Nhp2p and Nop10p are essential for the function of H/ACA snoRNPs

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The small nucleolar ribonucleoprotein particles containing H/ACA-type snoRNAs (H/ACA snoRNPs) are crucial *trans*-acting factors intervening in eukaryotic ribosome biogenesis. Most of these particles generate the site-specific pseudouridylation of rRNAs while a subset are required for 18S rRNA synthesis. To understand in detail how these particles carry out these functions, all of their protein components have to be characterized. For that purpose, we have affinity-purified complexes containing epitope-tagged Gar1p protein, previously shown to be part of H/ACA snoRNPs. Under the conditions used, three polypeptides of 65, 22 and 10 kDa apparent molecular weight specifically copurify with epitope-tagged Gar1p. The 22 and 10 kDa polypeptides were identified as Nhp2p and a novel protein we termed Nop10p, respectively. Both proteins are conserved, essential and present in the dense fibrillar component of the nucleolus. Nhp2p and Nop10p are specifically associated with all H/ACA snoRNAs and are essential to the function of H/ACA snoRNPs. Cells lacking Nhp2p or Nop10p are impaired in global rRNA pseudouridylation and in the A1 and A2 cleavage steps of the pre-rRNA required for the synthesis of mature 18S rRNA. These phenotypes are probably a direct consequence of the instability of H/ACA snoRNAs and Gar1p observed in cells deprived of Nhp2p or Nop10p. Our results suggest that Nhp2p and Nop10p, together with Cbf5p, constitute the core of H/ACA snoRNPs.

Keywords: Nhp2p/Nop10p/box H/ACA snoRNPs/pre-rRNA processing/snoRNA stability

Introduction

Synthesis of ribosomes in all eukaryotes requires extensive modification of a primary rRNA transcript that contains spacer regions flanking the rRNA sequences found in cytoplasmic ribosomes. Maturation of the pre-rRNA involves essentially two types of events: modifications of specific nucleotides confined to regions corresponding to

mature rRNAs, and endo- and exonucleolytic cleavages that remove the transcribed spacers (reviewed in Venema and Tollervey, 1995; Sollner-Webb *et al.*, 1996). Most of the nucleotide modifications appear on the pre-rRNA before nucleolytic processing; these are methylations at the 2'-*O*-hydroxyl position of riboses, and conversions of uridine residues into pseudouridines (Maden, 1990). Interestingly, most of these modifications are clustered in the universal core regions of rRNAs and have been remarkably conserved during evolution, suggesting they play an important role(s) in ribosome function (Lane *et al.*, 1995).

An ever increasing number of *trans*-acting factors required for pre-rRNA maturation are being characterized (reviewed in Venema and Tollervey, 1995; Sollner-Webb *et al.*, 1996). Several of these are small nucleolar RNAs (snoRNAs), most of which can be classified into two families based on their sharing sequence motifs and displaying structural homologies (Balakin *et al.*, 1996). The members of the family of C/D snoRNAs all contain, as their name implies, two short motifs termed the C (consensus 5'-UGAUGA-3') and D boxes (5'-GUCUGA-3'). The second family is constituted by snoRNAs which all share the so-called H (consensus 5'-ANANNA-3') and ACA boxes. C/D and H/ACA snoRNAs display peculiar modes of expression. In metazoans, although some snoRNAs are independently transcribed, the vast majority are produced from introns of pre-mRNAs by 5'→3' and 3'→5' exonucleolytic processing (Kiss and Filipowicz, 1995; Caffarelli *et al.*, 1996; Cavaillé and Bachellerie, 1996; Watkins *et al.*, 1996; Ganot *et al.*, 1997a; reviewed in Sollner-Webb, 1993; Tollervey and Kiss, 1997). In yeast and plants, some C/D snoRNAs are processed from di- or polycistronic transcripts by endo- and exonucleolytic activities (Leader *et al.*, 1997; Chanfreau *et al.*, 1998; Petfalski *et al.*, 1998). In all cases examined, and irrespective of whether the snoRNAs were of intronic or non-intronic origin, the C, D, H and ACA conserved boxes, as well as the stem structures present in their vicinity, have been shown to be essential for normal snoRNA processing and accumulation (Terns *et al.*, 1995; Balakin *et al.*, 1996; Caffarelli *et al.*, 1996; Cavaillé and Bachellerie, 1996; Watkins *et al.*, 1996; Ganot *et al.*, 1997a).

A few C/D and H/ACA snoRNAs are required for certain pre-rRNA cleavage events. The C/D box snoRNAs U3 (Hughes and Ares, 1991), U14 (Li *et al.*, 1990) and U22 (Tycowski *et al.*, 1994), as well as the H/ACA snoRNAs snR10 (Tollervey, 1987) and snR30 (Morrissey and Tollervey, 1993) are necessary for pre-rRNA processing steps leading to the production of mature 18S rRNA. In contrast, U8 (C/D snoRNA) intervenes in 5.8S and 28S rRNA maturation (Peculis and Steitz, 1993). It was shown recently, however, that the remaining known

members of both families are required for site-specific modifications of the pre-rRNA: they function, by way of specific snoRNA/pre-rRNA Watson–Crick base-pairing interactions, as guide RNAs selecting the ribose moieties that will undergo methylation of the 2' oxygen in the case of C/D snoRNAs (Cavaillé *et al.*, 1996; Kiss-László *et al.*, 1996, 1998; Nicoloso *et al.*, 1996; Tycowski *et al.*, 1996; Cavaillé and Bachellerie, 1998; reviewed in Tollervey, 1996; Bachellerie and Cavaillé, 1997, 1998; Maden and Hughes, 1997; Tollervey and Kiss, 1997) or the uridine residues to be converted into pseudouridines in the case of H/ACA snoRNAs (Ganot *et al.*, 1997b; Ni *et al.*, 1997; reviewed in Smith and Steitz, 1997).

In the nucleolus, snoRNAs are not free but found tightly associated with proteins in ribonucleoprotein complexes (snoRNPs) of ~10–20S in size. It is likely that only the complexes possess correctly regulated biological activity (Bousquet-Antonelli *et al.*, 1997). So far only few proteins of C/D and H/ACA snoRNPs have been characterized. The Nop1p/fibrillarin protein is specifically associated with all C/D snoRNAs (Tyc and Steitz, 1989; Baserga *et al.*, 1991; Peculis and Steitz, 1994; Ganot *et al.*, 1997a) and is required for pre-rRNA methylation and processing (Tollervey *et al.*, 1993), consistent with the involvement of C/D snoRNAs in both processes. The yeast Nop58p/Nop5p protein (Gautier *et al.*, 1997; Wu *et al.*, 1998) has also been shown to interact with all tested C/D snoRNAs (Wu *et al.*, 1998; D.L.J.Lafontaine and D.Tollervey, personal communication) and to be required for their stability (D.L.J.Lafontaine and D.Tollervey, personal communication). In addition, a mouse protein of 65 kDa (Watkins *et al.*, 1998) and a *Xenopus laevis* protein with an apparent mol. wt of 68 kDa (Caffarelli *et al.*, 1998) have been found to interact with the box C/D-terminal-stem core motif of U14 and U16, respectively, but their identities have not yet been determined. H/ACA snoRNPs are known to contain the proteins Gar1p and Cbf5p. Gar1p is an essential glycine/arginine rich (GAR) domain-containing nucleolar protein that is required both for 18S rRNA production and rRNA pseudouridylation (Girard *et al.*, 1992; Bousquet-Antonelli *et al.*, 1997). The essential nucleolar protein Cbf5p (Jiang *et al.*, 1993; Cadwell *et al.*, 1997) is also necessary for rRNA pseudouridylation (Lafontaine *et al.*, 1998) and 18S rRNA formation (Cadwell *et al.*, 1997; Lafontaine *et al.*, 1998). Cbf5p is strongly homologous to known pseudouridine synthases (Koonin, 1996; Becker *et al.*, 1997) and, therefore, it probably provides the actual catalytic activity. It is highly probable that H/ACA snoRNPs contain other proteins in addition to Gar1p and Cbf5p. Indeed, the snR30-containing snoRNP has been purified, and four proteins of apparent mol. wts 65, 25, 23 and 10 kDa appear to be tightly associated with snR30 (Lübber *et al.*, 1995). Only the 25 kDa protein was positively identified as Gar1p by Western blot analysis (Lübber *et al.*, 1995).

