A small nucleolar RNP protein is required for pseudouridylation of eukaryotic ribosomal RNAs

Cécile Bousquet-Antonelli,
Yves Henry, Jean-Paul Gélugne,
Michèle Caizergues-Ferrer and Tamás Kiss¹

Laboratoire de Biologie Moléculaire Eucaryote du CNRS, Université
Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex, France

¹Corresponding author
e-mail: tamas@ibcg.biotoul.fr

Eukaryotic rRNAs possess numerous post-transcriptionally modified nucleotides. The most abundant modifications, 2'-O-ribose methylation and pseudouridylation, occur in the nucleolus during rRNA processing. The nucleolus contains a large number of small nucleolar RNAs (snoRNAs) most of which can be classified into two distinct families defined by conserved sequence boxes and common associated proteins. The C and D box-containing snoRNAs are associated with fibrillarin, and most of them function as guide RNAs in site-specific ribose methylation of rRNAs. The nucleolar function of the other class of snoRNAs, which share box H and ACA elements and are associated with a glycine- and arginine-rich nucleolar protein, Gar1p, remains elusive. Here we demonstrate that the yeast *Saccharomyces cerevisiae* Gar1 snoRNP protein plays an essential and specific role in the overall pseudouridylation of yeast rRNAs. These results establish a novel function for Gar1 protein and indicate that the box H/ACA snoRNAs, or at least a subset of these snoRNAs, function in the site-specific pseudouridylation of rRNAs.

Keywords: Gar1 protein/nucleolus/pseudouridine formation/RNA modification/snoRNP

Introduction

In eukaryotic cells, synthesis and maturation of cytoplasmic rRNAs take place in the nucleolus (Hadjiolov, 1985). The 18S, 5.8S and 25/28S rRNAs are synthesized as a single precursor RNA (pre-rRNA) which contains long external and internal transcribed spacer sequences that are removed by complex nucleolytic reactions (Eichler and Craig, 1995; Venema and Tollervey, 1995; Sollner-Webb *et al.*, 1996). Before or during processing, many nucleotides in the rRNA-coding regions of pre-rRNA are methylated at the 2'-O-hydroxyl position, and numerous uridine residues are converted into pseudouridines (Maden, 1990; Eichler and Craig, 1995; Ofengand *et al.*, 1995). Since these modified nucleotides are confined to the universal core regions of rRNAs and their positions show remarkable evolutionary conservation, they are believed to contribute to the function of rRNAs (Maden, 1990; Lane *et al.*, 1995; Ofengand *et al.*, 1995).

The nucleolus contains a large number of small nucleolar RNAs (snoRNAs) (Filipowicz and Kiss, 1993; Maxwell and Fournier, 1995; Sollner-Webb *et al.*, 1996; Tollervey and Kiss, 1997). Almost 100 distinct snoRNA species have been identified in mammalian and yeast cells (Maxwell and Fournier, 1995; Balakin *et al.*, 1996; Kiss-László *et al.*, 1996; Nicoloso *et al.*, 1996; Tycowski *et al.*, 1996a; Ganot *et al.*, 1997b). Many snoRNAs, such as U3 (Kass *et al.*, 1990; Savino and Gerbi, 1990; Hughes and Ares, 1991), U8 (Peculis and Steitz, 1993), U14 (Li *et al.*, 1990), U22 (Tycowski *et al.*, 1994), snR30 (Morrissey and Tollervey, 1993) and 7-2/MRP RNA (Schmitt and Clayton, 1993; Chu *et al.*, 1994; Lygerou *et al.*, 1996) have been demonstrated to play essential roles in the nucleolytic processing of pre-rRNA. Recently, it was shown that a large subset of snoRNAs containing conserved C and D/D' boxes and displaying extensive complementarities to rRNAs function as guide RNAs in the site-specific 2'-O-ribose methylation of pre-rRNA (Cavaillé *et al.*, 1996; Kiss-László *et al.*, 1996; Tycowski *et al.*, 1996b). Formation of a long (10–21 nucleotide) uninterrupted snoRNA–rRNA duplex in the immediate vicinity of the D or D' box of the snoRNA specifies the target nucleotide for ribose methylation (Cavaillé *et al.*, 1996; Kiss-László *et al.*, 1996). The methylation guide snoRNAs, like other C and D box-containing snoRNAs (Tyc and Steitz, 1989; Tycowski *et al.*, 1993), are associated with an evolutionarily conserved nucleolar protein, fibrillarin (Kiss-László *et al.*, 1996; Tycowski *et al.*, 1996a). Consistent with this, the yeast equivalent of fibrillarin, Nop1p, which is specifically associated with all yeast box C/D snoRNAs (Ganot *et al.*, 1997b), is fundamental to the global ribose methylation of rRNAs (Tollervey *et al.*, 1993).

To date, no factors required for site-specific pseudouridylation of eukaryotic rRNAs have been identified. In addition to the family of box C/D snoRNAs, the nucleolus of yeast and mammalian cells contains another distinct set of snoRNAs which are defined by two conserved sequence elements, boxes H and ACA (Balakin *et al.*, 1996; Ganot *et al.*, 1997b). The box H/ACA snoRNAs also share a common secondary structure; they fold into two hairpin structures that are connected and followed by short single-stranded regions containing the H and ACA elements, respectively (Ganot *et al.*, 1997b). The nucleolar function of this class of snoRNAs is largely unknown. All yeast box H/ACA snoRNAs tested, with the exception of snR30 which is required for 18S rRNA processing (Morrissey and Tollervey, 1993), are dispensable for viability (Maxwell and Fournier, 1995; Balakin *et al.*, 1996, and references therein). In yeast, all box H/ACA snoRNAs are associated with an essential glycine- and arginine-rich nucleolar protein, Gar1p (Balakin *et al.*, 1996; Ganot *et al.*, 1997b). Although the Gar1 protein is required for the accumulation of mature 18S rRNA (Girard

Fig. 1. Schematic structure of the Gar1.1 protein encoded by the *gar1.1* allele. The mutant protein is represented as a box ranging from position 1 (N-terminus) to position 207 (C-terminus). The glycine- and arginine-rich GAR domains (Girard *et al.*, 1992) are indicated by shaded boxes. The amino acid substitutions and their corresponding positions within the mutant Gar1.1p and the wild-type Gar1p are indicated. Introduction of a new *Bam*HI site into the *GAR1* ORF resulted in the addition of an aspartic acid and a proline between positions five and six of the wild-type protein. Consequently, the mutant protein is 207 rather than 205 (Girard *et al.*, 1992) amino acids long.

et al., 1992, 1993), its nucleolar function as a component of numerous non-essential snoRNP particles is largely unknown.

