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Elements essential for accumulation and function of small nucleolar RNAs directing site-specific pseudouridylation of ribosomal RNAs

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During site-specific pseudouridylation of eukaryotic rRNAs, selection of correct substrate uridines for isomerization into pseudouridine is directed by small nucleolar RNAs (snoRNAs). The pseudouridylation guide snoRNAs share a common 'hairpin–hinge–hairpin–tail' secondary structure and two conserved sequence motifs, the H and ACA boxes, located in the single-stranded hinge and tail regions, respectively. In the 5′- and/or 3′-terminal hairpin, an internal loop structure, the pseudouridylation pocket, selects the target uridine through formation of base-pairing interactions with rRNAs. Here, essential elements for accumulation and function of rRNA pseudouridylation guide snoRNAs have been analysed by expressing various mutant yeast snR5, snR36 and human U65 snoRNAs in yeast cells. We demonstrate that the H and ACA boxes that are required for formation of the correct 5′ and 3′ ends of the snoRNA, respectively, are also essential for the pseudouridylation reaction directed by both the 5′- and 3′-terminal pseudouridylation pockets. Similarly, RNA helices flanking the two pseudouridylation pockets are equally essential for pseudouridylation reactions mediated by either the 5′ or 3′ hairpin structure, indicating that the two hairpin domains function in a highly co-operative manner. Finally, we demonstrate that by manipulating the rRNA recognition motifs of pseudouridylation guide snoRNAs, novel pseudouridylation sites can be generated in yeast rRNAs.

Keywords: box H/ACA snoRNA/guide RNA/nucleolus/pseudouridine/RNA modification

Introduction

The nucleolar biosynthesis of eukaryotic rRNAs consists of three major steps. First, RNA polymerase I synthesizes a large precursor RNA (pre-rRNA), in which the 18S, 5.8S and 25/28S rRNAs are flanked and separated by external and internal spacer sequences (Hadjiolov, 1985). After transcription, the rRNA regions of the pre-rRNA undergo extensive covalent modifications. Many precisely selected nucleotides are methylated at the 2′-*O*-hydroxyl position, and several uridine residues are converted into pseudouridine (Maden, 1990; Eichler and Craig, 1994). Finally, the mature-sized rRNAs are nucleolytically processed from the modified pre-rRNA (Eichler and Craig, 1994; Venema and Tollervey, 1995; Sollner-Webb *et al*., 1996).

In vertebrates, the mature 18S, 5.8S and 28S rRNAs contain >100 2′-*O*-methyl groups and ~95 pseudouridines (Maden, 1990; Ofengand *et al*., 1995; Ofengand and Bakin, 1997). Although substantial evidence points to a catalytic role for rRNAs in protein synthesis (Green and Noller, 1997; Nitta *et al*., 1998; Schimmel and Alexander, 1998), the function of modified nucleotides remains entirely speculative. Since pseudouridylation and ribose methylation sites cluster on the universally conserved functional centres of rRNAs and their positions show significant conservation during evolution (Maden, 1990; Ofengand and Bakin, 1997), we can anticipate that the modified nucleotides contribute to ribosome assembly or/and function (Lane *et al*., 1995; Ofengand *et al*., 1995).

In the nucleolus of eukaryotic cells, 2′-*O*-methylation and pseudouridylation of pre-rRNA is accomplished by a large number of different small ribonucleoprotein particles (snoRNPs) (Smith and Steitz, 1997; Tollervey and Kiss, 1997). Each snoRNP contains a specific small nucleolar RNA (snoRNA) and a set of associated snoRNP proteins. The 2′-*O*-methylation and pseudouridylation guide RNAs possess distinctive structural elements and are associated with different sets of proteins (Maxwell and Fournier, 1995; Smith and Steitz, 1997; Tollervey and Kiss, 1997). The methylation guide snoRNAs carry the conserved C, C′, D and D′ box elements that are essential for both the nucleolar accumulation and function of snoRNAs (Caffarelli *et al*., 1996; Cavaillé and Bachellerie, 1996; Cavaillé *et al*., 1996; Kiss-László *et al*., 1996, 1998; Watkins *et al*., 1996). The methylation guide snoRNAs select the target ribosomal nucleotides by forming a 10–21 bp Watson–Crick helix with rRNA sequences. This snoRNA–rRNA interaction, in conjunction with the D and C or D′ and C′ boxes of the snoRNA, provides the structural information for the methyltransferase activity to methylate the correct ribosomal nucleotide (Cavaillé *et al*., 1996; Kiss-László *et al*., 1996, 1998; Tycowski *et al*., 1996).

Information on the structural requirements for accumulation and function of pseudouridylation guide snoRNAs is much more limited. This group of snoRNAs share a common secondary structure that consists of two major hairpins connected by a hinge and followed by a short tail (Ganot *et al*., 1997b). The single-stranded hinge and tail regions contain the conserved H (consensus AnAnnA) and ACA box elements, respectively (Balakin *et al*., 1996; Ganot *et al*., 1997b). In vertebrates, the box H/ACA snoRNAs, similar to the box C/D methylation guide snoRNAs, are processed from introns of pre-mRNAs, whereas in yeast, most H/ACA snoRNAs are transcribed from their independent genes by RNA polymerase II

(pol II) or, less frequently, are processed from pre-mRNA introns or polycistronic pre-snoRNA transcripts (Maxwell and Fournier; 1995; Balakin *et al*., 1996; Tollervey and Kiss, 1997). Previous studies suggested that the human intron-encoded U17 and U19 box H/ACA snoRNAs are processed from the removed and debranched host introns by 5′→3′ and 3′→5′ exonucleolytic activities (Cecconi *et al*., 1995; Kiss and Filipowicz, 1995; Kiss *et al*., 1996). Supporting this view, recent studies on the biogenesis of yeast box C/D snoRNAs demonstrated that debranching of host intron lariats by the Dbr1p RNA debranching enzyme (Ooi *et al*., 1998; Petfalski *et al*., 1998) or endonucleolytic cleavages of polycistronic pre-snoRNAs by endonuclease III (Chanfreau *et al*., 1998) are essential for snoRNA production. These cleavage reactions provide the entry sites for exonucleolytic activities responsible for processing of mature snoRNAs (Petfalski *et al*., 1998; C.Allmang, P.Mitchell, E.Petfalski and D.Tollervey, personal communication; Qu *et al*., 1999).

The ACA box motif that is located three nucleotides from the 3′ end of box H/ACA snoRNAs, together with the adjacent 3′-terminal stem, plays an essential role in the processing and/or stability of the RNA. This structural motif determines the correct 3′ terminus of the RNA, most likely by binding snoRNP proteins and thereby protecting the snoRNA sequences from the processing exonucleases (Balakin *et al*., 1996; Ganot *et al*., 1997b). The H box was proposed to contribute to the 5′ end formation of box H/ACA snoRNAs (Ganot *et al*., 1997b). Consistent with this, it is required for the accumulation of at least the intron-encoded members of H/ACA snoRNAs.

The pseudouridylation guide snoRNAs select the substrate uridines by forming two short base-pairing interactions with rRNA sequences that flank the target uridine (Ganot *et al*., 1997a). The two rRNA recognition motifs occupy the opposite strands of an internal loop, termed the pseudouridylation pocket, which is located in the 5′ and/or 3′ hairpin domain of the snoRNA. In this study, the essential elements for accumulation and function of pseudouridylation guide snoRNAs have been analysed by expressing various mutant yeast snR5, snR36 and human U65 snoRNAs in yeast cells. Our results demonstrate that the pseudouridylation guide snoRNAs, in marked contrast to the methylation guide snoRNAs, have to meet very strict and complex structural requirements to ensure efficient snoRNA accumulation and guide RNA function.