In the present study, we sought to characterize the common core protein components of H/ACA snoRNPs by affinity purification of complexes containing epitope-tagged Gar1p. This is clearly a necessary first step in determining the various protein–protein and RNA–protein interactions that govern both the structure and function of these particles. We show that in addition to Cbf5p and Gar1p, all H/ACA snoRNPs contain the Nhp2p protein

Fig. 1. Gar1p is associated with 3 proteins. Purification over an IgG–Sepharose column of complexes containing Gar1p–ZZ (lane 1) or Nhp2p–ZZ (lane 2). Proteins eluted from the columns were resolved by SDS–PAGE on a 15% polyacrylamide gel and revealed by Coomassie staining. Note that the extract used to purify epitope-tagged Nhp2p also contains wild-type Nhp2p. M, molecular weight markers. The identity of the bands is indicated by arrows. The contaminating IgGs are indicated by asterisks, the Gar1p–ZZ degradation product by a dot and the bands probably corresponding to Cbf5p by unfilled arrows.

(Kolodrubetz and Burgum, 1991) and a small novel protein we named Nop10p. These proteins are essential to the synthesis and function of H/ACA snoRNPs and are likely, together with Cbf5p, to constitute their core.

Results

Gar1p is associated with the Nhp2p and Nop10p proteins

We have previously reported that the Gar1p protein, tagged at its C-terminus with two synthetic IgG binding domains (ZZ domains) derived from *Staphylococcus aureus* protein A, is specifically associated with all H/ACA snoRNAs and fully functional (Ganot *et al.*, 1997a). In an attempt to quickly and efficiently purify H/ACA snoRNPs, we made use of a strain expressing Gar1p–ZZ. An extract prepared from a Gar1p–ZZ expressing strain under non-denaturing conditions was loaded onto an IgG–Sepharose column, and complexes retained along with Gar1p–ZZ were eluted with acetic acid (see Materials and methods for details). Under these conditions, three distinct major polypeptides other than contaminating IgGs are specifically coeluted with Gar1p–ZZ, migrating as proteins of ~65, ~22 and ~10 kDa (Figure 1, lane 1). The broad bands labelled with asterisks correspond to contaminating IgGs. The polypeptide with an apparent molecular weight of 33 kDa (indicated by a dot in Figure 1) is a Gar1p–ZZ degradation product since Western blot analysis revealed that it contains the ZZ tag (data not shown). Given its apparent mobility, the polypeptide migrating just ahead of

A

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S.cerevisiae Nhp2p      73-VKRGVKEVVKALRKGEKGLVVIAGDISPADVISHIPVLCEHDSVPYIFIPSKQDLGAAGATK-134
H.sapiens DJ0167F23.5 100-LRRGVKEVQKFNKGEKGLVLAEDTLPIEVYCHLPVMCEDRNLAIVSIPLR-----161
S.cerevisiae RPL7A-1   132-VKYGLNHVVALTENKKAKLVLIANDVDPIELVVFLPALCKKMGVPPYIAIVKQKARLGLTLVNQK-193
H.sapiens RPL7a        136-LRAGVNTVTTLVENKKAQLVVIADVDPIELVVFLPALCKKMGVPPYCIKQKARLGLRIVHRK-197
H.sapiens NHP2L1       35-LRKGANEATKILNRGISEFIVMAADAEPLIILHLPLLCEDKNVPYVVFVRSKQALGRACGVS-96
M.jannaschii RPL7AE    27-IKKGANEVTKAVERGIKLVIIAEDVKPEEVVAHLPLCEKGIPIYAYVASKQDLGKAAGLE-88
S.solfataricus RPHS6   35-IKKGINETTKAVERGOAKLVIIAEDVQPEEIVAHLPPLCDEKKIPYVYVSSKKALGEACGLQ-96
S.cerevisiae RPL32     23-YTLGYKSTVKSLRQCKSKLIIIAANTPVLRKSELEYAMLSKTKVYVYFQGGNNELGTAVGKL-84
M.musculus RPL30       27-YVLGYKQTLKMTIQCKAKLVILANNCPALRKSEIEYYAMLAKTGVHHYSGNNIELGTACGKY-88
CONSENSUS              ---G-nev-k---g-aklv!IA-#-p-e---hlp-lc--k--py-----k--lg-a-g--

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B

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S.cerevisiae Nop10p    MELMYTLGPDGKRITTYTLKKVTESGEITKSAHPARFSPDDKYSRQRTVLKKRFGLVPGQ----- 58
C.elegans              MFLRYFLDENQQRVYTLKRTAPSGEQITLTAHPARFSPEDKNSKYRIIIKKRFGLLPTQAKTVC 64
Human/Mouse           MFLQYYLNEQGDRVYTLKKFDPMGQQTCSAHPARFSPDDKYSRHRITIKKRFKVLMTQPRPVL 64
CONSENSUS             MfL-Y-L-e#g-R!YTLKk--psG#qT-sAHPARFSP#DKySr-R!tiKKRFgllptQ----v-

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Fig. 2. (A) Nhp2p belongs to a family of putative RNA binding proteins. Alignments of *S.cerevisiae* Nhp2p with related human proteins Nhp2L1p (Saito *et al.*, 1996) and DJ0167F23.5 (DDBJ/EMBL/GenBank accession No. AC004079; genome sequencing centre, Washington University) with L32 ribosomal proteins from yeast (Dabeva and Warner, 1987) and mouse (L30, Wiedemann and Perry, 1984), L7a ribosomal proteins from human (Colombo *et al.*, 1991), *S.cerevisiae* (Yon *et al.*, 1991) and *Methanococcus jannaschii* (Bult *et al.*, 1996), and HS6 ribosomal protein from *Sulfolobus solfataricus* (Sensen *et al.*, 1996). The alignment was obtained with the MultAlin program (Corpet, 1988). Only the region of a given protein displaying significant homologies with all the others is shown. (B) Nop10p is highly conserved. *Saccharomyces cerevisiae* Nop10p was aligned with its counterpart from *C.elegans* (Wilson *et al.*, 1994) and mouse/human (EST data). The alignment was obtained with the MultAlin program (Corpet, 1988). != I or V; # = D, E, N or Q.

the 67 kDa marker probably corresponds to Cbf5p (Figure 1, lane 1, unfilled arrow), already shown to be part of H/ACA snoRNPs (Lafontaine *et al.*, 1998). A partial N-terminal amino acid sequence was obtained for the 22 and 10 kDa polypeptides MGKDNKEHK and MHLMYTLGPD, respectively. BLAST analysis of the yeast protein database identified the 22 kDa protein as Nhp2p (Kolodrubetz and Burgum, 1991) (Figure 2A). A protein containing the sequence MHLMYTLGPD could not be found in the protein database. However, a search of the yeast genome against a nucleotide sequence corresponding to this peptide identified a small open reading frame (ORF) on chromosome VIII, situated between ORF YHR072W (*ERG7*) and YHR073W, encoding a protein of 58 amino acids (Figure 2B). We termed this protein Nop10p.

To confirm the specific association between Gar1p, Nhp2p and Nop10p, we repeated the affinity purification using an extract prepared from a wild-type strain expressing a Nhp2p protein tagged at its C-terminus with the two synthetic IgG-binding domains derived from *S.aureus* protein A. This Nhp2p-ZZ fusion rescues perfectly an *nhp2::TRP1* knock-out (data not shown) showing that it is functional. Apart from contaminating IgGs (indicated by asterisks next to lane 2 in Figure 1), four major polypeptides are specifically coeluted with Nhp2p-ZZ. One of these was unambiguously identified as Gar1p by Western blot analysis (not shown). The upper polypeptide of apparent mol. wt 65 kDa is very probably Cbf5p (Figure 1, lane 2, unfilled arrow). The polypeptides of 22 and 10 kDa apparent mol. wt precisely comigrate with, and thus almost certainly are, wild-type Nhp2p and Nop10p, respectively (Figure 1, compare lanes 1 and 2).

Thus, we conclude that Gar1p, Nhp2p and Nop10p are specifically associated with each other.

Nhp2p and Nop10p are essential, conserved proteins present in the dense fibrillar component of the nucleolus

Nhp2p was previously identified in a search for novel high mobility group (HMG)-like proteins in *Saccharomyces cerevisiae* (Kolodrubetz *et al.*, 1988; Kolodrubetz and Burgum, 1991) and its gene was shown to be essential (Kolodrubetz and Burgum, 1991). Even though Nhp2p shares physico-chemical properties with HMG proteins, it displays no significant sequence homology with them (Kolodrubetz and Burgum, 1991). However, these authors noted that Nhp2p displays significant homologies with the L7a ribosomal protein from rat. Moreover, Vilardell and Warner (1997) have noted that Nhp2p shares homologies with the *S.cerevisiae* L32 ribosomal protein, which is an RNA-binding protein, and predicted that Nhp2p would bind RNA as well. We also conducted a comprehensive BLAST search to identify Nhp2p homologues. The best matches were to two human proteins; one is encoded by the gene WUGSC:H DJ0167F23.5 (genome sequencing, Washington University, St Louis, MO; DDBJ/EMBL/GenBank accession No. AC004079), the other by the *NHP2L1* gene (Saito *et al.*, 1996). The former protein displays over a 77 amino acid C-terminal segment 49% identity (72% homology) with yeast Nhp2p. The protein encoded by *NHP2L1* displays 38% identity with yeast Nhp2p. Although these proteins are clearly highly related to yeast Nhp2p, we suspect that neither of them correspond to its bona fide human counterparts. We have aligned together Nhp2p, its two related human proteins, and HS6, L32 and L7a ribosomal proteins from various organisms (Figure 2A). These proteins display homologies over a region of 53 amino acids, which in *S.cerevisiae* L32 has been implicated in RNA binding (Vilardell and Warner, 1997). Most notable is the conservation of spacing between blocks of homologous amino acids. These proteins prob-

Fig. 3. Nhp2p and Nop10p are found in the DFC of the nucleolus. Immunolocalization of Gar1p-ZZ (A), Nhp2p-ZZ (B) and Nop10p-ZZ (C) by electron microscopy. Tagged proteins are detected by treatment with anti-protein A antibodies followed by incubation with colloidal gold conjugated protein A. The positions of some gold particles in the dense fibrillar component of the nucleolus are indicated by arrowheads. No, nucleolus; Nu, nucleoplasm.

ably constitute a novel family of RNA binding polypeptides.

