipitation of RNAs in untagged, Shq1p-TAP, Naflp-TAP, and Nop1p-TAP strains whole-cell extracts. After precipitation on IgG beads, RNAs were extracted, fractionated on a 6% acrylamide gel, and detected by Northern blot using oligonucleotide probes. Immunoprecipitations were performed at either 150 or 500 mM NaCl. T represents 10% of the total RNAs in the extract before IgG precipitation. B, sedimentation profiles of Shq1p, Naflp, and of box H/ACA snoRNPs. Total extracts made from yeast strains carrying a TAP-tag versions of Shq1p or Naflp or a double-tag (Naflp-TAP, Shq1p-TAP, Nop1p-TAP) were loaded. The profiles shown for the snR42 snoRNA are from fraction samples of the Naflp-TAP gradient, whereas the profiles shown for the Nhp2p and U3 are from fraction samples of the Shq1p-TAP gradient, but similar profiles were obtained in both gradients.

In vitro Translated Shq1p Interacts with Naflp and Nhp2p in Reticulocyte Lysates—Two-hybrid studies suggested a direct interaction may be stabilized in the double-tagged strain. Overall these experiments suggest that Shq1p and Naflp exist as a complex of two or more proteins that are probably not associated with box H/ACA snoRNPs and would correspond to fraction 18 of the gradient. In addition, the result obtained with the double-tagged strain, which showed a cosedimentation of Naflp with box H/ACA snoRNPs, suggested that Naflp may interact transiently with box H/ACA snoRNPs and that this interaction may be stabilized in the double-tagged strain.
interaction between Shq1p and Naf1p and between Naf1p and both Nhp2p and Cbf5p (30). In addition, genome-scale protein purification and mass spectrometry studies showed that Shq1p as well as Naf1p copurified with overexpressed FLAG-tagged Cbf5p, the pseudouridyl synthetase (31). This result as well as our glycerol gradient sedimentation results (Fig. 5B) suggested an association between these proteins in vitro. We tested for in vitro interactions between Shq1p and either Naf1p, Nhp2p, or other box H/ACA snoRNP proteins (Cbf5p and Gar1p) using an in vitro translation pull-down approach. We translated Naf1p, Nhp2p, Cbf5p, and Gar1p in vitro in the presence of [35S]methionine and incubated these labeled proteins with unlabeled Shq1p, either untagged or tagged at the C terminus with a calmodulin-binding protein tag. The proteins were then mixed with calmodulin beads and extensively washed, and the presence of the radiolabeled proteins associated with the beads and hence with Shq1p was revealed by autoradiography (Fig. 6). This experiment showed that both Naf1p and Nhp2p bind to Shq1p when these proteins are generated in vitro by translation in reticulocyte lysates. In contrast, in vitro translated Gar1p did not bind to in vitro translated Shq1p, although we do not know whether in vitro translated Gar1p is folded properly and, therefore, active in this assay. The interaction between Shq1p and both Nhp2p and Naf1p was specific since no binding was detected with beads and reticulocyte lysate alone or with untagged in vitro translated Shq1p. When in vitro translated Cbf5p was used in these studies, it bound non-specifically to beads alone (data not shown), precluding any analysis of the interaction between Shq1p and this particular protein. These data demonstrate that Naf1p interacts with Shq1p in vitro in reticulocyte lysates, as suggested from published two-hybrid studies (30). Although proteins present in the cell lysate may bridge two proteins and result in indirect interactions, our results also suggest that Shq1p can bind directly to Nhp2p, showing an additional novel link between Shq1p and a component of the mature box H/ACA snoRNP.