Results

The 5′-terminal cap structure contributes to the stability of box H/ACA snoRNAs

The H and ACA box-containing snoRNAs are either synthesized from their own genes by pol II or are processed from longer RNA transcripts, such as pre-mRNAs or polycistronic pre-snoRNAs (see Introduction). The latter group of snoRNAs undergo exonucleolytic 5′ and 3′ end maturation, whereas the 5′ terminus of snoRNAs synthesized by pol II from independent genes is delineated by the polymerase itself and co-transcriptionally acquires a 7-methylguanosine cap structure that is modified later to 2,2,7-trimethylguanosine (Maxwell and Fournier, 1995).

It has been demonstrated that elements directing the processing of human and yeast intron-encoded H/ACA snoRNAs are located within the snoRNA sequence itself (Cecconi *et al*., 1995; Kiss and Filipowicz, 1995; Kiss *et al*., 1996; Ganot *et al*., 1997a,b). To assess whether box H/ACA snoRNAs that are synthesized by pol II from independent genes also possess the elements essential for processing from pre-mRNA introns, a cDNA of the yeast snR5 snoRNA was inserted into the intron of the yeast actin gene that had been placed under the control of the promoter and terminator of the yeast alcohol dehydrogenase (ADH) gene (Figure 1A; Kiss-László *et al*., 1996). The resulting ACT/SNR5 expression construct or the wild-type *SNR5* gene were inserted into the pFL45 yeast shuttle vector (Bonneaud *et al*., 1991) and transformed into the ΔsnR5 yeast strain that lacks a functional *SNR5* locus (Ganot *et al*., 1997a). Northern analyses showed that, as expected, the ΔsnR5 cells transformed with the wild-type *SNR5* gene (Figure 1A, lane 3) accumulated the snR5 RNA at the levels of the control cells (Figure 1A, lane 1). However, in cells transformed with the ACT/SNR5 construct, no snR5 accumulation was observed (Figure 1A, lane 5). Since the actin mRNA was expressed correctly (data not shown), we concluded that the snR5 RNA lacks elements that are essential for RNA accumulation. In fact, the major difference between the wild-type and the intronic snR5 RNA is that the latter lacks a 5′-terminal cap, suggesting that the cap is crucial to the stability of snR5.

To investigate this assumption further, the snR5-coding regions of the pFL45/SNR5 and the pFL45/ACT/SNR5 expression constructs were replaced with a cDNA of the yeast snR36 box H/ACA snoRNA that had been shown to be processed correctly from the yeast actin pre-mRNA (Ganot *et al*., 1997a). The resulting pFL45/SNR36 and pFL45/ACT/SNR36 constructs were transformed into the ΔsnR36 yeast strain (Ganot *et al*., 1997a). Northern analysis showed that, either processed from the actin pre-mRNA (Figure 1B, lane 1) or transcribed from the *SNR5* promoter (lane 6), the snR36 was expressed efficiently. The differences in the levels of RNA accumulation probably reflect the fact that the *SNR5* promoter supports transcription more efficiently than does the *ADH* promoter.

Integrity of the 3′-terminal helical stem of box H/ACA snoRNAs is essential for RNA accumulation (Balakin *et al*., 1996). To test whether the 5′-terminal stem is of similar importance, two mutant snR36 RNAs were constructed; snR36-*S1* and snR36-*S2*, which contain two base substitutions in the 5′ stem (Figure 1B). When the mutant snR36 cDNAs were inserted into the pFL45/ACT intronic snoRNA expression construct and transformed into the ΔsnR36 strain, neither snR36-*S1* (Figure 1B, lane 3) nor snR36-*S2* (lane 4) was expressed. However, accumulation of the intron-encoded snR36 was restored when the *S1* and *S2* base substitutions were combined to restore the base-pairing of the 5′-terminal stem (lane 5), indicating that integrity of this stem, rather than its nucleotide composition, is essential for snoRNA accumulation. Interestingly, both snR36-*S1* and snR36-*S2*, as well as snR36-*S1*+*S2*, were expressed under the control of the *SNR5* promoter (Figure 1B, lanes 7–9). Immunoprecipitations with antibodies directed against the m^3G cap demonstrated that all snR36 transcripts generated from the *SNR5* promoter contained the 5′-terminal m^3G cap (data not shown). Collectively, our results demonstrate that the cap