To determine whether the ORF encoding Nop10p is essential, a *nop10::TRP1* knock-out allele was integrated in a *trp1*– diploid strain and tetrads obtained from *nop10::TRP1/NOP10* diploids were dissected. In all cases, only two spores from each tetrad gave rise to colonies which were unable to grow on a medium lacking tryptophan (data not shown), showing that the *NOP10* gene is essential.

To identify Nop10p homologues, we again carried out a comprehensive BLAST search. Nop10p displays 56% identity (74% homology) with a putative protein from *Caenorhabditis elegans* (Wilson *et al.*, 1994) (Figure 2B). Moreover, numerous protein sequences derived from ESTs from mouse and human sources display 60% identity (70% homology) with yeast Nop10p. The vast majority of mouse and human ESTs code for the same polypeptide and contain start and stop codons. In addition, in the human case, the third in-frame codon upstream of the putative initiator methionine codon is a stop. We therefore believe that the mouse/human protein sequence deduced from ESTs is both correct and full length. We conclude that Nop10p is a highly conserved factor.

To determine the subcellular localization of Nhp2p, we made use of the strain expressing Nhp2p-ZZ (see above). For the same purpose, we produced a strain expressing Nop10p tagged at its C-terminus with the two ZZ IgG-binding domains. Immunodetection of Nhp2p-ZZ (Figure 3B) and Nop10p-ZZ (Figure 3C) by electron microscopy shows that both proteins are present in the dense fibrillar component of the nucleolus. Wild-type Gar1p (I.Léger-Silvestre, S.Trumtel, J.Noaillac-Depeyre and N.Gas, submitted) and Gar1p-ZZ (Figure 3A) show the same localization. In addition, some Nhp2p-ZZ and Nop10p-ZZ molecules can also be found in the nucleoplasm.

Nhp2p and Nop10p are specifically associated with H/ACA snoRNAs

The fact that Nhp2p and Nop10p are nucleolar proteins specifically associated with Gar1p strongly suggested that

they are components of H/ACA snoRNPs. To confirm this, the association of Nhp2p-ZZ and Nop10p-ZZ with H/ACA snoRNAs was tested by immunoprecipitation experiments using IgG–Sephacrose. Extracts were prepared under nondenaturing conditions from strains expressing either Nhp2p-ZZ or Nop10p-ZZ and, as controls, from strains expressing ZZ–Nop1p, Gar1p-ZZ, Cbf5p-ZZ or free ZZ domains. RNAs purified from the initial extracts or from the pellets following immunoprecipitations were either 3' end-labelled with [³²P]pCp and separated on a sequencing gel (Figure 4A) or used in Northern blot experiments using probes specific for a number of H/ACA or C/D snoRNAs (Figure 4B). All members of the family of H/ACA snoRNAs are specifically coprecipitated with Nhp2p-ZZ or Nop10p-ZZ at 500 mM KAc (Figure 4A, lanes 8 and 10). This was confirmed by the Northern blot results (see Figure 4B). Phosphoimager quantitation of the Northern blots revealed that the efficiency of precipitation of H/ACA snoRNAs with either tagged protein ranges from ~25 to 35% of input snoRNA at 500 mM KAc. In contrast, only a weak association of Nhp2p-ZZ or Nop10p-ZZ with C/D snoRNAs was detected at 150 mM KAc, which was even reduced when the salt concentration was increased (Figure 4B, compare lanes 2 and 3, 5 and 6, panels U24 and U14). At 500 mM KAc, at the most, 2% of input C/D snoRNAs are precipitated with either Nhp2p-ZZ or Nop10p-ZZ. Similar results were obtained with Gar1p-ZZ (Ganot *et al.*, 1997a). We conclude that Nhp2p and Nop10p are bona fide components of H/ACA snoRNPs.

Nhp2p and Nop10p are required for pre-rRNA processing

The two previously characterized components of H/ACA snoRNPs, Gar1p and Cbf5p, are necessary for pre-rRNA processing (Girard *et al.*, 1992; Cadwell *et al.*, 1997; Lafontaine *et al.*, 1998). This is, at least partly, a consequence of the inactivation of the snR10 and snR30 snoRNPs. If Nhp2p and Nop10p are also essential to the function of H/ACA snoRNPs, they should display pre-rRNA processing defects. To test this, strains conditionally expressing Nhp2p or Nop10p were constructed (see Mat-

erials and methods). These strains contain *NHP2* or *NOP10* chromosomal alleles transcribed from a *pGAL1-10/CYC1* hybrid promoter, which is induced by galactose but repressed by glucose. To be able to follow the depletion of either protein, strains containing *NHP2-ZZ* or *NOP10-ZZ* chromosomal alleles transcribed from the *pGAL1-10/CYC1* promoter were also constructed. The *pGAL-NHP2*, *pGAL-NHP2-ZZ*, *pGAL-NOP10* and *pGAL-NOP10-ZZ* strains can be propagated on medium containing galactose. Following transfer to glucose-containing medium, however, their growth was impaired. The doubling time of these strains, which behaved similarly, increased significantly after 16 h of growth on glucose to reach a value of 12–16 h after 48 h, whereas these strains have a doubling

time of 3 h on galactose (data not shown). The decline in growth rate was accompanied by the gradual disappearance of Nhp2p-ZZ or Nop10p-ZZ which became almost undetectable after 16 h (data not shown).

Northern blot experiments were performed with RNAs prepared from the conditional-lethal strains grown in permissive galactose-containing medium or shifted to non-permissive glucose-containing medium for from 4 h up to 48 h. The Northern blots were hybridized with several oligonucleotide probes detecting mature rRNAs or pre-rRNA processing intermediates (see Figure 5A for a cartoon of the processing pathway, and 5B for the Northern blot autoradiographs). The disappearance of Nhp2p or Nop10p is correlated with a very strong accumulation of the 35S pre-rRNA and the appearance of an aberrant 23S intermediate (Figure 5B) which extends from the 5' end of the 5' external transcribed spacer to cleavage site A3 in internal transcribed spacer 1. In contrast, the levels of the 32S and 27SA2 intermediates are strongly diminished (Figure 5B). Steady-state levels of the 20S intermediate are also strongly reduced in Nop10p-depleted cells but less so in Nhp2p-depleted cells (Figure 5B, panel 20S, compare lanes 6–9 with lanes 15–18). Probably as a direct consequence of this, mature 18S rRNA levels are more diminished in the former than in the latter case (Figure 5B, panel 18S, compare lanes 6–9 with lanes 15–18). Contrary to what is seen for the 32S, 27SA2 and 20S intermediates, no obvious reduction in the levels of the 27SB and 7S species can be detected in cells lacking Nhp2p or Nop10p (Figure 5B). Primer extensions were performed with the RNA samples used for the Northern blot experiments. Results of primer extension experiments show that cleavages at sites A0, A3, B1(L) and B1(S) occur at the normal positions (data not shown). They also confirm the disappearance of the 27SA2 intermediate as indicated by the progressive reduction in the levels of the cDNA products extending to site A2 (data not shown).