In this study, we have investigated the possible involvement of the Gar1 snoRNP protein in post-transcriptional modification of rRNAs in the yeast *Saccharomyces cerevisiae*. We show that depletion of Gar1 protein, in addition to inhibiting 18S rRNA production, also prevents the global pseudouridylation of 35S and 25S rRNAs, but not of tRNAs. Genetic restoration of Gar1p production re-establishes pseudouridylation of rRNAs. These results identify a novel cellular function for Gar1p and, more importantly, indicate that the Gar1p-associated box H/ACA snoRNAs, or a subset of these snoRNAs, function in the site-specific pseudouridylation of rRNAs.

Results

Construction of a yeast strain carrying a thermosensitive allele of the *GAR1* gene

The recent notion that the majority of fibrillarin-associated snoRNAs function as guide RNAs in site-specific ribose methylation of pre-rRNA raised the intriguing hypothesis that the other major group of snoRNAs, the Gar1p-associated box H/ACA snoRNAs, might be involved in formation of ribosomal pseudouridines (Ganot *et al.*, 1997b). This idea is reinforced by the observation that all yeast box H/ACA snoRNAs tested, with the exception of snR30 (Morrissey and Tollervey, 1993), are dispensable for viability (Maxwell and Fournier, 1995; Balakin *et al.*, 1996). Since yeast Gar1p interacts specifically and intimately with all box H/ACA snoRNAs (Balakin *et al.*, 1996; Ganot *et al.*, 1997b), the above hypothesis leads to the prediction that in a yeast strain deficient in functional Gar1p, the overall pseudouridylation of rRNAs should be inhibited while ribose methylation should remain unaffected.

To assay for rRNA pseudouridylation in a yeast strain lacking functional Gar1p, we made use of the *gar1.1* temperature-sensitive allele. This allele codes for a mutant protein, Gar1.1p, that is degraded rapidly at the non-permissive temperature of 37°C. As compared with the wild-type protein, the mutant Gar1.1p carries six amino acid substitutions (Figure 1). The two extra amino acids in the N-terminal region of Gar1.1p, an aspartic acid (D6) and a proline (P7) resulting from the introduction of a new restriction site (see Materials and methods), do not contribute to the temperature-sensitive phenotype of Gar1.1p (data not shown). A yeast strain (*gar1::LEU2*)

Fig. 2. Characterization of the *gar1.1* thermosensitive allele of the yeast *S.cerevisiae* *GAR1* gene. (A) Expression of the *gar1.1* allele. Accumulation of the Gar1.1, Nop1 and Srp21 proteins in the *gar1.1* yeast strain grown for 3 h at either 37 (lane 1) or 25°C (lane 2) was measured by Western blot analyses. The same blot was reacted with anti-GAR1, anti-SRP21 or anti-NOP1 antibodies. Molecular size markers are indicated on the left. (B) Pulse labelling of cellular RNAs. The *gar1.1* strain was grown at 25°C in minimal medium to mid-exponential phase. Cells were washed and resuspended in rich medium containing glucose but lacking inorganic phosphate, and were grown for 3 h at either 25 or 37°C. The cultures were labelled with [³²P]orthophosphate for 15 min before total RNA was extracted and analysed by electrophoresis on a 1.2% agarose-formaldehyde gel. The positions of the rRNA species are indicated.

lacking the endogenous wild-type *GAR1* gene was complemented with a centromeric vector bearing the *gar1.1* allele. Western blot analysis was used to monitor the accumulation of intracellular Gar1.1p in the *gar1.1* strain grown at the permissive (25°C) or non-permissive (37°C) temperature (Figure 2A). The amount of Gar1.1p was strongly reduced after 3 h growth at 37°C, whereas the levels of the nucleolar Nop1 snoRNP protein (Schimmang *et al.*, 1989; Maxwell and Fournier, 1995), and the cytoplasmic Srp21p, a protein component of yeast signal recognition particle (Brown *et al.*, 1994), remained unaffected. Since accumulation of the mRNA encoding the mutant Gar1.1p was not altered by shifting the *gar1.1* strain to non-permissive temperature (data not shown), we concluded that the instability of Gar1p at 37°C is due to conformational changes in the protein structure.

Depletion of Gar1p inhibits pseudouridylation of 35S and 25S rRNAs

To test for rRNA pseudouridylation, the *gar1.1* strain was grown on rich medium without inorganic phosphate for 3 h either at the permissive (25°C) or non-permissive (37°C) temperature before addition of [³²P]orthophosphate. After 15 min of labelling, total RNAs were extracted and analysed by gel electrophoresis (Figure 2B). Consistent with previous studies on Gar1p-depleted yeast cells (Girard *et al.*, 1992), the *gar1.1* strain impaired production of mature 18S rRNA at non-permissive temperature (lane 1). The 35S, 25S rRNAs and tRNAs were electroeluted and their pseudouridine content was determined by RNase T2 digestion followed by two-dimensional thin-layer chromatography (TLC) and PhosphorImager quantification (Figure 3). At the non-permissive temperature that does not support accumulation of the Gar1 protein, the global pseudouridylation of 35S and 25S rRNAs was almost completely abolished (Figure 3A and B). While the wild-type yeast 18S and 25S rRNAs are known to contain 13