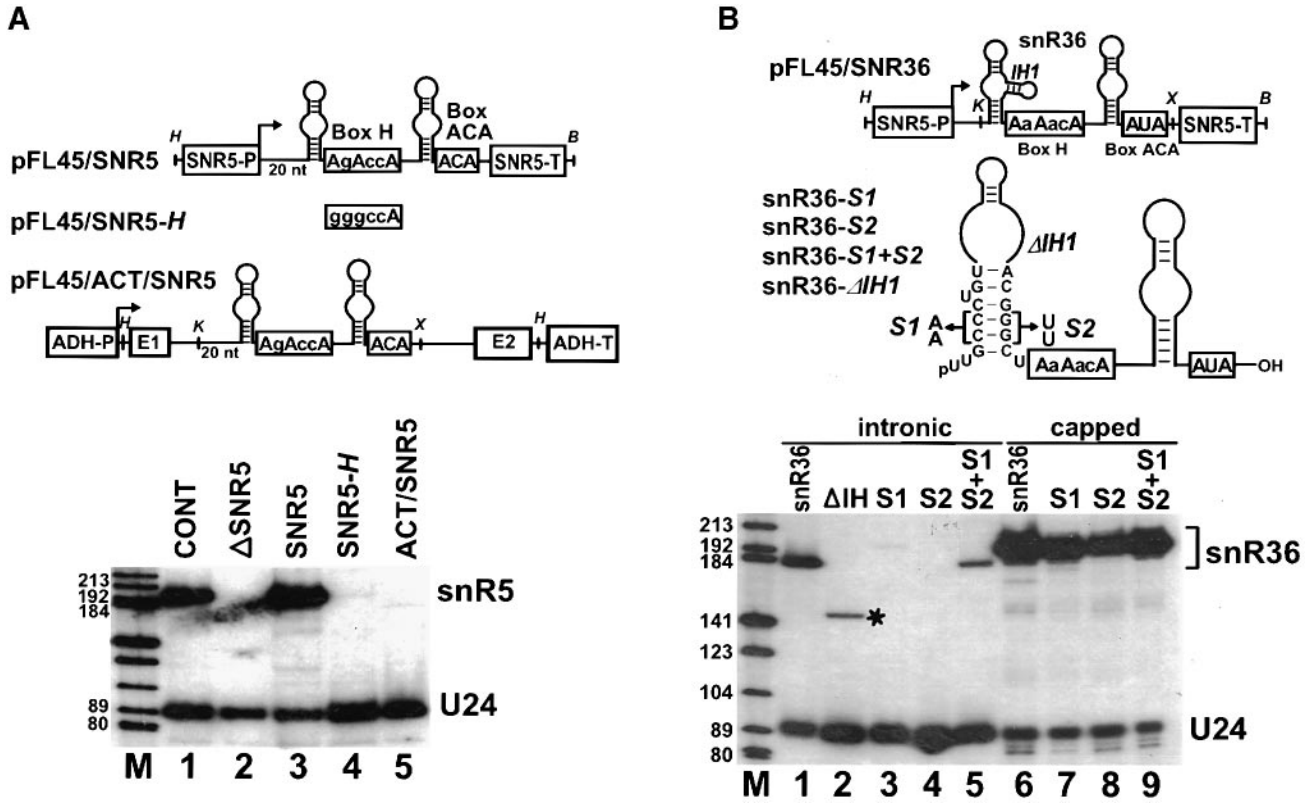

Fig. 1. Northern analyses of expression of snR5 and snR36 snoRNAs in yeast cells. (**A**) Expression of the wild-type and mutant yeast *SNR5* gene carrying an altered H box (*SNR5-H*) and processing of the wild-type snR5 RNA from the intron of the yeast actin pre-mRNA. Structures of the yeast *SNR5* gene and the ACT/SNR5 expression construct are shown, together with the schematic secondary structure of the snR5 RNA. The nucleotide sequences of the ACA and H boxes of the wild-type snR5 and the mutant snR5-*H* RNA are shown. Nucleotides conserved in most H/ACA snoRNAs are in upper case. Promoter (P) and terminator (T) regions of the *SNR5* (SNR5) and the yeast ADH genes are indicated. Arrows denote transcription initiation sites. E1 and E2 indicate the two exons of the yeast actin gene. The relevant restriction sites are indicated (H, *Hin*dIII; B, *Bam*HI; K, *Kpn*I; X, *Xho*I). RNAs isolated from the control CMY133 (CONT, lane 1) and ΔsnR5 (lane 2) strains and from derivatives of the ΔsnR5 strain transformed with either the pFL45/SNR5 (lane 3), pFL45/SNR5-*H* (lane 4) or pFL45/ACT/SNR5 (lane 5) construct were probed with ^{32}P-labelled oligodeoxynucleotides specific to the snR5 and U24 snoRNAs. Lane M, size markers (a mixture of *Hae*III- and *Taq*I-digested pBR322). (**B**) Expression of yeast snR36 RNA. Structures of the wild-type and mutant (*S1*, *S2*, *S1*+*S2* and Δ*IH1*) snR36 RNAs inserted between the promoter and terminator of the *SNR5* gene as well as into the intron of the yeast actin gene are shown. *IH1* indicates an inserted hairpin element. RNAs obtained from the ΔsnR36 strain transformed with pFL45/ACT (lanes 1–5) or pFL45/SNR (lanes 6–9) expression constructs carrying either the snR36 (lanes 1 and 6), snR36-*S1* (lanes 3 and 7), snR36-*S2* (lanes 4 and 8), snR36-*S1* +*S2* (lanes 5 and 9) or snR36-Δ*IH1* (lane 2) RNAs were probed with snR36- and U24-specific oligo probes. The asterisk indicates the processed snR36-Δ*IH1* RNA that lacks the 32 nucleotide *IH1* element. Lane M, size markers.

structure, most likely by protecting the 5′ end of the RNA, plays an important role in the accumulation of the wild-type snR5 and the mutant snR36-*S1* and snR36-*S2* box H/ACA snoRNAs.

The H box was shown to be essential for the accumulation of intron-encoded H/ACA snoRNAs (Ganot *et al*., 1997b). We tested whether this motif is also required for accumulation of box H/ACA snoRNAs which are transcribed from their independent genes. When the wild-type H motif (AGACCA) of the yeast *SNR5* was replaced with a mutant H box (gGgCCA) (Figure 1A), no snR5 accumulation was detected in the transformed ΔsnR5 strain (lane 4). This demonstrates that the H box is equally important for accumulation of both the intron-borne and indepedently transcribed box H/ACA snoRNAs and that the 5′-terminal cap structure cannot compensate the lack of an intact H box. The yeast snR36, like many other yeast H/ACA snoRNAs, carries a short stem–loop structure (*IH1*) inserted into the major 5′ hairpin domain (Figure 1B) (Ganot *et al*., 1997b). However, deletion of the *IH1* element of the intron-encoded snR36 (snR36-Δ*IH1*) did not interfere with maturation of snR36 (Figure 1B, lane 2), indicating that the *IH1* element does not contribute to the correct processing of the snoRNA.

Position of the H box determines the 5′ terminus of intron-encoded box H/ACA snoRNAs

In H/ACA snoRNAs that are processed from pre-mRNAs or polycistronic pre-snoRNAs, the 5′-terminal stem is either followed by the H box immediately or they are separated by one or two nucleotides (Figure 2A). SnoRNAs that lack spacer nucleotides always carry one unpaired 5′-terminal nucleotide, while snoRNAs with one or two spacer nucleotides feature two free 5′-terminal nucleotides, suggesting that the position of the H box relative to the 5′-terminal stem determines the 5′ terminus of the snoRNA. The snR36 possesses two potential H box motifs that are separated by one or two nucleotides from the 5′ stem (Figure 2A). Nevertheless, utilization of either one of these motifs is expected to result in two unpaired 5′-terminal nucleotides. Wild-type and mutant snR36 RNAs carrying H box motifs with altered positions

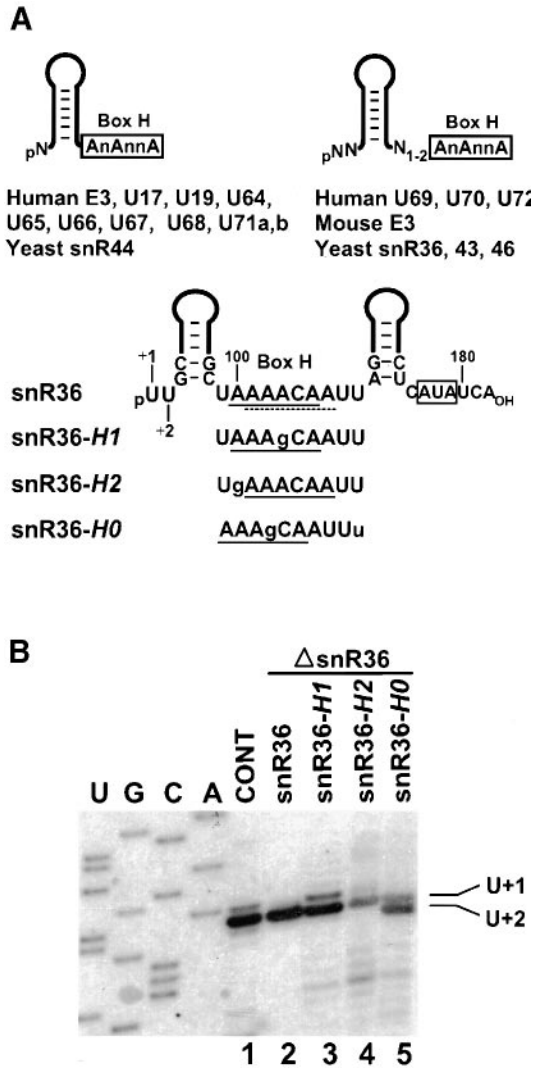

Fig. 2. The position of the H box determines the 5′ terminus of intron-encoded snR36 RNA. (**A**) Compilation of the 5′-terminal structures of 5′ end-processed human and yeast H/ACA snoRNAs. The 5′ termini of the listed snoRNAs have been mapped by primer extension. Schematic structure of the wild-type and modified snR36 RNAs inserted into the intron region of the pFL45/ACT expression construct. The putative H box sequences are underlined by continuous or dashed lines. (**B**) Analysis of the 5′ termini of snR36 RNAs processed from the actin pre-mRNA. RNAs extracted from the control CMY133 strain (lane 1) or from the ΔsnR36 strain transformed with either the pFL45/ACT/SNR36 (lane 2), pFL45/ACT/SNR36-*H1* (lane 3), pFL45/ACT/SNR36-*H2* (lane 4) or pFL45/ACT/SNR36-*H0* (lane 5) construct were analysed by primer extension using a primer complementary to the wild-type snR36 RNA from position 34 to 53. Dideoxynucleotide sequencing ladders generated with the same primer on the pFL45/ACT/SNR36 expression plasmid were run in parallel.

(Figure 2A) were expressed in the ΔsnR36 strain and their 5′ termini were mapped by primer extension (Figure 2B). As expected, for the wild-type snR36 and the mutant snR36-*H1* and snR36-*H2* RNAs in which the H box was placed unambiguously one or two nucleotides far from the 5′ stem, respectively, the major stop was observed at position U +1 (Figure 2B, lanes 2–4). However, when the H box was placed in the immediate vicinity of the 5′ stem (snR36-*H0*), the major transcription stop identified the U +2 residue as the 5′-terminal nucleotide (Figure 2B, lane 5). Northern analyses showed that the 3′ terminus of the U65-*H0* snoRNA was processed correctly (data not shown). This demonstrates that the H box, most likely in concert with the 5′-terminal helix, delineates the correct 5′ terminus of box H/ACA snoRNAs that are processed from precursor RNAs.