**Shq1p and Naf1p Show Nuclear Localization**—To better understand the function of Shq1p and Naf1p, we analyzed their subcellular localization. We used the GAL-HA-tagged strains described above to perform immunolocalization using the anti-hemagglutinin 12CA5 monoclonal antibody. When compared with 4,6-diamidino-2-phenylindole (DAPI) staining, the two proteins clearly showed a nuclear localization pattern (Fig. 7). Shq1p seemed to be excluded from a crescent-shaped nuclear territory, which was reminiscent of the nucleolus, suggesting that it was not localized in the nucleolus. The exclusion from the nucleolus would help to fit the result described above that Shq1p and Naf1p are not core components of mature box H/ACA snoRNP, which are mainly localized in the nucleolus at the steady state. Our results also fit with the observation that Naf1p is nucleoplasmic but does not localize to the nucleolus (36, 37). The localization of Shq1p does not fit with the whole-genome localization studies from the Snyder group (ygac.med.yale.edu/ygac/cgi/loc_oe_qry.pl?gene_name=yil104c&localization=), who reported that a V5-tagged version of Shq1p is cytoplasmic. However, given the function of Shq1p in box H/ACA snoRNA metabolism, it is more likely that the nuclear localization that we observe is more representative of its biological function and that the V5 tag used may have interfered with proper localization of the protein.

**DISCUSSION**

We have described two proteins, Shq1p and Naf1p, that are required for box H/ACA snoRNA accumulation. In the absence of these proteins, box H/ACA snoRNAs become depleted, resulting in a dramatic inhibition of pre-ribosomal RNA processing and presumably in the inhibition of ribosomal RNA pseudouridylation, as described previously for protein members of the box H/ACA snoRNPs (12, 13). However, in contrast to these core proteins, Shq1p and Naf1p are not associated with precursor or mature box H/ACA snoRNAs. Moreover, although they are clearly localized in the nucleus, they seem to be absent from the nucleolus. Our data are consistent with results obtained in Dr. Yves Henry, Dr. Michelle Caizergues-Ferrer, and Dr. David Tollervey’s groups who showed that Naf1p is a nucleoplasmic protein required for box H/ACA snoRNA accumulation (36, 37).

We originally studied the function of Shq1p based on a two-hybrid interaction with yeast RNase III, Rnt1p. Rnt1p has been shown to process three box H/ACA snoRNAs at their 5’ end (19). We failed to find an interaction between Rnt1p and Shq1p by other means than the two-hybrid assay, and the search of various yeast proteomics databases did not indicate an interaction between these two proteins. It is possible that the two-hybrid interaction between Rnt1p and Shq1p is fortuitous and cannot be reproduced by other ways than the two-hybrid assay. Another possibility is that Rnt1p interacts only with a C-terminal domain of Shq1p but that interaction with the full-length Shq1p protein cannot be observed. However, we think that given the functions of both Rnt1p and Shq1p in early box H/ACA snoRNA metabolism, it is likely that this two-hybrid interaction indicates a weak but significant interaction, which could be relevant in early box H/ACA snoRNA biogenesis. One possibility would be that Rnt1p recruits Shq1p at the vicinity of the box H/ACA snoRNAs and that this recruitment would help to enhance the rate of packaging of just-processed.
snoRNAs into snoRNPs. This interaction would be relevant to only a few snoRNAs, since most box H/ACA snoRNAs do not seem to be processed by Rnt1p (19). Another possibility would be that Shq1p helps to recruit Rnt1p at the vicinity of the snoRNA precursors processed by Rnt1p. In this case, we would expect to detect an accumulation of unprocessed snR36, snR43, and snR46 upon Shq1p depletion. These unprocessed species were not detected in RNAs extracted from Shq1p-depleted cells (data not shown). However, since depletion of Shq1p results in a rapid depletion of snoRNPs, it is possible that the rapid turnover of snoRNAs precursors when they are no longer packaged into snoRNPs may mask any effect on the processing efficiency. Thus, the potential role of the Rnt1p–Shq1p interaction in the processing of box H/ACA snoRNAs may be difficult to demonstrate. Finally, the recent demonstration of an interaction between Rnt1p and Gar1p (33) strengthens the potential link between Rnt1p and proteins of box H/ACA snoRNPs.