Fig. 3. Depletion of Gar1p inhibits pseudouridylation of rRNAs. *In vivo* labelled 35S pre-rRNA, 25S rRNA and tRNA originating from the *gar1.1* temperature-sensitive strain grown at permissive (25°C) or restrictive (37°C) temperature were isolated by electroelution from a preparative gel similar to the one shown in Figure 1C. The purified RNAs were digested by RNase T2 and analysed by two-dimensional TLC. Spots corresponding to the Ap, Gp, Cp and Up residues are indicated in (A). The position of the pseudouridine 3'-monophosphate residue is indicated in each panel (Ψp). Weak spots represent 2'-O-methylated dinucleotides resistant to RNase T2 digestion (Keith, 1995). The diagram indicates the relative pseudouridine contents of 35S (open columns) and 25S (closed columns) rRNAs obtained from the *gar1.1* strain grown at either 25 or 37°C and from a control wild-type strain grown at 25°C. The levels of pseudouridines and uridines were measured by PhosphorImager quantification and the percentage of uridine residues converted into pseudouridines was calculated. Values represent means $\pm$ SEM from at least three independent experiments. Yeast 35S and 25S rRNAs contain 1847 and 868 uridines, respectively.

and 30 pseudouridine residues, respectively (Ofengand *et al.*, 1995), the 35S pre-rRNA and the 25S rRNA obtained from cells grown at 37°C were calculated to carry only 2–4 and 1–3 pseudouridines, respectively. At 25°C, pseudouridylation of the 35S pre-rRNA and 25S rRNA was much more efficient (Figure 3D and E), although the measured pseudouridine content of the 25S rRNA (20–25 residues per molecule) never reached the wild-type levels. This indicates that the mutant Gar1.1p

cannot fully support rRNA pseudouridylation even at permissive temperature. The fact that the 35S pre-rRNA at 25°C contained ~30–35 pseudouridine residues confirms that most or all conversions of uridine residues into pseudouridines occur already on the 35S primary transcript (Brand *et al.*, 1979).

Pulse-chase labelling of pre-rRNAs in yeast cells lacking a functional *GAR1* gene unambiguously demonstrated that the Gar1 protein is not required for global 2'-O-ribose methylation of pre-rRNA (Girard *et al.*, 1992). Consistent with this observation, quantification of ribose-methylated nucleotides in 25S and 35S rRNAs by P1 nuclease digestion followed by two-dimensional TLC analyses showed that the 2'-O-ribose methylation of pre-rRNA was not altered in the *gar1.1* strain grown at the non-permissive temperature (data not shown). Hence, weak spots representing ribose-methylated dinucleotides resistant to RNase T2 digestion (Smith and Dunn, 1959) were always detected during the RNase T2–TLC analyses of 25S or 35S rRNAs obtained from the *gar1.1* or its derivative strains (Figures 3 and 4C). We also tested the possible involvement of Gar1 protein in pseudouridylation of tRNAs (Figure 3C and F). In marked contrast to rRNAs, the global pseudouridylation of tRNAs was not affected by shifting the *gar1.1* strain to non-permissive temperature, indicating that the Gar1 protein is required specifically for rRNA pseudouridylation.

Expression of wild-type Gar1p restores rRNA pseudouridylation at non-permissive temperature

To demonstrate unambiguously the specific involvement of the Gar1 snoRNP protein in pre-rRNA pseudouridylation, we tested whether genetic restoration of wild-type Gar1p production in the *gar1.1* strain at non-permissive temperature would restore normal levels of rRNA pseudouridylation. For this purpose, we constructed a centromeric vector, called pGAL, where the wild-type *GAR1* open reading frame (ORF) was placed under the control of the *GAL1-10* promoter. This plasmid was introduced into the *gar1::LEU2* strain complemented with plasmids bearing either the mutant *gar1.1* or the wild-type *GAR1* gene. The resulting *gar1.1+pGAL* and *GAR1+pGAL* strains were grown on glucose-containing medium for 3 h at 25 or 37°C. Half of the cultures grown at 37°C were resuspended in galactose-containing medium and grown for an additional 8 h at 37°C to induce expression of the wild-type *GAR1* gene from the *GAL1-10* promoter. Accumulation of the Gar1 proteins and the state of rRNA pseudouridylation was monitored for each sample as described above for the *gar1.1* strain. The strong reduction in Gar1p levels observed in the *gar1.1+pGAL* strain grown on glucose at 37°C (Figure 4A, lane 5) was correlated with an inhibition of 18S rRNA production (Figure 4B, lane 5) and a strong reduction of 25S rRNA pseudouridylation (Figure 4C, panel e). Shifting of the *gar1.1+pGAL* strain to galactose medium at 37°C restored normal levels of Gar1p (Figure 4A, lane 6), production of 18S rRNA (Figure 4B, lane 6) as well as pseudouridylation of 25S rRNA (Figure 4C, panel f). In the control *GAR1+pGAL* strain that expresses the wild-type *GAR1* gene constitutively (Figure 4A, lanes 1–3), processing (Figure 4B, lanes 1–3) and pseudouridylation (Figure 4C, panels a–c) of rRNAs remained unaffected, regardless of growth conditions. These results

Fig. 4. Genetic restoration of normal levels of Gar1p protein in the thermosensitive *gar1.1* strain restores pseudouridylation of 25S rRNA at non-permissive temperature. (A) Western blot analysis. Yeast strains lacking the intact chromosomal *GAR1* gene, but expressing plasmid-born wild-type Gar1 (*GARI* strain) or thermosensitive Gar1.1 (*gar1.1* strain) proteins, were transformed with a centromeric vector (pGAL) carrying the wild-type *GAR1* gene under the control of the *GALI-10* promoter, resulting in strains *GARI*+pGAL and *gar1.1*+pGAL. Proteins extracted from the *GARI*+pGAL and *gar1.1*+pGAL strains grown either in glucose-containing medium for 3 h at 25 (lanes 1 and 4) or 37°C (lanes 2 and 5), or in glucose-containing medium for 3 h and for an additional 8 h in galactose-containing medium at 37°C (lanes 3 and 6) were analysed by Western blotting. To facilitate cloning, we have introduced linker sequences into the *GAR1* ORF. As a consequence, the Gar1 protein encoded by pGARI is 23.5 kDa and the Gar1.1 protein encoded by the *gar1.1* allele on pGARI.1 is 21.7 kDa. The pGAL vector contains an unmodified *GAR1* ORF coding for a 21.5 kDa protein. For other details, see legend to Figure 1B. (B) Denaturing agarose gel electrophoresis of *in vivo* labelled total RNAs extracted from the *GARI*+pGAL and *gar1.1*+pGAL strains grown under the conditions described above. Pulse labelling, extraction and separation of cellular RNAs were performed as described for the *gar1.1* strain in the legend to Figure 1. (C) Analysis of the pseudouridine content of 25S rRNAs obtained from the *GARI*+pGAL or *gar1.1*+pGAL strains. *In vivo* labelled 25S rRNAs eluted from preparative gels similar to the one shown in (B) were hydrolysed by RNase T2 and analysed by two-dimensional TLC. Culture conditions are indicated above the panels. The position of the pseudouridine 3'-monophosphate residue is indicated in each panel (Ψ_p).