Human U65 snoRNA directs site-specific pseudouridylation of yeast 25S rRNA

The 5′ and 3′ hairpins of yeast snR34 and human U65 snoRNAs carry two pseudouridylation pockets that are predicted to direct pseudouridylation of yeast 25S and human 28S rRNAs at two equivalent positions, at Ψ2822/Ψ2876 and Ψ4374/Ψ4428, respectively (Ganot *et al*., 1997a; Ni *et al*., 1997) (Figure 3A). To test whether the human U65 can support pseudouridylation of yeast 25S rRNA, the U65 RNA was placed under the control of the *SNR5* promoter and transformed into the ΔsnR34 yeast strain (Ni *et al*., 1997). Northern analysis of cellular RNAs isolated from the resulting U65 (Figure 3B, lane 4) and the control CMY133 and ΔsnR34 yeast strains (lanes 2–3), as well as from human HeLa cells (lane 1), showed that the human U65 snoRNA was expressed efficiently in yeast. Note that the 5′-terminal region of U65 transcribed from the *SNR5* promoter carries 11 leader nucleotides.

The state of pseudouridylation of the 25S rRNA was monitored by primer extension performed on CMC-treated cellular RNAs. CMC reacts irreversibly with pseudouridines, and the modified pseudouridine-CMC residue stops reverse transcriptase one nucleotide before the pseudouridylation site (Bakin and Ofengand, 1993). Predictably enough (Ganot *et al*., 1997a; Ni *et al*., 1997), disruption of the *SNR34* locus abolished pseudouridylation of the 25S rRNA at Ψ2822 (Figure 3B, compare lanes 1 and 2) and Ψ2876 (compare lanes 4 and 5). However, expression of the human U65 RNA in the ΔsnR34 cells restored pseudouridylation of 25S rRNA at Ψ2822 (Figure 3B, lane 3) and Ψ2876 (lane 6). These results demonstrate that the U65 snoRNA represents the human orthologue of yeast snR34 and, more importantly, structural elements required for the function of pseudouridylation guide snoRNAs are conserved between humans and yeast.

Neither the 5′ nor the 3′ hairpin domain of U65 can direct rRNA pseudouridylation alone

Since the H and ACA boxes and the 5′- and 3′-terminal helical stems are essential for accumulation of H/ACA snoRNAs, the function of these elements in the rRNA pseudouridylation reaction cannot be investigated directly by mutational analyses. Recently, we have shown that the human intron-encoded U24 box C/D snoRNA, when expressed in yeast cells, is processed correctly and packaged into a functional snoRNP particle (Kiss-László *et al*., 1998). Therefore, a U65/U24 fusion snoRNA was constructed and placed under the control of the *SNR5* promoter (Figure 4A), anticipating that the U65/U24 transcript, even if carrying mutant U65 sequences, would be stable in yeast cells due to the presence of the 5′-terminal cap structure and the 3′-terminal U24 snoRNP particle. Indeed, Northern blot analyses of cellular RNAs obtained from the ΔsnR34 strain transformed with the pFL45/SNR/65/U24 construct showed that the U65/U24 snoRNA accumulated efficiently (Figure 4B, lane 2). Moreover, mapping of rRNA pseudouridylation demonstrated that

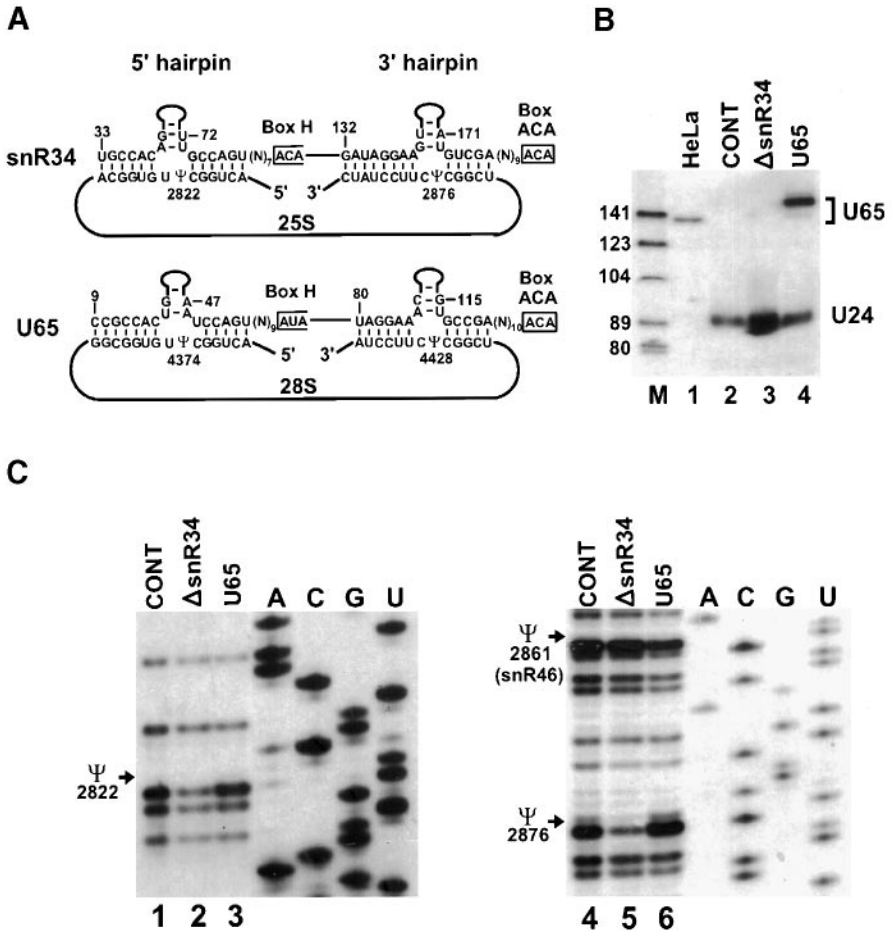

Fig. 3. Expression of human U65 snoRNA in yeast. (**A**) Selection of ribosomal pseudouridine residues (Ψ) by the human U65 and yeast snR34 snoRNAs. The upper parts of the 5′ and 3′ hairpins of U65 and snR34 are shown schematically. (**B**) Northern analysis of expression of human U65 snoRNA. RNAs extracted from human HeLa (lane 1) and yeast CMY133 cells (lane 2) as well as from yeast ΔsnR34 cells either transformed (lane 4) or not (lane 3) with the pFL45/SNR/U65 construct were separated on a 6% sequencing gel and probed with a mixture of labelled oligonucleotides specific to the human U65 and yeast U24 snoRNAs. Lane M, size markers. (**C**) Primer extension mapping of pseudouridine residues. CMC-alkali-modified cellular RNAs obtained from the control CMY133 (lanes 1 and 4) and ΔsnR34 cell lines (lanes 2 and 5) or from ΔsnR34 cells expressing the human U65 snoRNA (lanes 3 and 6) were annealed with labelled oligonucleotides complementary to the 25S rRNA from positions 2835 to 2855 (lanes 1–3) or 2889 to 2910 (lanes 4–6). The extended products were separated on a 6% sequencing gel. Lanes A, C, G and U represent dideoxy sequencing reactions.

expression of the U65/U24 snoRNA in the ΔsnR34 strain restored the wild-type levels of pseudouridylation of yeast 25S rRNA at Ψ2822 (Figure 4C, lanes 1–3) and Ψ2876 (lanes 4–6). This demonstrates that the U65/U24 fusion snoRNA is not only stable but also functional in yeast cells.

The 5′-terminal hairpin (5′hp) followed by the H box motif and the 3′-terminal hairpin (3′hp) with the ACA box seem to represent two elements that are structurally and perhaps functionally equivalent. To test whether either of these domains can direct rRNA pseudouridylation independently, the 5′- or 3′-terminal domain of the human U65 RNA, U65-5′hp and U65-3′hp, respectively, were fused to the U24 RNA and expressed in the ΔsnR34 strain (Figure 5A). Although the two chimeric RNAs accumulated efficiently, in contrast to the U65/U24 RNA (Figure 5B, lanes 3 and 8), neither U65-5′hp/U24 (lanes 4 and 9) nor U65-3′hp/U24 (lanes 5 and 10) could restore pseudouridylation of 25S rRNA at Ψ2822 or Ψ2876. This demonstrates that the presence of both the 5′hp and 3′hp is essential not only for snoRNA accumulation, but also for the rRNA pseudouridylation reaction.