Taken together, these results indicate that the main pre-rRNA processing defects occurring in cells depleted of Nhp2p or Nop10p are inhibitions of cleavages at sites A1 and A2, whose consequences are an inhibition of 18S

Fig. 4. Nhp2p and Nop10p interact with all H/ACA snoRNAs. (A) Patterns of 3' end-labelled snoRNAs associated with Nhp2p-ZZ or Nop10p-ZZ. Yeast extracts were obtained from cells expressing ZZ-Nop1p (lanes 1 and 2), Cbf5p-ZZ (lanes 3 and 4), Gar1p-ZZ (lanes 5 and 6), Nhp2p-ZZ (lanes 7 and 8), Nop10p-ZZ (lanes 9 and 10) or the two ZZ domains alone (lanes 11 and 12). RNAs purified from the total extracts (T, lanes 1, 3, 5, 7, 9 and 11) or from the pellets obtained after immunoprecipitations performed in 500 mM KAc (P, lanes 2, 4, 6, 8, 10 and 12) were 3' end-labelled with [³²P]pCp and separated on a 6% sequencing gel. Positions of some of the C/D and H/ACA snoRNAs inferred from their size are indicated. M, molecular weight markers (pBR322 digested with *Hae*III-*Taq*I). (B) Detection by Northern analysis of snoRNAs associated with Nhp2p-ZZ or Nop10p-ZZ. Yeast extracts were obtained from cells expressing Nhp2p-ZZ (lanes 1–3) or Nop10p-ZZ (lanes 4–6). RNAs purified from the total extracts (T, lanes 1 and 4) or from the pellets obtained after immunoprecipitations performed in 150 mM KAc (P150, lanes 2 and 5) or 500 mM KAc (P500, lanes 3 and 6) were separated by PAGE, transferred onto nylon membranes and hybridized with various antisense oligodeoxynucleotide probes detecting various snoRNAs. Note that the amount of RNA loaded in the 'T' lanes corresponds to one-tenth of the input extract used in the immunoprecipitations.

Fig. 5. pre-rRNA processing defects detected in cells lacking Nhp2p or Nop10p. **(A)** Schematic representation of the pre-rRNA processing pathway. The structure of the 35S pre-rRNA is depicted above. The positions of the oligonucleotide probes used in Northern analyses and primer extension experiments are indicated by small filled boxes. In wild-type cells, the 35S pre-rRNA is first cleaved at site A₀ within the 5' external transcribed spacer (5' ETS) producing intermediate 33S which is very rapidly cleaved at site A₁, the 5' end of 18S rRNA, to produce intermediate 32S. 32S is then cleaved at site A₂ within the internal transcribed spacer 1 (ITS1) releasing 20S, the immediate precursor to 18S rRNA, and 27SA₂. 27SA₂ is then processed via two alternative pathways. It is either cut at site A₃ to produce 27SA₃, which is then trimmed by 5' to 3' exonucleases up to site B1(S), producing 27SB(S). Alternatively, it can be processed into 27SB(L) by an as yet unknown mechanism. 27SB(S) and 27SB(L) are then processed in the same manner to produce 25S and 5.8S(S) or 5.8S(L), respectively. In cells lacking Nhp2p or Nop10p, cleavages at sites A₁ and A₂ are inhibited, as revealed by the diminished levels of the 32S, 27SA₂ and 20S intermediates. 35S is instead directly cut at site A₃, producing 23S which is degraded. For other details and references, see the review by (Venema and Tollervy, 1995). **(B)** Detection by Northern analysis of pre-rRNA processing intermediates produced in cells progressively depleted of Nhp2p or Nop10p. Total RNAs were extracted from wild-type cells (WT, lanes 1–3 and 10–12) or from cells possessing *NHP2* (GAL::nhp2, lanes 4–9) or *NOP10-ZZ* (GAL::nop10, lanes 13–18) alleles transcribed from a hybrid *GAL-CYC1* promoter. Cells were grown in galactose- (Gal, lanes 1, 4, 10 and 13) or in glucose-containing medium (Glu, lanes 2, 3, 5–9, 11, 12, 14–18) for the times indicated. Total RNAs were separated on 1% agarose-formaldehyde gels to detect the 35S, 32S, 27SA₂, 27SB, 25S, 23S, 20S and 18S species, or on 8% polyacrylamide gels to detect the 7S(L), 7S(S), 5.8S(L) and 5.8S(S) species. The 35S and 32S species were revealed by probe b, 27SA₂ and 27SB by probe e, 23S and 20S by probe b, 25S by probe f, 18S by probe a, 7S(L) and 7S(S) by probe e, and 5.8S(L) and 5.8S(S) by probe d.

Fig. 6. Nhp2p and Nop10p are required for 25S rRNA pseudouridylation. TLC analysis of RNase T2 digestion products of *in vivo*-labelled 25S rRNAs purified from cells possessing *NHP2* [GAL::nhp2, (A) and (B)] or *NOP10* [GAL::nop10, (C) and (D)] alleles transcribed from a hybrid *GAL-CYC1* promoter. The cells were grown in galactose [Gal, (A) and (C)] or 24 h in glucose-containing medium [Glu, (B) and (D)]. Spots corresponding to Ap, Cp, Gp, Up and Ψ p are indicated.

rRNA production. Cleavage at site A₀ is also at least delayed since we observe an accumulation of the 23S intermediate.

Cells depleted of Nhp2p or Nop10p are defective in pre-rRNA pseudouridylation

Most H/ACA snoRNAs function as guides selecting uridine residues in the pre-rRNA that will undergo conversion to pseudouridines (Ganot *et al.*, 1997b; Ni *et al.*, 1997). Two of their associated proteins, Gar1p and Cbf5p, are needed for pre-rRNA pseudouridylation (Bousquet-Antonelli *et al.*, 1997; Lafontaine *et al.*, 1998). The requirement of Nhp2p and Nop10p for pre-rRNA pseudouridylation was therefore tested. *pGAL-NHP2* and *pGAL-NOP10* strains were grown in permissive galactose-containing medium or in glucose-containing medium for 24 h. These strains were then labelled with [³²P]orthophosphate for 15 min. Labelled 35S pre-rRNAs and 25S rRNAs purified from these strains were digested by RNase T2 and the digestion products were resolved by two-dimensional thin-layer chromatography. Autoradiographs of the TLC plates corresponding to the 25S samples are shown in Figure 6. While the Ψ p spots are clearly apparent in the case of the strains grown on galactose-containing medium, they can barely be seen on the plates corresponding to the strains grown on glucose (the same results were obtained for the 35S samples; data not shown). The level of pseudouridine synthesis was assessed by determining the ratio of incorporation into Ψ p, compared with Ap. Ψ p:Ap ratios obtained for the 25S rRNA or 35S pre-rRNA samples are essentially identical for the *pGAL-NHP2*, *pGAL-NOP10* strains and for a wild-type strain (Lafontaine *et al.*, 1998) grown on galactose-containing medium, showing that pseudouridine synthesis was normal under permissive conditions. The Ψ p:Ap ratios corresponding to the 25S rRNAs from the *pGAL-NHP2* and *pGAL-NOP10* strains grown on glucose are ~22–25% of the value obtained for the same strains grown on galactose.

This would correspond to a residual level of ~6–7 pseudouridines per 25S molecule (wild-type 25S contains 30 pseudouridines) (Ofengand *et al.*, 1995). The ψ p/Ap ratios corresponding to the 35S samples from the *pGAL-NHP2* and *pGAL-NOP10* strains grown on glucose are ~25–29% of the value obtained for the same strains grown on galactose. This would correspond to a residual level of ~11–12% pseudouridines per 35S molecule (wild-type 35S contains 43 pseudouridines) (Ofengand *et al.*, 1995). We cannot say whether these values reflect low level of ψ synthesis at all sites or whether some sites remain preferentially modified under non-permissive conditions, although we think the former possibility more likely. Pseudouridine synthesis in 18S rRNA could not be directly assessed under non-permissive conditions since 18S is not being synthesized in strains lacking Nhp2p or Nop10p. However, the strong inhibition of pseudouridine formation on 35S rRNA in depleted strains indicates that 18S rRNA also is undermodified. We conclude from these results that Nhp2p and Nop10p are both required for global pseudouridine synthesis in ribosomal RNAs.

Nhp2p and Nop10p are required for the stability of H/ACA snoRNPs

A number of reasons could explain why Nhp2p and Nop10p are essential to the function of H/ACA snoRNPs: they could play a role in the uridine selection or isomerization processes, be required for the association of the particles with the pre-rRNA and/or be required for the synthesis and/or stability of the particles, for example because they contact the conserved boxes of the snoRNA component. The steady-state levels of snoRNAs in cells depleted of Nhp2p or Nop10p were thus assessed by Northern analysis. In Nop10p-depleted cells, levels of all H/ACA snoRNAs fall dramatically while levels of C/D snoRNAs, U1 or MRP are not diminished (Figure 7A, lanes 10–18). The situation in Nhp2p-depleted cells is essentially similar except that, curiously, levels of snR30 are far less affected (Figure 7A, lanes 1–9, panel snR30). snR30 is substantially longer than other H/ACA snoRNAs and may fold into a structure somewhat different from the canonical stem–hinge–stem structure. This may explain the greater stability of snR30 in cells depleted of Nhp2p compared with other H/ACA snoRNAs.