demonstrate that Gar1p plays a specific and fundamental role in the overall pseudouridylation of yeast rRNAs.

Pseudouridylation of pre-rRNA is independent of 18S rRNA processing

In addition to the Gar1 (Girard *et al.*, 1992) and Nop1 (Tollervey *et al.*, 1991) snoRNP proteins, several Nop1p-associated snoRNAs (U3, U14, U22) and at least one

Fig. 5. Depletion of snR30 and U3 snoRNAs has no effect on the global pseudouridylation of 35S pre-rRNA. Yeast strains D190 and JH88 were grown for 18 h on rich medium lacking inorganic phosphate and supplemented either with galactose or with glucose in order to deplete cells of snR30 (D190) or U3 (JH88) snoRNAs. Cells were labelled with [32 P]orthophosphate for 15 min and total RNA was extracted. Purified 35S rRNAs were digested with RNase T2 and analysed by two-dimensional TLC. For other details see the legend to Figure 3.

Gar1p-associated snoRNA, snR30, are required for the production of 18S rRNA (Maxwell and Fournier, 1995; Venema and Tollervey, 1995; Sollner-Webb *et al.*, 1996; Tollervey and Kiss, 1997). Since processing events in the 5' external transcribed spacer (ETS) and internal transcribed spacer 1 (ITS1) regions of pre-rRNA are coupled, the snoRNPs essential for mature 18S rRNA production are believed to assemble into a large multi-RNP complex, called the processome (Fournier and Maxwell, 1993; Maxwell and Fournier, 1995; Venema and Tollervey, 1995). In principle, it is imaginable that formation of an active processome on the pre-rRNA may be crucial for pseudouridylation of pre-rRNA, and inhibition of ribosomal pseudouridine formation in the Gar1p-depleted strain may reflect a general property of mutants impaired in 18S production.

To exclude this possibility, we checked rRNA pseudouridylation in yeast strains depleted of snR30 and U3 snoRNAs. We used strains D190 (Morrissey and Tollervey, 1993) and JH88 (Hughes and Ares, 1991), in which the disrupted chromosomal *snR30* or *U3a/U3b* genes are complemented with the *snR30* and *U3b* genes placed under the control of the *GALI-10* promoter, respectively. In the D190 and JH88 strains, after 18 h growth on glucose-containing medium, snR30 and U3 snoRNAs were hardly detectable, and accumulation of 18S rRNA was completely abolished (Hughes and Ares, 1991; Morrissey and Tollervey, 1993; and data not shown). However, as compared with the control cells grown on galactose-containing medium (Figure 5A and C), pseudouridylation of 35S pre-rRNAs in the snR30- (Figure 5B) or U3-depleted (Figure 5D) cells was globally unaffected. Similar results were obtained for 25S rRNAs as well (data not shown). These results prove that pseudouridylation of pre-rRNA is independent of the processing of 18S RNA and, more importantly, demonstrate that inhibition of rRNA

pseudouridylation is restricted to strains deficient in *GAR1* function.

Discussion

Recently, it became evident that the great majority of snoRNPs fall into two distinct structurally and probably functionally well-defined families (Balakin *et al.*, 1996; Ganot *et al.*, 1997b). Many snoRNAs share two conserved sequence elements, boxes C and D, and are associated with a common antigen, fibrillarin. Some fibrillarin-associated snoRNAs are required for processing of rRNAs, but most of them function as guide RNAs in site-specific ribose methylation of pre-rRNA. The other major group of snoRNAs share an evolutionarily conserved 'hairpin-hinge-hairpin-tail' secondary structure and two conserved sequence elements positioned in the single-stranded hinge (box H) and tail (box ACA) regions of the snoRNA (Balakin *et al.*, 1996; Ganot *et al.*, 1997b). In yeast, all box H/ACA snoRNAs are associated with Gar1p, which is an abundant nucleolar protein required for accumulation of mature 18S rRNA (Girard *et al.*, 1992). In this study, using a thermosensitive allele of the *GAR1* gene, we have demonstrated that Gar1p plays a specific and fundamental role in global pseudouridylation of yeast rRNAs. These results establish a novel function for Gar1p and identify the first factor involved in rRNA pseudouridylation.

The fact that an snoRNP protein common to all box H/ACA snoRNPs is required for global pseudouridylation of rRNAs strongly suggests that the box H/ACA snoRNAs, or at least a subset of these RNAs, function in the site-specific pseudouridylation of pre-rRNA. Since the overwhelming majority of the intracellular pool of yeast Gar1 snoRNP protein is associated with snoRNPs or higher order nucleolar structures (our unpublished data), it seems unlikely that the observed function of Gar1p in pre-rRNA pseudouridylation is independent of snoRNA association. This leads to the conclusion that selection of specific uridine residues in the pre-rRNA and/or isomerization of the selected uridines to pseudouridines is mediated by box H/ACA snoRNPs. Indeed, recent genetic depletion and reconstitution experiments on two yeast box ACA snoRNAs, snR5 and snR36, proved that these snoRNAs function in the site-specific pseudouridylation of pre-rRNA (Ganot *et al.*, 1997a). The yeast snR5 is required for the pseudouridylation of 25S rRNA at positions Ψ 1003 and Ψ 1123, and snR36 plays a crucial and specific role in the formation of Ψ 1185 in the 18S rRNA. In the same study, inspection of mammalian, yeast and *Tetrahymena* box H/ACA snoRNAs revealed a general model by which box H/ACA snoRNAs can specifically select ribosomal pseudouridylation sites. According to this model, the pseudouridylation guide snoRNAs, similarly to the 2'-O-ribose methylation guide snoRNAs, function through direct Watson-Crick interactions with pre-rRNA sequences. Single-stranded sequences in an internal loop structure of the 5'- or 3'-terminal hairpin of box H/ACA snoRNAs select the rRNA sequences that precede and follow the target uridine residue (Ganot *et al.*, 1997a).