Intact H and ACA boxes are required for rRNA pseudouridylation

In the rRNA–guide RNA interaction, the distance between the pseudouridylation site and the H or ACA box of the snoRNA shows a remarkable conservation. Normally, they are separated by 14 nucleotides (Ganot *et al*., 1997a; Ni *et al*., 1997). Alteration of the distance between the ACA box of the yeast snR8 and the target uridine residue resulted in either reduced pseudouridine synthesis at the correct site and/or partial modification of an adjacent uridine (Ni *et al*., 1997). To test directly the importance of the H and ACA boxes in the pseudouridylation reaction, the wild-type H (AUAGUA) or ACA box sequences of the U65/U24 fusion snoRNA were replaced with the gggGgg and cCc mutant sequences, respectively (Figure 6A). The resulting U65-*H*/U24 and U65-*ACA*/U24 RNAs accumulated efficiently when expressed in the ΔsnR34 strain (Figure 6A, lanes 3 and 4).

The state of pseudouridylation of 25S rRNA derived from the control CMY133, the ΔsnR34, U65/U24, U65-*H*/U24 and U65-*ACA*/U24 strains was monitored by primer

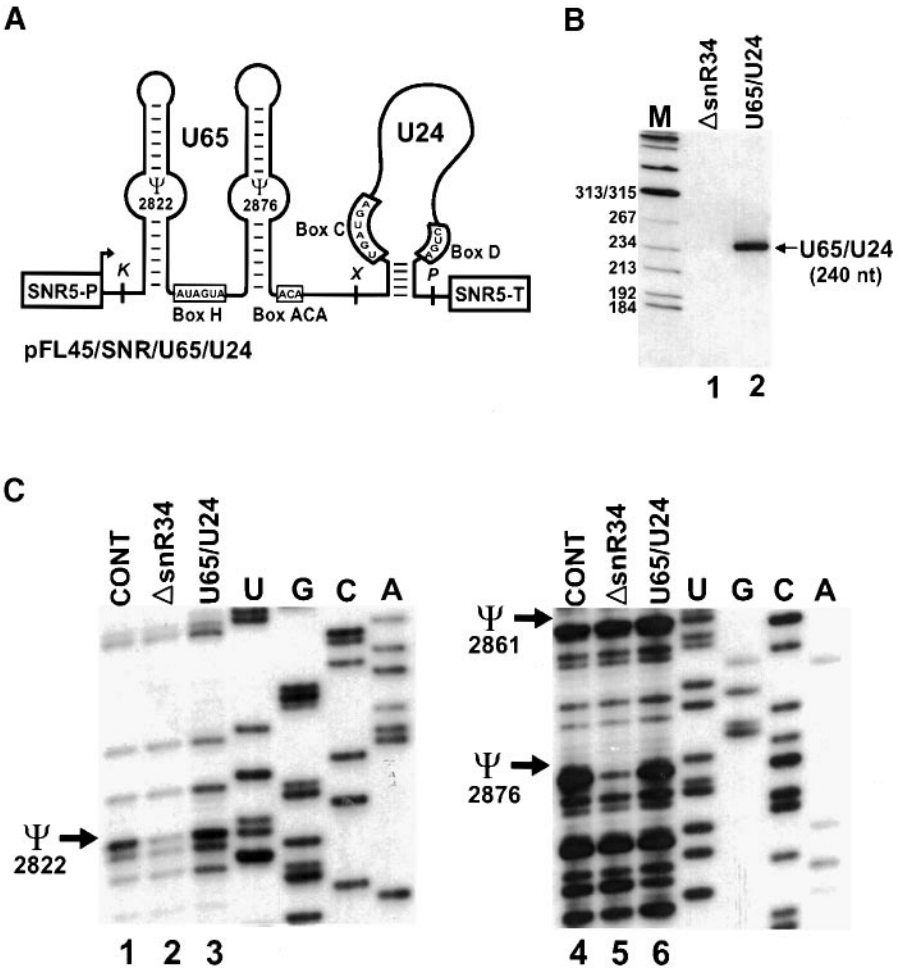

Fig. 4. Expression of fused U65 and U24 snoRNAs in yeast. (**A**) Structure of the pFL45/SNR/U65/U24 expression construct. The schematic secondary structure of human U65 and U24 snoRNAs together with the conserved H, ACA, C and D boxes are shown. Relevant restriction sites are indicated (K, *Kpn*I; X, *Xho*I; P, *Pst*I). The promoter (SNR5-P) and terminator (SNR5-T) regions of the expression construct are from the yeast *SNR5* gene. (**B**) Northern analysis of expression of human U65/U24 fusion RNA. Cellular RNAs obtained from the ΔsnR34 (lane 1) or U65/U24 yeast strain (lane 2) were probed with a U65-specific oligonucleotide. (**C**) Mapping of pseudouridylation of yeast 25S rRNA at Ψ2822 and Ψ2876. For other details, see legend to Figure 3C.

extension (Figure 6B). The U65-*H*/U24 RNA that carried a mutant H box motif failed to direct pseudouridylation of the 25S rRNA at Ψ2822 and, to our surprise, also at Ψ2876 (Figure 6B, lane 9). Likewise, lack of an intact ACA box in the U65-*ACA*/U24 RNA inhibited the pseudouridine synthesis at both Ψ2876 (Figure 6B, lane 10) and Ψ2822 (lane 5), demonstrating that the H and ACA boxes play equally important roles in the pseudouridylation reactions directed by both the 5′- and 3′-terminal pseudouridylation pockets.

Helical stems bracketing the pseudouridylation pockets are essential for rRNA pseudouridylation

In the two hairpins of box H/ACA snoRNAs, the pseudouridylation pockets are always flanked by two well-defined stem structures (Ganot *et al.*, 1997b) (Figure 7A). While the stems at the bases of the 5′ and 3′ hairpins (stems B) are essential for snoRNA accumulation (Balakin *et al.*, 1996; this study), helices above the pseudouridylation pockets (stems U) do not contribute significantly to the stability of the snoRNA (M.-L.Bortolin, P.Ganot and T.Kiss, unpublished results). Nonetheless, we investigated whether the lower (B) and upper (U) helical stems flanking the 5′ and 3′ pseudouridylation pockets of the U65 snoRNA are essential for rRNA pseudouridylation. To this end, these stems were destroyed in the U65/U24 fusion snoRNA by substitution of the descending (3′ side) strands of each stem with non-complementary sequences (d). In the next step, the base-pairing for each helix was restored by substitution of the 5′ side of the stems with appropriate complementary sequences (r). Northern analysis demonstrated that upon transformation of the resulting pFL45/SNR/U65/U24 constructs into the ΔsnR34 strain, all the mutant U65/U24 snoRNAs were expressed.