Based on the data presented in this study and on proteomics studies, we can propose the following model to describe the function of Shq1p and Naf1p in box H/ACA snoRNP biogenesis. Proteomics studies and our in vitro interaction studies show that Shq1p and Naf1p associate with each other and with some protein components of the box H/ACA snoRNP such as Cbf5p, the putative pseudouridylyl synthase, and Nhp2p. Our glycerol gradient cosedimentation data are also consistent with the idea that Shq1p and Naf1p interact with each other and form a complex. Naf1p was independently identified by Fatica and Tollervey as a protein with an RNA binding motif and was shown to possess RNA binding activity in vitro (37). The sequence similarities between Naf1p and Gar1p and Nrd1p (Fig. 4A) also suggest a role for Naf1p in box H/ACA snoRNA metabolism. Because Shq1p and Naf1p are mainly localized in the nucleoplasm, we propose that they may associate weakly and transiently with newly transcribed box H/ACA snoRNAs in the nucleoplasmic compartment. In favor of this model, Dez et al. (36) could show a weak but significant association of Naf1p with box H/ACA snoRNAs. In addition, a double-tagged strain, which shows a significant growth defect, also results in cosedimentation of Naf1p with the box H/ACA snoRNPs, suggesting that transient interactions between Naf1p and components of the box H/ACA snoRNP may be stabilized in that strain. In this model Naf1p would remain bound to partially or fully assembled snoRNPs, resulting in partially dysfunctional snoRNPs and, therefore, in a growth defect.

In favor of a model of co-transcriptional recruitment, Fatica et al. showed that Naf1p interacts with the phosphorylated CTD of RNA polymerase II using GST-pull down and two-hybrid studies (37). In addition to its RNA binding properties, the association of Naf1p with the elongation form of RNA polymerase II further supports the hypothesis that Naf1p and possibly Shq1p play an early role in box H/ACA snoRNA biogenesis. The association of Shq1p and Naf1p with newly transcribed box H/ACA snoRNAs would occur in a complex with Cbf5p and Nhp2p (30, 31). This interaction could bring Cbf5p and Nhp2p at the vicinity of box H/ACA snoRNAs to form the initial core of the box H/ACA snoRNP and, thus, nucleate the formation of the mature snoRNP. In this model, Cbf5p and Nhp2p would bind first and would then recruit Gar1p and Nop10p. This model would fit in vitro reconstitution results showing that the binding of human Gar1p is not a prerequisite for binding of other box H/ACA snoRNP proteins (16) and with the fact that Gar1p is not required for box H/ACA snoRNA stability in yeast cells (11).

Whatever the role of these proteins, it seems likely that their function is conserved among eukaryotes. Both proteins are essential in Saccharomyces cerevisiae, and orthologs can be found in other eukaryotic genomes. Shq1p seems to be the most conserved protein of the two, since orthologs of Shq1p/Yil104c can be found in humans (FLJ10539), Drosophila melanogaster (CG10655), Caenorhabditis elegans (Y48A5A.1), and Schizosaccharomyces pombe (Pi052p), whereas orthologs of Naf1p/Shq2p are present in human (ALF), mice (SMPD3), and S. pombe (Spbc30d10.15p), but no obvious orthologs are indicated in the databases for D. melanogaster or C. elegans.

Whether a protein similar to Naf1p is truly absent from these two organisms or whether an ortholog with a very weak sequence similarity is present is not clear. Strikingly, Naf1p shows limited similarities with ALF, a transcription factor with similarity to the transcription factor TFIIA-α/β. This sequence similarity between Naf1p and a putative transcription factor further supports the model that Naf1p may associate with the transcription machinery to interact with newly transcribed box H/ACA snoRNAs. It seems likely that given the conservation of Shq1p and Naf1, similar functions will be found for its orthologs in box H/ACA snoRNA metabolism in higher eukaryotes. It would also be interesting to test whether the human orthologs of Shq1p and Naf1p have any putative function in telomerase RNA metabolism, since the human telomerase shows motifs that are characteristic of box H/ACA snoRNAs (15, 16, 34, 35). Thus, the function of the Shq1p and Naf1p proteins could be extended not only to box H/ACA snoRNA metabolism but also to human telomerase RNA biogenesis.

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