Yeast strains depleted of Gar1p impair both processing of 18S rRNA (Figure 2B; Girard *et al.*, 1993) and formation of ribosomal pseudouridines (Figure 3 and 4C). At the moment, it is unclear whether inhibition of 18S

rRNA production in the *gar1.1* strain at restrictive temperature is due to the lack of global rRNA pseudouridylation. Pseudouridylation of eukaryotic rRNAs occurs predominantly on the primary rRNA transcripts before nucleolytic processing (Jeanteur *et al.*, 1968; Brand *et al.*, 1979; this study). This raised the intriguing idea that pseudouridine residues in the pre-rRNA may facilitate the processing of rRNAs (reviewed in Maden, 1990; Eichler and Craig, 1995). Our results show that pseudouridylation of pre-rRNA is not required for the efficient and correct processing of 25S rRNA (Figure 2B, lane 1, and Figure 4B, lane 5). However, the importance of ribosomal pseudouridines for 18S processing is less well understood. It is conceivable that depletion of Gar1p may hinder assembly of functional box H/ACA snoRNPs involved in the synthesis of ribosomal pseudouridines that are essential for 18S formation. According to an alternative explanation, some box H/ACA snoRNPs may function directly or indirectly in the nucleolytic processing of 18S rRNA. Supporting this scenario, genetic depletion of yeast snR30, the only known essential box H/ACA snoRNA that lacks apparent pseudouridine recognition motifs (Ganot *et al.*, 1997a), disrupts 18S production (Morrissey and Tollervey, 1993), but does not affect the global pseudouridylation of pre-rRNA (Figure 5). Of course, we cannot rule out the formal possibility that snR30, by using a mechanism distinct from the majority of box H/ACA snoRNAs, functions in the formation of a specific pseudouridine residue that plays a fundamental role in the maturation or accumulation of yeast 18S rRNA.

Consistent with the notion that pre-rRNA pseudouridylation happens before nucleolytic processing of pre-rRNA, correct processing of pre-rRNA or formation of a pre-rRNA-multi-snoRNP complex competent in rRNA processing does not seem to be crucial for global pseudouridylation of pre-rRNA (Figure 5). Moreover, depletion of yeast Nop1 snoRNP protein, although it abolishes both the formation of mature 18S rRNA and the 2'-O-methylation of pre-rRNA (Tollervey *et al.*, 1993), does not inhibit the overall pseudouridylation of pre-rRNA (our unpublished data). This further corroborates that site-specific ribose methylation and pseudouridylation of pre-rRNA are independent of each other and rely on different sets of nucleolar factors. Consistent with this, we recently have shown that yeast Nop1p, contrary to previous reports (Schimmang *et al.*, 1989; Balakin *et al.*, 1996), is specifically and exclusively associated with box C/D snoRNAs while the box H/ACA snoRNAs are specifically complexed with the Gar1 snoRNP protein (Balakin *et al.*, 1996; Ganot *et al.*, 1997b).

Recently, it was discovered that all known pseudouridine synthases can be grouped into four distinct protein families (Koonin, 1996). Three of these families share three similarly positioned conserved motifs, one of which is present in two other families of enzymes that also bind uridine, namely deoxycytidine triphosphate deaminases and deoxyuridine triphosphatases. Since Gar1p contains none of these conserved motifs and shows no significant similarities to any known pseudouridine synthase, it seems unlikely that Gar1p itself provides the catalytic activity for pseudouridine formation. Although Gar1p seems to be an integral component of box H/ACA snoRNPs (Lübben *et al.*, 1995), depletion of the cellular pool of Gar1p does

not alter the levels of snR30 and snR36 snoRNAs (Girard *et al.*, 1992; our unpublished observation). However, we found that depletion of Gar1p in the thermosensitive *gar1.1* strain after 3 h of growth at the non-permissive temperature resulted in snR36-containing small RNPs that are unable to associate with higher order nucleolar particles (our unpublished observation). Therefore, we assume that Gar1 protein plays a crucial role in pre-rRNA pseudouridylation by structuring box H/ACA snoRNPs and making them competent for association with nucleolar RNP structures containing pre-rRNAs. In the future, it will be interesting to investigate the fate of the newly synthesized box H/ACA snoRNAs in the Gar1p-depleted yeast cells. Are these snoRNAs correctly processed in the absence of Gar1p? Do they accumulate in the nucleolus? Clearly, dissection of the precise function of Gar1p and understanding of the molecular mechanism by which box H/ACA snoRNPs accomplish site-specific pseudouridylation of eukaryotic pre-rRNA will be an interesting task.