The fate of H/ACA snoRNP proteins in cells depleted of Nhp2p or Nop10p was assessed by Western blot analysis. In cells depleted of Nhp2p or Nop10p, levels of Gar1p detected by affinity-purified anti-Gar1p antibodies (Girard *et al.*, 1992) fall dramatically (Figure 7B), while levels of Nop1p are not affected. The time-course of disappearance of Gar1p exactly parallels that of Nhp2p or Nop10p (data not shown). To assess the fate of Nhp2p in cells deprived of Nop10p and vice versa, centromeric plasmids containing *NHP2-ZZ* or *NOP10-ZZ* alleles under the control of their original promoters were transformed into the *pGAL-NOP10-ZZ* and the *pGAL-NHP2-ZZ* strains, respectively. In cells depleted of Nop10p-*ZZ*, Nhp2p-*ZZ* levels remain unaffected (data not shown). The same is true for Nop10p-*ZZ* levels in cells lacking Nhp2p-*ZZ* (data not shown).

We conclude that in cells depleted of Nhp2p or Nop10p, most H/ACA snoRNAs as well as one of their associated proteins, Gar1p, are unstable. This itself is sufficient to

Fig. 7. Levels of H/ACA snoRNAs and Gar1p are greatly diminished in Nhp2p and Nop10p depleted cells. (A) Northern analysis of snoRNA steady-state levels in cells progressively depleted of Nhp2p or Nop10p. Total RNAs were extracted from wild-type cells (lanes 1–3 and 10–12) or from cells possessing *NHP2* (GAL::nhp2, lanes 4–9) or *NOP10-ZZ* (GAL::nop10, lanes 13–18) alleles transcribed from an hybrid *GAL-CYC1* promoter. Cells were grown in galactose (Gal, lanes 1, 4, 10 and 13) or in glucose-containing medium (Glu, lanes 2, 3, 5–9, 11, 12, 14–18) for the times indicated. Extracted RNAs were separated by PAGE and transferred onto nylon membranes, which were incubated with antisense oligonucleotide probes detecting various snoRNAs. (B) Western analysis of Gar1p protein levels in cells lacking Nhp2p or Nop10p. Total cellular extracts were prepared from wild-type cells (WT, lanes 1–3 and 10–12), or from cells possessing *NHP2* (GAL::nhp2, lanes 4–9) or *NOP10* (GAL::nop10, lanes 13–18) alleles transcribed from an hybrid *GAL-CYC1* promoter. Cells were grown in galactose (Gal, lanes 1, 4, 10 and 13) or in glucose-containing medium (lanes 2, 3, 5–9, 11, 12 and 14–18) for the times indicated. Proteins from total cellular extracts were separated by SDS-PAGE and transferred onto Hybond-C super membranes, which were treated with an anti-Gar1p antibody which also reacts weakly with Nop1p.

explain the 18S synthesis and rRNA pseudouridylation defects previously described observed in cells lacking Nhp2p or Nop10p.

Nhp2p and Nop10p associate with H/ACA snoRNA precursors

Results of the previous paragraph strongly suggest that Nop10p and Nhp2p are components of the core of H/ACA snoRNPs. They are thus likely to associate early with the snoRNA component during synthesis of H/ACA snoRNPs. *Saccharomyces cerevisiae* contains both capped and uncapped H/ACA snoRNAs (Balakin *et al.*, 1996). Some uncapped species are processed from introns (e.g. snR44), probably by exonucleolytic activities that digest from both ends of the debranched lariat, as has been shown for the U24 C/D snoRNA (Petfalski *et al.*, 1998). Uncapped H/ACA snoRNAs that are not encoded in introns (e.g. snR36, snR46) are probably processed from a primary transcript by endo- and exonucleolytic activities, like the snR190 and U14 or the Z2 to Z8 C/D snoRNAs which are processed from polycistronic precursors by the endonuclease RNase III (Chanfreau *et al.*, 1998; L.-H. Qu, A. Henras, Y.-J. Lu, H. Zhou, W.-X. Zhou, Y.-Q. Zhu, J. Zhao,

Fig. 8. H/ACA snoRNA processing intermediates detected in *rnt1Δ* cells are unstable in the absence of Nhp2p or Nop10p. RNAs were extracted from *pGAL-NHP2-ZZ/rnt1Δ* (*GAL::nhp2/rnt1Δ*, lanes 3–9) or *pGAL-NOP10-ZZ/rnt1Δ* (*GAL::nop10/rnt1Δ*, lanes 12 to 18) strains grown in galactose (Gal, lanes 3, 4, 12 and 13) or in glucose-containing medium (Glu, lanes 5–9 and 14–18) for the times indicated. Lane 2, control RNA extracted from the *rnt1Δ* strain; lane 1, control RNA extracted from the isogenic wild-type *RNT1* strain. RNAs were fractionated by PAGE, transferred to nylon membranes and hybridized with probes specific for the snR36 (A) or snR46 (B) snoRNAs.

Y.Henry, M.Caizergues-Ferrer and J.-P.Bachellerie, submitted) and the exonucleases Rat1p and Xrn1p (Ptfalski *et al.*, 1998; L.-H.Qu, A.Henras, Y.-J.Lu, H.Zhou, W.-X.Zhou, Y.-Q.Zhu, J.Zhao, Y.Henry, M.Caizergues-Ferrer and J.-P.Bachellerie, submitted). Indeed, we have observed that in a strain lacking RNase III (*rnt1Δ*), production of high molecular weight processing intermediates revealed by probes hybridizing within the mature snR36 and snR46 sequences strongly accumulate (Figure 8, lane 2). These processing intermediates possess a Me₃G cap and contain a 5' extension compared with the mature species (Y.Henry, unpublished data).

We were interested to determine whether the stability of these precursors to snR36 and snR46 require their association with snoRNP proteins. To test this, the wild-type *NHP2* and *NOP10* alleles were replaced by the *pGAL-NHP2-ZZ* and *pGAL-NOP10-ZZ* alleles in the *rnt1Δ* strain. The *pGAL-NHP2-ZZ/rnt1Δ* and *pGAL-NOP10-ZZ/rnt1Δ* strains were depleted of Nhp2p-ZZ and Nop10p-ZZ, respectively, by prolonged growth on glucose-containing medium. Northern analysis shows that these strains deprived of Nhp2p-ZZ or Nop10p-ZZ lack both the mature and precursor forms of snR36 and snR46 (Figure 8A and B, compare lanes 3 and 9, 12 and 18). In contrast, levels of the polycistronic transcript containing the Z2 to Z8 C/D snoRNAs are not affected (data not shown). Thus these results indicate that: (i) Nhp2p and Nop10p associate with the snR36 and snR46 processing intermediates accumulating in cells lacking RNase III, and it is likely that they do so in wild-type cells as well; and (ii) this association is required for the accumulation of these intermediates. This strongly suggests that the disappearance of the mature snoRNAs observed in cells lacking Nhp2p or Nop10p is due to degradation occurring during processing of the snoRNAs.

Discussion

H/ACA snoRNPs play pivotal roles in eukaryotic ribosome biogenesis. At least two particles, containing the snR10 and snR30 RNAs, are required for the pre-rRNA processing steps leading to mature 18S rRNA production while the remainder are necessary for site-specific pseudouridylation of rRNAs. It is clear that both processes require base pairing of the snoRNA component to the pre-rRNA (Morrissey and Tollervey, 1993; Ganot *et al.*, 1997b; Ni *et al.*, 1997). In the case of H/ACA snoRNAs intervening in pseudouridylation, the nature of the base-pairing interaction between the pre-rRNA and the H/ACA snoRNA has been clearly defined (Ganot *et al.*, 1997b; Ni *et al.*, 1997). Far less is known of the identity and function of the protein components of H/ACA snoRNPs. Until now, only two proteins of H/ACA snoRNPs had been identified: Gar1p (Girard *et al.*, 1992; Balakin *et al.*, 1996; Ganot *et al.*, 1997a) and Cbf5p (Jiang *et al.*, 1993), the putative rRNA pseudouridine synthase (Koonin, 1996; Lafontaine *et al.*, 1998). In the present study, we have attempted to identify the common core proteins of H/ACA snoRNPs by biochemical means. Complexes containing Gar1p tagged with two IgG binding domains (Gar1p-ZZ) were purified by affinity chromatography. Three polypeptides specifically copurify with Gar1p-ZZ, of apparent mol. wt 65, 22 and 10 kDa. The 65 kDa band probably corresponds to Cbf5p while the 22 and 10 kDa polypeptides were positively identified by partial protein sequencing as Nhp2p (Kolodrubetz and Burgum, 1991) and a novel protein termed Nop10p. Gar1p and two polypeptides with apparent mol. wts of 65 and 10 kDa, probably corresponding to Cbf5p and Nop10p, respectively, also copurify specifically with epitope-tagged Nhp2p. All H/ACA snoRNAs are specifically coprecipitated with epitope-tagged Nhp2p or Nop10p, showing that both proteins are components of H/ACA snoRNPs. Altogether, these results suggest that H/ACA snoRNPs contain only four common core proteins, namely Cbf5p, Gar1p, Nhp2p and Nop10p. Analysis of the protein components present in the snR30 snoRNP after a stringent isolation procedure (Lübbers *et al.*, 1995) supports our suggestion that only four proteins are intimately associated with H/ACA snoRNAs. After anti-Me₃G immunoaffinity chromatography followed by MonoQ anion-exchange chromatography and isopycnic centrifugation in a cesium sulfate gradient, only four proteins remain associated with the snR30 RNA, of 10, 23, 25 and 65 kDa apparent mol. wt (Lübbers *et al.*, 1995). Western blot analysis conclusively showed that the 25 kDa polypeptide is Gar1p and it is highly probable that the 65, 23 and 10 kDa bands correspond to Cbf5p, Nhp2p and Nop10p, respectively. We cannot, of course, rule out the possibility that some common protein component other than Cbf5p, Gar1p, Nhp2p and Nop10p dissociated from H/ACA snoRNPs during our purification procedure. Moreover, a subset of H/ACA snoRNPs may contain specific polypeptides, particularly those particles involved in pre-rRNA processing. This has already been established in the case of the U3 snoRNP, belonging to the family of particles containing C/D type snoRNAs, which contains at least two polypeptides, Sof1p and Mpp10p, not shared with other C/D snoRNPs (Jansen *et al.*, 1993; Dunbar *et al.*, 1997; Westendorf *et al.*, 1998). In the case of