We tested whether the mutant U65 snoRNAs can restore the pseudouridylation of 25S rRNA at Ψ2822 and Ψ2876 in the ΔsnR34 strain (Figure 7B). When the upper or lower stem in the 5′ hairpin of U65 was destroyed (U65-5′Ud and U65-5′Bd), pseudouridylation of 25S rRNA was abolished at Ψ2822 (Figure 7B, lanes 4 and 6) and, to our surprise, also at Ψ2876 (lanes 11 and 13). Restoration of these stem structures in the U65-5′Ur and U65-5′Br RNAs re-established the pseudouridylation of 25S rRNA both at Ψ2822 (Figure 7B, lanes 5 and 7) and at

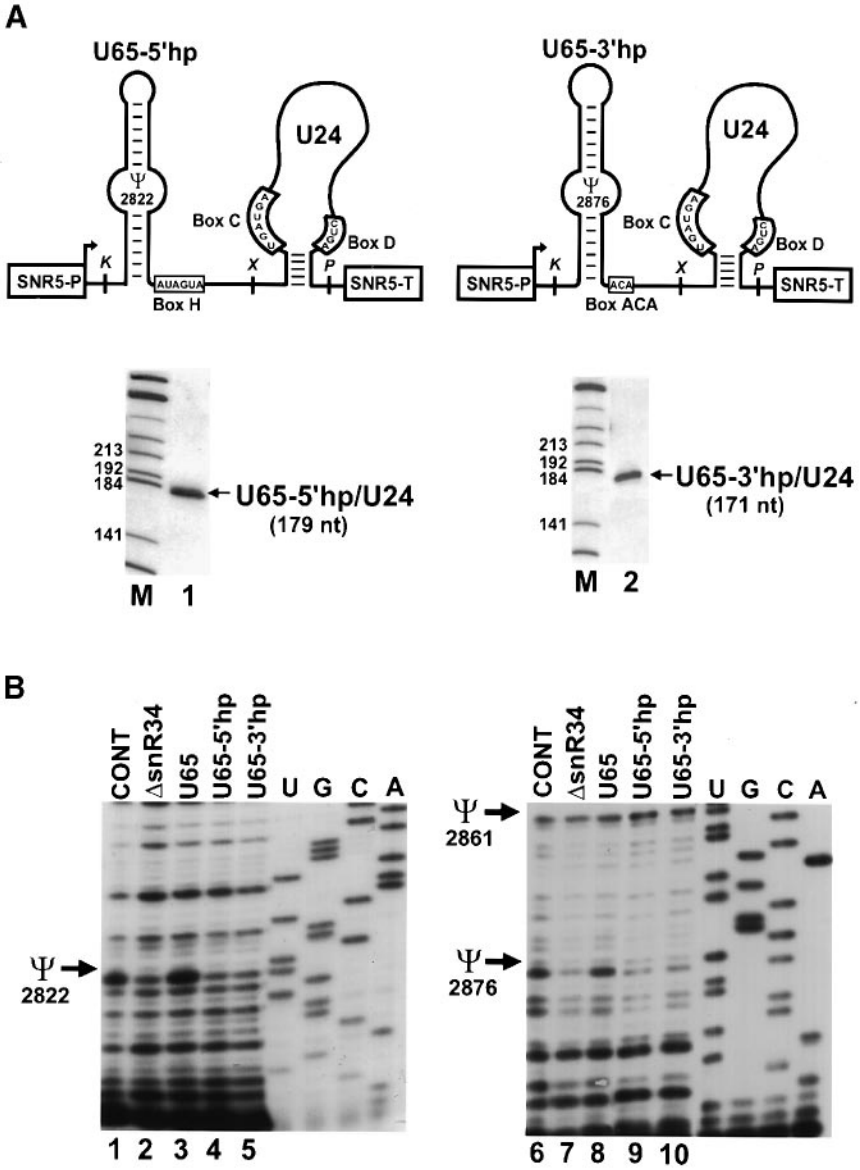

Fig. 5. Neither the 5′- nor the 3′-terminal hairpin of U65 mediates rRNA pseudouridylation. (**A**) Expression of U65-5′hp/U24 and U65-3′hp/U24 chimeric snoRNAs in the ΔsnR34 yeast strain. The structures of the pFL45/SNR/U65-5′hp/U24 and pFL45/SNR/U65-3′hp/U24 expression constructs are shown. While the leader sequences of the U65-5′hp/U24 and U65-3′/U24 RNAs are 10 nucleotides long, the spacer regions including the H and ACA boxes consist of 23 or 19 nucleotides, respectively. Accumulation of the chimeric snoRNAs was detected by Northern analysis. (**B**) Mapping of pseudouridylation of 25S rRNAs at Ψ2822 and Ψ2876. Pseudouridylation of 25S rRNAs obtained from the CMY133 (lanes 1 and 6) and ΔsnR34 (lanes 2 and 7) control strains and the U65 (lanes 3 and 8), U65-5′hp/U24 (lanes 4 and 9) or U65-3′hp/U24 (lanes 5 and 10) strains were monitored by primer extension analyses. For other details, see legends to Figures 3C and 4C.

Ψ2876 (lanes 12 and 14). Similar results were obtained upon mutation of the stem structures flanking the 3′ pseudouridylation pocket of U65. Disruption of either the upper (U65-3′Ud) or the lower stem (U65-3′Bd) had detrimental effects on pseudouridine formation at both Ψ2822 (Figure 7B, lanes 18 and 20) and Ψ2876 (lanes 25 and 27). When base-pairing interactions were re-established, the resulting U65-3′Ur and U65-3′Br RNAs restored pseudouridine formation at Ψ2822 (Figure 7B, lanes 19 and 21) and Ψ2876 (lanes 26 and 28). These observations demonstrate that the helical stems bracketing the 5′ and 3′ pseudouridylation pockets of box H/ACA snoRNAs are essential for the rRNA pseudouridylation reaction.

Targeted pseudouridylation of yeast 25S rRNA directed by artificial guide snoRNAs

To select a ribosomal pseudouridylation site, two short sequence motifs in the pseudouridylation pocket of the guide RNA were proposed to form double helices with rRNA sequences that flank the substrate uridine (Ganot *et al*., 1997a). The importance of this interaction has been confirmed by showing that alteration of rRNA recognition motifs of the yeast snR36 and snR8 interferes with the rRNA pseudouridylation reactions directed by these snoRNAs (Ganot *et al*., 1997b; Ni *et al*., 1997). However, it remains unclear whether the two short rRNA recognition motifs provide all the information for selection of a unique pseudouridylation site in pre-rRNA. To address

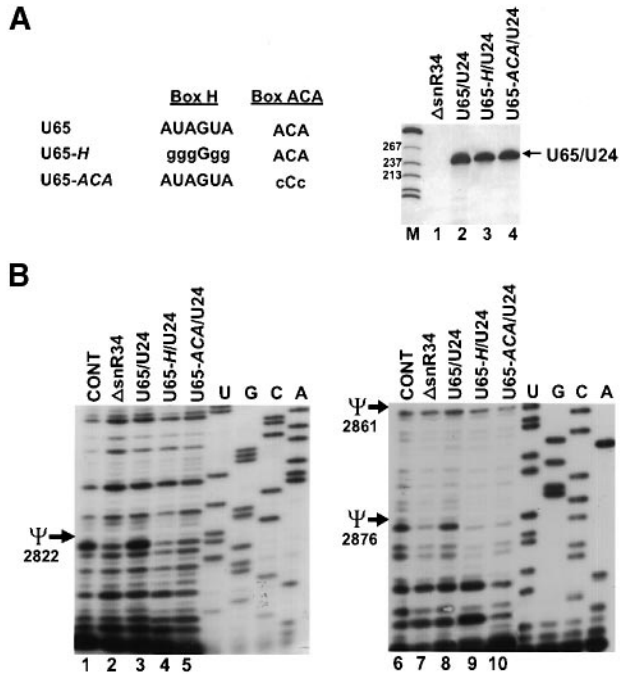

Fig. 6. Effects of alteration of box H and ACA sequences of U65. (**A**) Expression of U65/U24 fusion snoRNAs carrying altered box H or ACA motifs. The altered nucleotides in the H box of U65-*H* and in the ACA box of U65-*ACA* RNAs are indicated by lower case letters. RNAs from the ΔsnR34 (lane 1) and its derivative strains transformed with either the pFL45/SNR/U65/U24 (lane 2), pFL45/SNR/U65-H/U24 (lane 3) or pFL45/SNR/U65-ACA/U24 (lane 4) construct were subjected to Northern analysis. (**B**) Primer extension mapping of pseudouridylation of 25S rRNAs extracted from the CMY133 (lanes 1 and 6) and ΔsnR34 control strains and from the derivatives of the ΔsnR34 strain expressing either the U65/U24 (lanes 3 and 8), U65-*H*/U24 (lanes 4 and 9) or U65-*ACA*/U24 (lanes 5 and 10) fusion snoRNAs. Other details are identical to Figures 3C and 4C.

this question, two slightly altered versions of the yeast snR36 pseudouridylation guide snoRNA, snR36-m1 and snR36-m2, were created (Figure 8A). The rRNA recognition motif of snR36 that directs the synthesis of Ψ1185 in the 18S rRNA (Ganot *et al*., 1997a) was substituted for sequences that, in principle, could select the U2871 residue in the 25S rRNA. The snR31-m1 and snR36-m2 RNAs carried identical recognition motifs except that the potential snoRNA–rRNA interaction region was extended by one base pair for the snR36-m2 RNA. The wild-type snR36 and the mutant snR36-m1 and snR36-m2 snoRNA were expressed from the SNR5 promoter in the ΔsnR36 strain (Figure 8A).