Materials and methods

Plasmids and yeast strains

Unless otherwise indicated, all techniques used for manipulating DNA were as described by Sambrook *et al.* (1989). A null allele of the yeast *GAR1* gene was created in the diploid strain 510 (*Mata/α*, *ade2*, *ade3*, *leu2*, *trp1.1*, *lys2*, *his3*, *ura3.52*, *tyr7.1/TYR7*, *met8.1/MET8*, *ile1.1/ILE1*, *can1-100*) as follows. The 0.6 kb *Bam*HI fragment of pJG67 (Girard *et al.*, 1994) containing the *GAR1* ORF was replaced, after Klenow polymerase treatment, with the 1.8 kb *Hpa*I fragment of YEpl3 (Broach *et al.*, 1979) encompassing the *LEU2* gene. From the resulting recombinant plasmid, a 3.7 kb *Pvu*II–*Hind*III fragment containing the interrupted *GAR1* gene (*gar1::LEU2*) was purified and used to transform strain 510. Recombinants were selected on minimal medium without leucine and the correct genomic integration of the *LEU2* gene was verified by Southern analysis. The resulting *gar1::LEU2/GAR1*⁺ strain was transformed by a *TRP1*–*GAR1* centromeric vector. After sporulation and tetrad dissection, we selected a *LEU*⁺/*TRP*⁺ haploid designated 535-2d (*Mata*, *ade2*, *ade3*, *trp1.1*, *ura3.52*, *lys2*, *his3*, *leu2*, *ile1*, *can1-100*, *gar1::LEU2/pGAR1-TRP1*). Strains D190 (*ura3.52*, *leu2*, *ade1*, *his4*, *URA3-GAL::snR30*) (Morrissey and Tollervey, 1993) and JH88 (*his3*, *trp1*, *lys1*, *ade2*, *can1-100*, *URA3-GAL::U3a*, *u3b::LEU2*) (Hughes and Ares, 1991) were kindly provided by D.Tollervey and J.M.X.Hughes, respectively.

To obtain pGAR1, the 1.2 kb *Bgl*III fragment of pFL38 (DDBJ/EMBL/GenBank accession No. X70482) containing the *URA3* gene was replaced by the 4.8 kb *Sal*I–*Sma*I fragment of YDpK (Berben *et al.*, 1991) carrying the *LYS2* gene. The vector and insert were filled in by Klenow polymerase. The 2.0 kb *Eco*RI–*Sal*I cassette of pJPG67 (Girard *et al.*, 1994) containing the wild-type *GAR1* gene was inserted into the *Eco*RI–*Sal*I sites of the resulting construct. In pGAR1.1, the *Bam*HI cassette of pGAR1 containing the *GAR1* ORF was replaced by the temperature-sensitive *gar1.1* allele produced by random PCR mutagenesis (see below). To construct pGAL, the *URA3*–pGAL1.10–*GAR1* cassette prepared for construction of the *GAL::GAR1* strain (Girard *et al.*, 1994) was excised by *Nhe*I–*Sal*I digestion and, after Klenow fill-in, inserted into the *Sma*I–*Sal*I sites of pCH1122 (Kranz and Holm, 1990). The resulting expression constructs were used to complement the haploid strain 535-2d. Transformation of the 535-2d strain with GAR1.1 yielded strain *gar1.1*; transformation with pGAR1 and pGAL yielded strain *GAR1* + pGAL; transformation with pGAR1.1 and pGAL yielded strain *gar1.1* + pGAL. The pTRP1–*GAR1* present in the 535-2d strain (see above) was cured from each strain.

Isolation and characterization of a thermosensitive allele of the GAR1 gene

A 620 bp fragment of the *GAR1* gene encompassing the *GAR1* ORF was PCR amplified using pJPG1 as a template (Girard *et al.*, 1992) and primers specific for the 5' end (5'-CCCGGATCCTGGTAATAGAGGTG-GCCG-3') and the 3' end (5'-CCCGGATCCTATTATCTTCTACCTCCT-CTGA-3') regions of the *GAR1* ORF. Use of these primers resulted in

inclusion of *Bam*HI sites in the N-terminal region and behind the translation stop codon of the *GAR1* ORF. The amplified *Bam*HI cassette was inserted into the same site of pBluescribe (KS+). To introduce random mutations into the *GAR1* ORF, the resulting pBKS-*GAR1* was used as a template for PCR mutagenesis using pBluescribe-specific M13 primers (–20 and reverse primers). The amplification reaction was performed in a mixture of 10 mM Tris–HCl, pH 8.4, 50 mM KCl, 7 mM MgCl₂, 0.5 mM MgCl₂, 0.2 mM dATP and dGTP, 1 mM dCTP and dTTP and 5 U of *Taq* polymerase (Boehringer) as described by Cadwell *et al.* (1992). The PCR-amplified fragment was digested by *Bam*HI and inserted into the same site of pJGP53 (Girard *et al.*, 1992). Transformation of the pJG53-*GAR1* recombinant plasmid into ultracompetent XL2-Blue cells (Stratagene) resulted in ~60 000 colonies, 90% of which carried the *GAR1* insert. Colonies were pooled and plasmid was extracted to transform yeast strain YO24 (*URA3*–pGAL::*GAR1*) (Girard *et al.*, 1994). The transformant colonies, selected on YPD at 25°C, were replica plated and grown at 37°C to identify temperature-sensitive mutants. Among 16 000 yeast transformants, three mutants were identified which exhibited the temperature-sensitive phenotype.

Accumulation of Gar1 and control Srp21 and Nop1 proteins was monitored by Western blot analysis using standard techniques. The same blot was reacted with anti-GAR1 antibody (Girard *et al.*, 1992) at 1/200 dilution O/N at 4°C, anti-SRP21 antibody (kindly provided by D.Jullien and E.Käs) at 1/500 dilution O/N at 4°C or anti-NOP1 RP1-4 antibody (kindly provided by E.C.Hurt) at 1/40 000 dilution for 1 h at room temperature.

Determination of the pseudouridine content of rRNAs

For each experiment, specific growth conditions are described in the text and figure legends. Generally, the *gar1.1* and its derivative strains were grown at 25°C in minimal medium to mid-exponential phase. Cells were washed and resuspended (OD₆₀₀ = 0.3) in rich medium containing either 2% glucose or 2% galactose, but lacking inorganic phosphate (Warner, 1991), and were grown for 3 h either at 25 or 37°C. Fifty ml aliquots of each culture were centrifuged and resuspended in 25 ml of pre-warmed medium supplemented with 5 mCi of [³²P]orthophosphate (9000 Ci/mmol). After 15 min of labelling, cells were collected and total RNA was extracted (Tollervey and Mattaj, 1987) and analysed by electrophoresis on a 1.2% agarose–formaldehyde gel. The 35S pre-rRNA and 25S rRNA were isolated by electroelution, digested with RNase T2 in 50 mM Na acetate, pH 5.5 at 37°C and analysed by two-dimensional cellulose TLC in System B (Filipowicz and Shatkin, 1983).