H/ACA snoRNPs, antibodies against the nucleolar protein Ssb1p were found to immunoprecipitate the snR10, and to a lesser extent the snR11 H/ACA snoRNAs (Clark *et al.*, 1990). Thus, Ssb1p may be associated with a subset of H/ACA snoRNAs, although the functional relevance of the association detected with snR10 and snR11 was not assessed. In H/ACA snoRNP preparations such as ours, proteins unique to some particles or present in only a subset may be expected to be less abundant than common proteins and thus would not have been readily detected by our approach.

Like Gar1p (Girard *et al.*, 1992; Bousquet-Antonelli *et al.*, 1997) and Cbf5p (Cadwell *et al.*, 1997; Lafontaine *et al.*, 1998), Nhp2p and Nop10p are required for normal 18S rRNA production and rRNA pseudouridylation. This is readily explained by the fact that in cells lacking Nhp2p or Nop10p, levels of snoRNA components required for 18S rRNA production or site-specific pseudouridylation are diminished. Moreover, as was previously observed in cells lacking Cbf5p (Lafontaine *et al.*, 1998), the Gar1p protein becomes almost undetectable in cells deprived of Nhp2p or Nop10p. This could be specific to Gar1p, however, since levels of Nhp2p are not affected in cells depleted of Nop10p and vice versa. The drastic reduction in the steady-state levels of most H/ACA snoRNAs and Gar1p most probably is the consequence of their rapid degradation once synthesized, rather than an inhibition of *de novo* synthesis, since there is no lag between the onset of the depletion of Nhp2p or Nop10p and that of H/ACA snoRNAs and Gar1p. Such degradations could occur after assembly of the particles because these are partially misfolded and their RNA component more susceptible to attack by nucleases. Alternatively, and more probable, the degradation process could take place during snoRNP biogenesis by the very nucleases involved in the final steps of snoRNA processing. Indeed, it has been proposed that production of mature H/ACA, as well as of C/D snoRNAs, relies on 3'→5', and 5'→3' when the mature 5' ends are not capped, exonucleolytic trimming of snoRNA precursors (reviewed in Tollervey and Kiss, 1997). 5'→3' maturation by the exonucleases Rat1p and Xrn1p of some C/D snoRNAs processed from introns or from polycistronic transcripts has in fact been demonstrated recently (Petfalski *et al.*, 1998; L.-H.Qu, A.Henras, Y.-J.Lu, H.Zhou, W.-X.Zhou, Y.-Q.Zhu, J.Zhao, Y.Henry, M.Caizergues-Ferrer and J.-P.Bachellerie, submitted).

Because the integrity of the conserved boxes (H and ACA or C and D) is essential for the stability of the H/ACA or C/D snoRNAs (Terns *et al.*, 1995; Balakin *et al.*, 1996; Caffarelli *et al.*, 1996; Cavaillé and Bachellerie, 1996; Watkins *et al.*, 1996; Ganot *et al.*, 1997a), it is now widely accepted that they bind proteins which would stop the progression into the sequences found in the mature snoRNAs of the exonucleases responsible for the processing of snoRNA precursors. Since they are all required for the stability of H/ACA snoRNAs, Cbf5p, Nhp2p and Nop10p are all possible candidates for binding the H and/or the ACA box. As the following discussion will make it clear, the other so far available evidence does not allow us to rule out any of these factors as H- and/or ACA-box binders.

According to the pre-snoRNA processing model just described, it is expected that the proteins binding the

conserved boxes will interact not just with the mature snoRNAs but also with their precursors. We have shown that Nop10p and Nhp2p are required for the stability of pre-snoRNAs accumulating in cells lacking the endonuclease RNase III. It is highly likely that Nop10p and Nhp2p will also interact with these precursors in wild-type cells. Somewhat similarly, it has been found that in *Xenopus* oocytes, fibrillarin is already associated with the L1 ribosomal protein pre-mRNA, prior to its processing to produce mature C/D type U16 snoRNA (Caffarelli *et al.*, 1996). Recent data suggest that maturation of at least some C/D snoRNA precursors occur in the nucleoplasm at the sites of transcription (Samarski *et al.*, 1998). If this is also the case for precursors of H/ACA snoRNAs, we would expect some H/ACA box binding protein(s) to be present in the nucleoplasm. In this respect it is interesting to note that, although most Nhp2p and Nop10p proteins are found concentrated in the dense fibrillar component of the nucleolus, consistent with their roles in early ribosome biogenesis events, some are also localized in the nucleoplasm.

H- and ACA-box binding proteins, needless to say, are expected to possess RNA binding domains. Among the three proteins required for H/ACA snoRNA stability, only Nhp2p seems related to a known RNA binding protein, the ribosomal protein L32 (Figure 2A), which is able to bind to its own pre-mRNA and mRNA, and presumably also to 25S rRNA (Eng and Warner, 1991; Dabeva and Warner, 1993; Vilardell and Warner, 1994, 1997). The L32 binding site consists of a short stem-loop structure interrupted by an internal pocket, one side of which is composed of the sequence 5'-AGAGAU-3'. Intriguingly, this sequence resembles that of the H box while the overall structure of the L32 binding site is reminiscent of that of the stems containing the 'pseudouridylation' pockets found in H/ACA snoRNAs. It seems highly probable that Nhp2p binds directly to the H/ACA snoRNAs. It could directly interact with one or with both conserved boxes; alternatively, it could bind to the stem(s) and/or the pseudouridylation pocket(s) of H/ACA snoRNAs. We cannot exclude the possibility that it may need additional factors, Cbf5p and/or Nop10p, for efficient RNA binding. Moreover, although Nop10p is not obviously related to any known RNA binding protein, the intriguing observation that snR30 remains fairly stable in Nhp2p-depleted cells but is barely detectable in Nop10p-depleted cells could be explained by proposing that Nop10p, rather than Nhp2p, contacts one of the conserved boxes.

Contrary to what is observed in cells depleted of either Cbf5p, Nhp2p or Nop10p, H/ACA snoRNAs remain stable in cells lacking Gar1p (Girard *et al.*, 1992; Bousquet-Antonelli *et al.*, 1997). Thus, even though Gar1p has been shown to interact directly with snR10 and snR30 *in vitro* (Bagni and Lapeyre, 1998), it is unlikely to bind either the H or ACA box *in vivo*. Rather, Gar1p is likely to interact directly with Cbf5p since it was selected in an exhaustive two-hybrid screen conducted with Gar1p as a bait (M.Fromont, Y.Henry and P.Legrain, unpublished results). We envisage that the interaction of Gar1p with Cbf5p places the former in contact with the pseudouridylation pocket such that it could modulate the pre-rRNA/snoRNA associations, consistent with the findings that Gar1p has RNA binding potential (Ghisolfi *et al.*, 1992;

Bagni and Lapeyre, 1998) and that in cells deprived of Gar1p, H/ACA snoRNPs are no longer bound to the pre-rRNA (Bousquet-Antonelli *et al.*, 1997). Gar1p could play such a role in association with the putative Rok1p helicase since it has been shown that Rok1p is required for 18S rRNA synthesis and that some mutant alleles of *ROK1* and *GAR1* produce synthetic lethal phenotypes when combined (Venema *et al.*, 1997).