The state of pseudouridylation of 25S rRNA obtained from the control and ΔsnR36 strains as well as from the ΔsnR36 strain expressing either the wild-type snR36 or the mutant snR36-m1 or snR36-m2 snoRNA was assayed (Figure 8B). Expression of the snR36-m2 (Figure 8B, lane 1) and snR36-m1 (lane 2) snoRNAs resulted in conversion of the U2871 residue into pseudouridine. Clearly, this pseudouridine was not detectable in the control (Figure 8B, lane 5), ΔsnR36 (lane 4) and snR36 (lane 3) strains. Contrary to expectations, the extended rRNA recognition motif of the snR36-m2 snoRNA did not improve the efficiency of pseudouridine synthesis. However, as compared with a known pseudouridylation site in the yeast 25S rRNA at Ψ2861, it was apparent that the U2871 residue was converted into pseudouridine with only 40–50% efficacy. Similar results were obtained when another novel pseudouridylation site was introduced into the U2976 position of the yeast 25S rRNA (data not shown). Since snoRNA-guided pseudouridylation sites are found in both helical and single-stranded regions of rRNAs, it seems unlikely that the local structural environment of the substrate uridine would greatly alter the efficiency of the pseudouridine synthesis reaction (Ofengand *et al*., 1995; Ofengand and Bakin, 1997). We therefore propose that in the catalytic centre of these artificial snoRNPs, the substrate uridines occupy a suboptimal sterical position that impairs the isomerization reaction. It is also unlikely that additional, not yet identified snoRNA–rRNA interactions would contribute to the substrate recognition event. This conclusion is supported by the facts that, apart from the short rRNA recognition motifs in the pseudouridylation pockets, no obvious conservation can be found between the yeast snR34 and the human U65 snoRNAs and, more importantly, ribosomal target sequences as short as 12 nucleotides are pseudouridylated efficiently when expressed in the nucleolus (Ganot *et al*., 1997a). Hence, we conclude that the substrate specificity of the rRNA pseudouridylation reaction mediated by the box H/ACA snoRNAs is provided exclusively by the rRNA recognition motif of the snoRNA.

Discussion

The nucleolar maturation of eukaryotic rRNAs is assisted by an unexpectedly complex population of snoRNAs (Smith and Steitz, 1997; Tollervey and Kiss, 1997). While a few snoRNAs are required for the nucleolytic formation of mature-sized rRNAs (Maxwell and Fournier, 1995; Sollner-Webb *et al*., 1996), most of them direct the site-specific 2′-*O*-ribose methylation (reviewed in Maden, 1996; Peculis and Mount, 1996; Tollervey, 1996; Bachellerie and Cavaillé, 1997; Tollervey and Kiss, 1997) or pseudouridylation (reviewed by Maden, 1997, Peculis, 1997; Smith and Steitz, 1997) of rRNAs. We report here a comprehensive analysis of the structural elements essential for accumulation and function of rRNA pseudouridylation guide snoRNAs.

The pseudouridylation guide snoRNAs feature a highly conserved ‘hairpin–hinge–hairpin–tail’ secondary structure with two conserved sequence motifs, the H and ACA boxes. Previous works (Balakin *et al*., 1996; Ganot *et al*., 1997b) together with this study (Figure 1A) demonstrate that the box H and ACA motifs are absolutely required for accumulation of both mammalian and yeast H/ACA snoRNAs. These single-stranded sequence motifs most likely represent protein-binding signals that are recognized by snoRNP proteins common to this class of snoRNPs (Henras *et al*., 1998; Watkins *et al*., 1998). However, it seems very unlikely that either the H (consensus AnAnna) or the ACA (consensus AcA) motif alone could provide sufficient information for binding of snoRNP proteins. Supporting this assumption, the H and ACA boxes are always located in the close vicinity of the 5′- or 3′-terminal helical stems, respectively, that are also required for snoRNA accumulation (Balakin *et al*., 1996; Figure 1B). Alteration of the distance between the ACA box and the 3′-terminal stem interferes with the accumula-

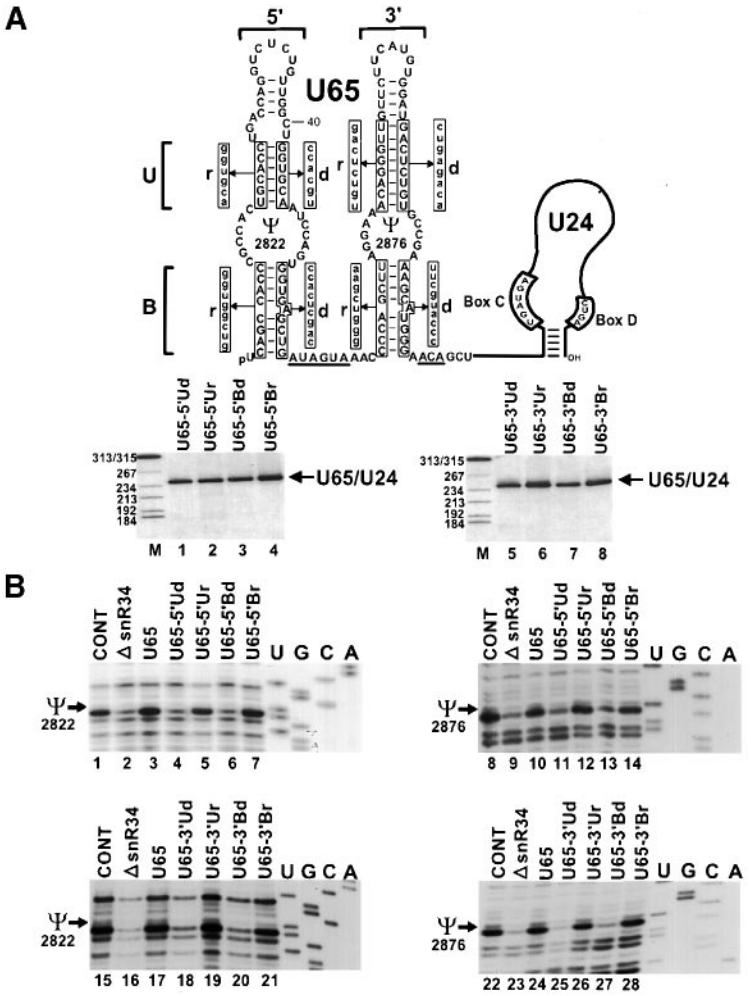

Fig. 7. Effects of destruction of helical stems delineating the 5′- and 3′-terminal pseudouridylation pockets of U65. (**A**) Expression of modified U65/U24 fusion snoRNAs in the ΔsnR34 yeast strain. The sequence and secondary structure of the wild-type U65 snoRNA are shown. Sequences introduced to destroy (d) or restore (r) the lower (B) or upper (U) helical stems in the 5′- or 3′-terminal hairpins of U65 are shown in lower case letters. The H and ACA boxes of U65 are underlined. Expression of the U65/U24 fusion snoRNAs was verified by Northern analysis. (**B**) Mapping of pseudouridylation of 25S rRNA. Pseudouridine formation in the yeast 25S rRNA at positions Ψ2822 (lanes 1–7 and 15–21) and Ψ2876 (lanes 8–14 and 22–28) was monitored by primer extension analysis of cellular RNAs obtained from the control CMY133 and ΔsnR34 strains or from derivatives of the ΔsnR34 strain expressing various U65/U24 fusion snoRNAs as indicated above the lanes. For other details, see legend to Figure 3.

tion of yeast snR11 RNA (Balakin *et al*., 1996). In this study, we demonstrate that the position of the H box relative to the 5′-terminal stem determines the 5′ end of the intron-encoded yeast snR36 (Figure 2). These observations strongly support the notion that the H box together with the 5′-terminal stem, and the ACA box in concert with the 3′-terminal stem, constitute the recognition signals for snoRNP proteins. Most probably, snoRNP proteins associated with the 5′- and 3′-terminal 'stem–box' structural motifs protect the snoRNA sequence from the processing exonucleases and, thereby, control the correct 5′ and 3′ end formation (Balakin *et al*., 1996, Ganot *et al*., 1997b).