Acknowledgements

We are grateful to D.Tollervey, J.M.X.Hughes, E.C.Hurt, E.Käs and D.Jullien for providing us with yeast strains and antibodies. We thank L.Poljak for critical reading of the manuscript. C.B.-A. was supported by the Association pour la Recherche Contre le Cancer. Our work was supported by the Centre National de la Recherche Scientifique, Université Paul Sabatier, Toulouse, and by grants from the Région Midi-Pyrénées, Association pour la Recherche Contre le Cancer (to M.C.-F.), la Ligue Nationale Contre le Cancer and la Fondation pour la Recherche Médicale (to T.K.).

References

- Balakin, A.G., Smith, L. and Fournier, M.J. (1996) The RNA world of the nucleolus: two major families of small RNAs defined by different box elements with related functions. *Cell*, **86**, 823–834.
- Berben, G., Dumont, J., Gilliquet, V., Bolle, P.-A. and Hilger, F. (1991) The YDp plasmids: a uniform set of vectors bearing versatile gene disruption cassettes for *Saccharomyces cerevisiae*. *Yeast*, **7**, 475–477.
- Brand, R.C., Klootwijk, J., Sibum, C.P. and Planta, R.J. (1979) Pseudouridylation of yeast ribosomal precursor RNA. *Nucleic Acids Res.*, **7**, 121–134.
- Broach, J.R., Strathern, J.N. and Hicks, J.B. (1979) Transformation of yeast: development of a hybrid cloning vector and isolation of the *CAN1* gene. *Gene*, **8**, 121–133.
- Brown, J.D., Hann, B.C., Medzihradsky, K.F., Niwa, M., Burlingame, A.L. and Walter, P. (1994) Subunits of the *Saccharomyces cerevisiae* signal recognition particle required for its functional expression. *EMBO J.*, **13**, 4390–4400.
- Cadwell, R.C. and Joyce, G.F. (1992) In *PCR Methods and Applications*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 28–33.