With the identification of most, if not all, common core proteins of H/ACA snoRNPs, we are now in a position to discriminate between the models of H/ACA snoRNP structure outlined above and in so doing to acquire a more detailed understanding of how H/ACA snoRNPs function in pre-rRNA processing and modification. This goal has become all the more important with the recent finding that X-linked recessive dyskeratosis congenita, a human bone marrow failure syndrome, is caused by mutations in the human homologue of the *S.cerevisiae* *CBF5* gene (Heiss *et al.*, 1998).

Materials and methods

Strains and media

The yeast diploid strain JG337.1B (*MATa/Matα*, *ade2-1/ade2-1*, *his4-260/his4-260*, *leu2-2/leu2-2*, *lys2-1/lys2-1*, *met8-1/met8-1*, *trp1-1/trp1-1*, *ura3-52/ura3-52*, *can1-100/can1-100*) and the haploid strains YNN281 (*Mata*, *ade2-101*, *his3-Δ200*, *lys2-801*, *trp1-Δ1*, *ura3-52*, *CAN+*), JG540 (*Mata*, *ade2*, *ade3*, *leu2*, *lys2.1*, *trp1.1*, *tyr7.1*, *ura3*, *can1*), *rnt1-Δ* (Abou Elela *et al.*, 1996; strain SAE-52/1 kindly provided by M.Ares; *MATa*, *his3*, *leu2-3,112*, *lys2*, *trp1*, *ura3-52*, *pep4*, *prb1*, *pre1*, *rnt1::HIS3*) were used. The control *RNT1* strain (strain SAE-6 kindly provided by M.Ares) is the same as strain SAE-52/1 but transformed with plasmid pRS316-RNT1. *Saccharomyces cerevisiae* strains were grown in rich (YP) medium (1% yeast extract, 1% peptone, 2% glucose or galactose) or in YNB medium (0.67% yeast nitrogen base without amino acids, 2% glucose or 2% galactose, supplemented with appropriate amino acids). Strains containing the *rnt1::HIS3* allele were grown at 26°C. Yeast was transformed by the lithium acetate method. *Escherichia coli* DH5α strain [*F'*, *endA1*, *hsdR17* (*r_K⁻m_K⁺*), *supE44*, *thi-1*, *recA1*, *gyrA* (*Nal^r*), *relA1*, *Δ(lacZYA-argF)U169*, *deoR*, (φ80*dlacΔ(lacZ)M15*)] grown on LB (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) liquid or solid media was used for all cloning procedures.

Purification of proteins associated with Gar1p-ZZ or Nhp2p-ZZ by affinity chromatography

Yeast pellets corresponding to ~1000–1500 OD were lysed in a 'one shot cell disrupter' (Cell'D, 20 bis rue du Chapitre, 30150 Roquemaure, France) in 20 ml of 20 mM HEPES pH 7.8, 1 mM MgCl₂, 300 mM KCl supplemented with a complete protease inhibitor cocktail (Boehringer Mannheim). Yeast extracts containing Gar1p-ZZ were produced from a *pGAL-GAR1* strain grown on glucose-containing medium (Girard *et al.*, 1992) transformed with pMCGZ1, a centromeric plasmid containing *GAR1-ZZ* transcribed from the *GAR1* promoter (Ganot *et al.*, 1997a). Yeast extracts containing Nhp2p-ZZ were obtained from the wild-type strain JG540, transformed with pJPG250, a centromeric plasmid containing the *URA3* marker gene and *NHP2-ZZ* transcribed from the *GAR1* promoter (Bousquet-Antonelli, 1998). Extracts were centrifuged 20 min at 10 000 g, 4°C and supernatants were loaded on 1.8 ml IgG-Sepharose columns previously packed and equilibrated according to the manufacturer's recommendations (Pharmacia). The flow rate during the loading step was 0.5 ml/min. The columns were washed with lysis buffer until the ODs of the flow-through became close to zero and were then washed with 15 ml 5 mM NH₄Ac, pH 5. Bound complexes were eluted with 10 ml 0.5 M acetic acid, pH 2.5, and 1 ml fractions were collected. Proteins from the peak fractions were concentrated by TCA precipitation and analysed by SDS-PAGE followed by Coomassie Blue staining of the gel (Figure 1). For protein sequencing, the proteins were separated by SDS-PAGE, then transferred on a PVDF membrane (ProteinBlott, Applied Biosystems) in 50 mM Tris base, 50 mM boric acid. The proteins were stained in 0.2% amido-black, the bands of interest were excised and sent for N-terminal sequencing to the Laboratoire de Microséquence des Protéines of the Pasteur Institute, Paris, France.

Construction of the *nop10::TRP1/NOP10* diploid strain and the *pGAL-NHP2*, *pGAL-NHP2-ZZ*, *pGAL-NHP2-ZZ,rnt1Δ*, *pGAL-NOP10*, *pGAL-NOP10-ZZ*, *pGAL-NOP10-ZZ,rnt1Δ* haploid strains

To generate the *pGAL-NHP2*, *pGAL-NHP2-ZZ* and *pGAL-NHP2-ZZ,rnt1Δ* haploid strains, the following constructs were produced: An *EcoRI-EcoRV* genomic fragment containing the *NHP2* gene was cloned into a pBKS (+) derivative lacking a *HindIII* site digested with *EcoRI* and *Ecl136II*, creating pHA100. pHA100 was digested with *HindIII*, blunt-ended with Klenow polymerase, then digested with *NsiI* and blunt-ended with T4 DNA polymerase. An *HindIII* fragment from pYEDP60.2 (Urban *et al.*, 1990) containing the *URA3* marker gene, a *GAL/CYC1* hybrid promoter, multiple cloning sites and the *PGK* transcription termination sequences was blunt-ended with Klenow polymerase and inserted into pHA100 treated as described, producing pHA102. An *NHP2* gene cassette flanked by *BamHI* restriction sites was PCR amplified with oligos AH-Nhp2-5' (5'-CCCGGATCCAAATGGG-TAAAGACAACAA-3') and AH-Nhp2-3'-*BamHI* (5'-CCCGGATCCT-CATAAAGCTTGAAGCTTCTT-3'), digested with *BamHI* and inserted into pHA102 digested with the same enzyme, producing pHA103. An *NHP2-ZZ* gene cassette, containing the *NHP2* ORF followed by the coding sequence of the two synthetic IgG-binding domains derived from *S.aureus* protein A, and flanked by two *BamHI* restriction sites was produced by PCR amplification using oligos AH-Nhp2-5' and AH-Nhp2-ZZ-3'-*BamHI* (5'-CCCCGGATCCCTATTTCGGCGCCTGATCATCAT-3') and plasmid pJPG250 (Bousquet-Antonelli, 1998). This cassette was digested with *BamHI* and cloned into pHA102 cut with the same enzyme, producing pHA104. A fragment from pHA103 cut with *Bsu36I* and *BspEI* containing the *URA3* marker gene and the *pGAL-NHP2-TERM pgk* cassette flanked by genomic sequences of the *NHP2* locus was integrated into the haploid strain JG540. The corresponding fragment from pHA104 (containing the *pGAL-NHP2-ZZ-TERM pgk* cassette) was integrated into the haploid strains JG540 and SAE-52/1 (*rnt1::HIS3*). Correct integration at the *NHP2* locus was verified by Southern analysis and by plating on medium containing glucose (not shown).

To generate the *nop10::TRP1/NOP10* diploid strain, the following constructs were produced: an *EcoRI-Cac8I* fragment of genomic DNA containing the *NOP10* gene and flanking sequences was inserted into the pGEM1 vector digested with *EcoRI* and *HincII*, producing pGEM1-NOP10. A *BglIII* fragment from plasmid pFL39 (Bonneaud *et al.*, 1991) containing the *TRP1* marker gene was blunt-ended with Klenow polymerase and inserted into pGEM1-NOP10 cut with *HincII* and *BsaAI*, producing plasmid pFH29 where *NOP10* has been replaced by *TRP1*. An *AatII-SpeI* fragment from pFH29 containing the *nop10::TRP1* disrupted allele and flanking genomic sequences was integrated into the diploid strain JG337.1B. Correct integration at the *NOP10* locus of *TRP1+* transformants was verified by Southern analysis (not shown).

To generate the *pGAL-NOP10* strain, the following constructs were produced: an *HindIII* fragment from pYEDP60.2 (Urban *et al.*, 1990) containing the *URA3* marker gene, a *GAL/CYC1* hybrid promoter, multiple cloning sites and the *PGK* transcription termination sequences was blunt-ended with Klenow polymerase and inserted into pGEM1-NOP10 digested with *HincII* and *BsaAI*, producing plasmid pFH28. A *NOP10* gene cassette containing start and stop codons and flanked by *BglIII* and *BamHI* restriction sites was PCR amplified using oligonucleotides NOP10#4 (5'-CCCCCAGATCTAAGATGCATTGTGATGTACAC-TTTGGG-3') and NOP10#5 (5'-CCCCCGGATCCTATTGGCCTGG-TACCAAACC-3'). This cassette was digested with *BglIII* and *BamHI* and inserted into pFH28 cut with *BamHI*, creating pFH30. An *AatII/SpeI* fragment from pFH30 containing the *URA3* marker and the *pGAL-NOP10-TERM pgk* cassette flanked by the genomic sequences found at the *NOP10* locus was integrated into haploid strain JG540. The correct integration at the *NOP10* locus of the *URA+* transformants was checked by Southern analysis and by plating on medium containing glucose.