- Cavaillé, J., Nicoloso, M. and Bachellerie, J.-P. (1996) Targeted ribose methylation of RNA *in vivo* directed by tailored antisense RNA guides. *Nature*, **383**, 732–735.
- Chu, S., Archer, R.H., Zengel, J.M. and Lindahl, L. (1994) The RNA of RNase MRP is required for normal processing of ribosomal RNA. *Proc. Natl Acad. Sci. USA*, **91**, 569–663.
- Eichler, D.C. and Craig, N. (1995) Processing of eukaryotic ribosomal RNA. *Prog. Nucleic Acids Res. Mol. Biol.*, **49**, 179–239.
- Filipowicz, W. and Kiss, T. (1993) Structure and function of small nucleolar snRNPs. *Mol. Biol. Rep.*, **18**, 149–156.
- Filipowicz, W. and Shatkin, A.J. (1983) Origin of splice junction phosphate in tRNAs processed by HeLa cell extract. *Cell*, **32**, 547–557.
- Fournier, M.J. and Maxwell, E.S. (1993) The nucleolar snRNAs: catching up with the spliceosomal snRNAs. *Trends Biochem. Sci.*, **18**, 131–135.
- Ganot, P., Bortolin, M.-L. and Kiss, T. (1997a) Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. *Cell*, **89**, 799–809.
- Ganot, P., Caizergues-Ferrer, M. and Kiss, T. (1997b) The family of box ACA small nucleolar RNAs is defined by an evolutionarily conserved secondary structure and ubiquitous sequence elements essential for RNA accumulation. *Genes Dev.*, **11**, 941–956.
- Girard, J.-P., Lehtonen, H., Caizergues-Ferrer, M., Amalric, F., Tollervey, D. and Lapeyre, B. (1992) GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast. *EMBO J.*, **11**, 673–682.
- Girard, J.-P., Caizergues-Ferrer, M. and Lapeyre, B. (1993) The *SpGAR1* gene of *Schizosaccharomyces pombe* encodes the functional homologue of the snoRNP protein GAR1 of *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **21**, 2146–2155.
- Girard, J.-P., Bagni, C., Caizergues-Ferrer, M., Amalric, F. and Lapeyre, B. (1994) Identification of a segment of the small nucleolar ribonucleoprotein-associated protein GAR1 that is sufficient for nucleolar accumulation. *J. Biol. Chem.*, **269**, 18499–18506.
- Hadjiolov, A.A. (1985) The nucleolus and ribosome biogenesis. In Alfert, M., Beermann, W., Goldstein, L., Porter, K.R. and Sitte, P. (eds), *Cell Biology Monographs*. Springer-Verlag, Wien.
- Hughes, J.M.X. and Ares, M., Jr (1991) Depletion of U3 small nucleolar RNA inhibits cleavage in the 5' external transcribed spacer of yeast pre-ribosomal RNA and prevents formation of 18S ribosomal RNA. *EMBO J.*, **10**, 4231–4239.
- Jeanteur, P., Amaldi, F. and Attardi, G. (1968) Partial sequence analysis of ribosomal RNA from HeLa cells. II Evidence for sequences of non-ribosomal type in 45S and 32S ribosomal RNA precursors. *J. Mol. Biol.*, **33**, 757–775.
- Kass, S., Tyc, K., Steitz, J.A. and Sollner-Webb, B. (1990) The U3 small nucleolar ribonucleoprotein functions in the first step of preribosomal RNA processing. *Cell*, **60**, 897–908.
- Keith, G. (1995) Mobilities of modified ribonucleotides on two-dimensional cellulose thin-layer chromatography. *Biochimie*, **77**, 142–144.
- Kiss-László, Z., Henry, Y., Bachellerie, J.-P., Caizergues-Ferrer, M. and Kiss, T. (1996) Site-specific ribose methylation of preribosomal RNA: a novel function for small nucleolar RNAs. *Cell*, **85**, 1077–1088.
- Koonin, E.V. (1996) Pseudouridine synthases: four families of enzymes containing a putative uridine-binding motif also conserved in dUTPases and dCTP deaminases. *Nucleic Acids Res.*, **24**, 2411–2415.
- Kranz, J.E. and Holm, C. (1990) Cloning by function: an alternative approach for identifying yeast homologues of genes from other organisms. *Proc. Natl Acad. Sci. USA*, **87**, 6629–6633.
- Lane, B.G., Ofengand, J. and Gray, M.W. (1995) Pseudouridine and *O*²-methylated nucleotides. Significance of their selective occurrence in rRNA domains that function in ribosome-catalyzed synthesis of the peptide bonds in proteins. *Biochimie*, **77**, 7–15.
- Li, H.-V., Zagorski, J. and Fournier, M.J. (1990) Depletion of U14 small nucleolar RNA (snR128) disrupts production of 18S rRNA in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **10**, 1145–1152.
- Lübbers, B., Fabrizio, P., Kastner, B. and Lührmann, R. (1995) Isolation and characterization of the small nucleolar ribonucleoprotein particle snR30 from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **270**, 11549–11554.
- Lygerou, Z., Allmang, C., Tollervey, D. and Séraphin, B. (1996) Accurate processing of a eukaryotic precursor ribosomal RNA by ribonuclease MRP *in vitro*. *Science*, **272**, 268–270.
- Maden, B.E.H. (1990) The numerous modified nucleotides in eukaryotic ribosomal RNA. *Prog. Nucleic Acids Res.*, **39**, 241–303.
- Maxwell, E.S. and Fournier, M.J. (1995) The small nucleolar RNAs. *Annu. Rev. Biochem.*, **35**, 897–934.
- Morrissey, J.P. and Tollervey, D. (1993) Yeast snR30 is a small nucleolar RNA required for 18S rRNA synthesis. *Mol. Cell. Biol.*, **13**, 2469–2477.
- Nicoloso, M., Qu, L.-H., Michot, B. and Bachellerie, J.-P. (1996) Intronic, antisense small nucleolar RNAs: the characterization of nine novel species points to their direct role as guides for the 2'-O-ribose methylation of rRNAs. *J. Mol. Biol.*, **260**, 178–195.
- Ofengand, J., Bakin, A., Wrzesinski, J., Nurse, K. and Lane, B.G. (1995) The pseudouridine residues of ribosomal RNA. *Biochem. Cell Biol.*, **73**, 915–924.
- Peculis, B.A. and Steitz, J.A. (1993) Disruption of U8 nucleolar snRNA inhibits 5.8S and 28S rRNA processing in the *Xenopus* oocyte. *Cell*, **73**, 1233–1245.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Savino, R. and Gerbi, S.A. (1990) *In vivo* disruption of *Xenopus* U3 snRNA affects ribosomal RNA processing. *EMBO J.*, **9**, 2299–2308.
- Schimmang, T., Tollervey, D., Kern, H., Frank, R. and Hurt, E.C. (1989) A yeast nucleolar protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability. *EMBO J.*, **8**, 4015–4024.
- Schmitt, M.E. and Clayton, D.A. (1993) Nuclear RNase MRP is required for correct processing of pre-5.8S rRNA in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **13**, 7935–7941.
- Smith, J.D. and Dunn, D.B. (1959) An additional sugar component of ribonucleic acids. *Biochim. Biophys. Acta*, **31**, 573–575.
- Sollner-Webb, B., Tycowski, K.T. and Steitz, J.A. (1996) Ribosomal RNA processing in eukaryotes. In Zimmermann, R.A. and Dahlberg, A.E. (eds), *Ribosomal RNA. Structure, Evolution, Processing, and Function in Protein Biosynthesis*. CRC Press, Boca Raton, FL, pp. 469–490.
- Tollervey, D. and Kiss, T. (1997) Function and synthesis of small nucleolar RNAs. *Curr. Opin. Cell Biol.*, **9**, 337–342.
- Tollervey, D. and Mattaj, J.W. (1987) Fungal small nuclear ribonucleoproteins share properties with plant and vertebrate U-snRNPs. *EMBO J.*, **6**, 469–476.
- Tollervey, D., Lehtonen, H., Carmo-Fonseca, M. and Hurt, E.C. (1991) The small nucleolar RNP protein NOP1 (fibrillarin) is required for pre-rRNA processing in yeast. *EMBO J.*, **10**, 573–583.
- Tollervey, D., Lehtonen, H., Jansen, R., Kern, H. and Hurt, E.C. (1993) Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA methylation, and ribosome assembly. *Cell*, **72**, 443–457.
- Tyc, K. and Steitz, J.A. (1989) U3, U8 and U13 comprise a new class of mammalian snRNPs localized in the cell nucleolus. *EMBO J.*, **8**, 3113–3119.
- Tycowski, K.T., Shu, M.-D. and Steitz, J.A. (1993) A small nucleolar RNA is processed from an intron of the human gene encoding ribosomal protein S3. *Genes Dev.*, **6**, 1120–1130.
- Tycowski, K.T., Shu, M.-D. and Steitz, J.A. (1994) Requirement for intronic encoded U22 small nucleolar RNA in 18S ribosomal RNA maturation. *Science*, **266**, 1558–1561.
- Tycowski, K.T., Shu, M.-D. and Steitz, J.A. (1996a) A mammalian gene with introns instead of exons generating stable RNA products. *Nature*, **379**, 464–466.
- Tycowski, K.T., Smith, C.M., Shu, M.-D. and Steitz, J.A. (1996b) A small nucleolar RNA required for site-specific ribose methylation of rRNA in *Xenopus*. *Proc. Natl Acad. Sci. USA*, **93**, 14480–14485.
- Venema, J. and Tollervey, D. (1995) Processing of pre-ribosomal RNA in *Saccharomyces cerevisiae*. *Yeast*, **11**, 1629–1650.
- Warner, J.R. (1991) Labeling of RNA and phosphoproteins in *Saccharomyces cerevisiae*. In Guthrie, C. and Flink, G.R. (eds), *Guide to Yeast Genetics and Molecular Biology*. Academic Press, Vol. 194, pp. 423–428.

Received on March 17, 1997; revised on May 13, 1997