To generate the *pGAL-NOP10-ZZ* and *pGAL-NOP10-ZZ,rnt1Δ* haploid strains, the following constructs were produced: a *NOP10* gene cassette containing a start but lacking a stop codon and flanked by *BglIII* and *BamHI* restriction sites was PCR amplified using oligonucleotides NOP10#4 and NOP10#6 (5'-CCCCCGGATCCTTGGCCTGG-TACCAAACCAATC-3'). This cassette was digested with *BglIII* and *BamHI* and inserted into pFH28 cut with *BamHI*, creating pFH31. A cassette coding for two synthetic IgG-binding domains derived from *S.aureus* protein A containing a stop codon and flanked by *BamHI* restriction sites was PCR amplified using oligonucleotides NOP10ZZ#1 (5'-CCCCCGGATCCTCAGCATGCCTTGGCAACACGATG-3') and NOP10ZZ#2 (5'-CCCCCGGATCCTATTTCGGCGCCTGAGCATCA-

TTTAGC-3'). This cassette was digested with *Bam*HI and inserted into pFH31 also digested with *Bam*HI, creating pFH32. An *Aat*II-*Spe*I fragment from pFH32 containing the *URA3* marker and the *pGAL-NOP10-ZZ-TERM pgk* cassette flanked by the genomic sequences found at the *NOP10* locus was integrated into the haploid strains JG540 and SAE-52/1 (*mtl1::HIS3*). The correct integration at the *NOP10* locus of the *URA+* transformants was checked by Southern analysis and by plating on medium containing glucose.

Immunoelectron microscopy

The Gar1p-ZZ, Nhp2p-ZZ and Nop10p-ZZ expressing strains used were, respectively, strain *pGAL-GAR1* transformed with pMCGZZ1 grown on glucose-containing medium, WT strain JG540 transformed with pJPG250 grown on the same medium and strain *pGAL-NOP10-ZZ* grown on galactose-containing medium. Cell fixation, cutting and mounting of sections on nickel grids were performed as described in Léger-Silvestre *et al.* (1997). The grids were incubated for 2 h at room temperature with anti-protein A antibodies (Sigma) diluted (1:1000) in phosphate-buffered saline buffer containing 2% bovine serum albumin. Sections were washed, then incubated for 1 h with colloidal gold conjugated protein A (British Biocell International) diluted 1:20. Sections were contrasted with 5% aqueous uranyl acetate and imaged in a JEOL-1200 EX electron microscope operating at 80 kV.

Immunoprecipitations

To determine the snoRNA population associated with the Nop1p, Gar1p, Cbf5p, Nhp2p, Nop10p proteins tagged with two synthetic IgG-binding domains (ZZ), immunoprecipitations were performed as described in Ganot *et al.* (1997a). Strains expressing ZZ-Nop1p (Ganot *et al.*, 1997a) and Cbf5p-ZZ (Lafontaine *et al.*, 1998) were kindly provided by T.Bergès (Université de Poitiers, France) and D.Lafontaine (Edinburgh University, UK), respectively. Gar1p-ZZ-containing extracts were obtained from the *pGAL-GAR1* strain transformed with pMCGZZ1 grown on glucose (see above). Strains *pGAL-NHP2-ZZ* and *pGAL-NOP10-ZZ* (see above) grown on galactose-containing medium were used as sources of extracts containing Nhp2p-ZZ and Nop10p-ZZ, respectively. A control extract containing free ZZ domains was produced from strain *pGAL-GAR1* grown on galactose-containing medium and transformed with pMCGZZ3, a centromeric plasmid containing the ZZ sequence transcribed from the *GAR1* promoter (Bousquet-Antonelli, 1998).

RNA extractions, Northern hybridizations and 3' end-labelling of RNAs

RNA extractions were performed as described in Tollervey and Mattaj (1987). Five micrograms of total RNAs were fractionated on standard 1% agarose/6% formaldehyde gels to analyse high mol. wt RNA species; alternatively, they were fractionated on 6 or 8% polyacrylamide gels to analyse low mol. wt species. After electrophoresis, RNAs were transferred onto N-hybrid (+) membranes (Amersham). Membranes were hybridized with kinased oligodeoxynucleotide probes overnight at 35°C in 6× SSC, 5× Denhardt's, 0.5% SDS, 90 µg/ml heat denatured salmon sperm DNA. The following oligodeoxynucleotides hybridizing to snRNAs were used: anti-U14 5'-CTCAGACATCCTAGGAAGG-3'; anti-snR190 5'-CGA-GGAAGAAGAGACACCATATC-3'; anti-yU24 5'-ATTGGTA-TGTCTCATTCGGATCTCAAAGTTCCATCTGA-3'; anti-snR10 5'-CATGGGTCAAGAACGCCCGGAGGGG-3'; anti-snR11 5'-GACG-AATCGTGACTCTG-3'; anti-snR31 5'-GTAGAACGAATCATGACC-3'; anti-snR33 5'-GATTGTCCACACACTTCT-3'; anti-snR36 5'-TTA-CTCGAGTGATGAGACGTTCTAATTA-3'; anti-snR42 5'-TCAA-ACAATAGGCTCCCTAAAGCATCACA-3'; anti-snR46 5'-CCATA-AACCACGCAAAAATGC-3'; anti-MRP 5'-CAACCCAAAGCATA-TTACTGAG-3'; anti-U1 5'-CTGATATCTTAAGGTAAGTAT-3'. The oligonucleotides used to analyse pre-rRNA processing were as follows (Figure 5A): oligonucleotide a 5'-CATGGCTTAATCTTTGAGAC-3'; b 5'-TTAAGCGCAGGCCCGGCTGG-3'; c 5'-GATTGCTCGAATGCC-CAAAG-3'; d 5'-TGCGTTCAAAGATTCGATG-3'; e 5'-GGCCAGCA-ATTTCAAGTTA-3'; f 5'-CCATCTCCGGATAAAC-3'. To detect snR30, an *Eco*RI-*Hind*III fragment encompassing the entire *SNR30* gene from pT3/T7α18-snR30 (Morrissey and Tollervey, 1993) was used to synthesize complementary DNA probes by random priming using the megaprime kit reagents (Amersham). These probes were hybridized to RNA blots as described in Caizergues-Ferrer *et al.* (1989). In all cases membranes were washed twice in 2× SSC, 0.1% SDS at 35°C during a 15 min interval, and twice in 1× SSC, 0.1% SDS at 35°C during a 15 min interval. 3' end-labelling of RNAs with [⁵-³²P]pCp was performed as described in Ganot *et al.* (1997a).

Analysis of ψ levels

This analysis was performed as described in Lafontaine *et al.* (1998) except that 2% galactose rather than a combination of 2% raffinose, 2% sucrose and 2% galactose was used in the permissive medium.

Western blotting

Proteins from total extracts were separated by SDS-PAGE on 12 or 15% polyacrylamide gels and transferred on hybond-C super membranes (Amersham). Affinity-purified anti-Gar1p antibodies which weakly react with Nop1p were used as described (Lafontaine *et al.*, 1998). Epitope-tagged proteins (Nhp2p-ZZ and Nop10p-ZZ) were detected by use of Dako rabbit PAP followed by ECL (Amersham).

Acknowledgements

We are particularly grateful to Prof. M.Ares and Dr S.Abou Elela for the gifts of strains SAE-6 and SAE 52/1, and to Dr T.Bergès and Dr D.Lafontaine for gifts of ZZ-Nop1p- and Cbf5p-ZZ-expressing strains, respectively. We would like to thank Dr N.Watkins and Prof. R.Lührmann for sharing results prior to publication. We are grateful to Y.de Préval (LBME, Toulouse, France) for the synthesis of oligonucleotides and D.Villa for the photographs. We also thank members of the Ferrer lab for helpful discussions and Dr T.Kiss for critical reading of the manuscript. We are indebted to Prof. F.Amalric and Dr H.Richard-Foy for their support. Immunodetection by electron microscopy of epitope-tagged proteins was performed in the lab of Prof. N.Gas, LBME, Toulouse, France. This work was supported by the CNRS, the Université Paul Sabatier and grants from the Association pour la Recherche sur le Cancer, La Ligue Nationale contre le Cancer and the Région Midi-Pyrénées. A.H. was supported by a grant from the Ministère de l'Éducation Nationale, de l'Enseignement Supérieur et de la Recherche.

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Received September 16, 1998; revised October 6, 1998;
accepted October 7, 1998