In yeast, the majority of H/ACA snoRNAs are synthesized from independent transcription units by pol II. Selection of the transcription initiation site and the co-transcriptionally added 5′ cap determines the 5′ terminus of these RNAs and, therefore, they undergo maturation only at their 3′ ends. The other group of H/ACA snoRNAs that are processed from intronic or polycistronic pre-snoRNA transcripts undergo both 5′ and 3′ end maturation. Apparently, the basic structural requirements for accumulation, such as the presence of the 5′- and 3′-terminal stems and the H/ACA boxes, are identical for both groups of snoRNAs. However, our results demonstrate that the steric structure of the 5′ and 3′ end-processed snoRNAs has to conform to more rigorous requirements. When transcribed within the intron of the yeast actin pre-mRNA, neither the yeast snR5 that is normally transcribed by pol II from its own gene (Figure 1A) nor the human intron-encoded E3, U17, U19, U64 and U65 (our unpublished results) snoRNAs accumulated in yeast cells. However, when expressed under the control of the *SNR5* promoter, all these snoRNAs accumulated efficiently (Figures 1 and 3; data not shown). It is notable that the human U65, when it was synthesized by pol II and carried a 5′-terminal m^3G cap, not only accumulated, but also directed the pseudouridylation of yeast rRNA (Figure 3). Moreover, accumulation of a mutant version of the intron-processed snR36 RNA carrying two unpaired nucleotides in its 5′-terminal stem was rescued when it was transcribed from the *SNR5* promoter and possessed a m^3G cap (Figure

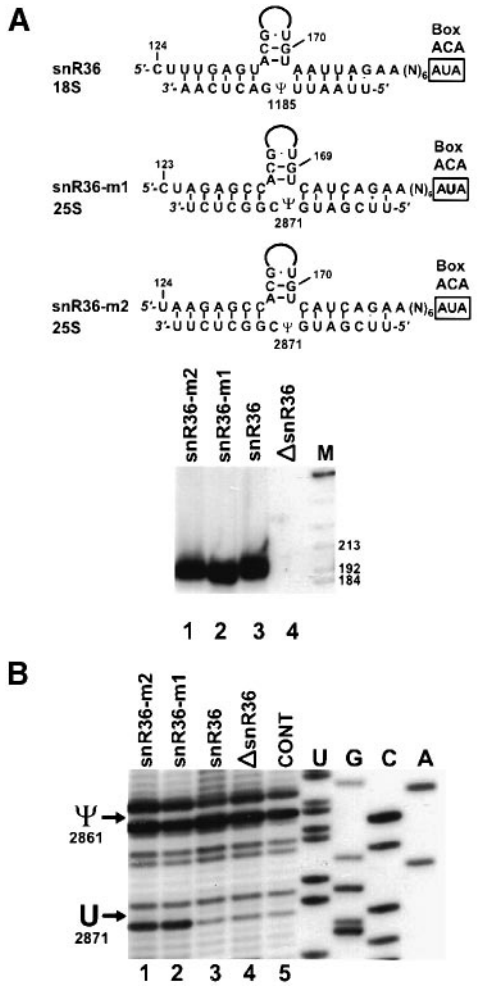

Fig. 8. Targeted pseudouridylation of yeast 25S rRNA. (**A**) Selection of ribosomal pseudouridylation sites by the wild-type and modified yeast snR36 snoRNA carrying altered rRNA recognition sequences. Ribosomal uridines known or expected to be converted into pseudouridine are indicated (Ψ). Accumulation of the wild-type snR36 (lane 3) and the modified snR36-m1 (lane 2) and snR36-m2 (lane 1) snoRNAs in the ΔsnR36 strain was verified by Northern analysis. (**B**) Mapping of pseudouridylation of yeast 25S rRNA. Isomerization of the U2871 residue to pseudouridine was assayed by primer extension analysis of CMC-treated cellular RNAs obtained from the CMY133 (lane 5), ΔsnR36 (lane 4), snR36 (lane 3), snR36-m1 (lane 2) or snR36-m1 yeast strains.

1B). Collectively, our observations suggest that the 5′-terminal cap structure, through stabilization of the snoRNA transcripts, contributes to the efficient accumulation of box H/ACA snoRNAs in yeast cells.

Thus far, four common snoRNP proteins, Gar1p, Nhp2p, Cbf5p and Nop10p, have been identified for yeast H/ACA snoRNPs (Balakin *et al*., 1996; Ganot *et al*., 1997b; Henras *et al*., 1998; Lafontaine *et al*., 1998; Watkins *et al*., 1998). Since the Cbf5 protein shows striking structural similarities to known pseudouridine synthases (Koonin, 1996; Watkins *et al*., 1998), it is most probably the enzyme that is responsible for the synthesis of ribosomal pseudouridines (Lafontaine *et al*., 1998). Therefore, each H/ACA snoRNP particle can be considered as a site-specific pseudouridine synthase. While the Cbf5p provides the catalytic activity, the snoRNA component of the particle provides the specificity for the rRNA pseudouridylation reaction. Indeed, demonstration that novel pseudouridines can be introduced into the yeast 25S rRNA by manipulating the rRNA recognition motif of pseudouridylation guide snoRNAs (Figure 8) proves that all the information necessary to select the correct pseudouridylation sites is carried by the RNA component of the snoRNP particle. These experiments also provide direct evidence that pseudouridylation guide snoRNAs select the target uridines by forming direct Watson–Crick base-pairing interactions with the target rRNA sequences.

The two major structural domains of box H/ACA snoRNAs, the 5′hp followed by the H box and the 3′hp together with the ACA box, share striking structural and functional similarities. Pseudouridylation pockets are found equally frequently in the 5′hp and 3′hp and, even more tellingly, many snoRNAs carry pseudouridylation pockets in both the 5′hp and 3′hp domains (Ganot *et al*., 1997a). It has been documented experimentally that the 5′ and 3′ pseudouridylation pockets of yeast snR5, snR34 and human U65 snoRNAs can direct pseudouridylation of rRNAs at two different positions (Ganot *et al*., 1997a; Figure 3). The H and the ACA boxes are located normally ~14 nucleotides downstream of the catalytic centre of the corresponding pseudouridylation pocket in the 5′ or 3′ hairpin, respectively (Ganot *et al*., 1997a; Ni *et al*., 1997). Alteration of the wild-type spacing between the ACA box and the 3′ catalytic centre of yeast snR8 impairs the efficiency and correctness of rRNA pseudouridylation directed by the 3′ pseudouridylation pocket of this snoRNA (Ni *et al*., 1997). This shows that for selection of the correct ribosomal uridine, in addition to the snoRNA–rRNA base-pairing interaction, the pseudouridine synthase activity also relies on the position of the ACA box relative to the catalytic centre of the snoRNA. Although not yet experimentally supported, it is easy to imagine that the H box that is located 14 nucleotides downstream from the 5′ pseudouridylation centre possesses a function analogous to that of the ACA box.

Demonstration that the yeast snR5, snR34 and the human U65 snoRNPs, and probably many others, possess two independent catalytic centres for rRNA pseudouridylation implies that these snoRNPs carry two copies of the Cbf5p pseudouridine synthase (Ganot *et al*., 1997a; Figure 3). Moreover, the notion that the 5′- and 3′-terminal domains of these snoRNAs are functionally equivalent presupposes that they bind the same set of snoRNP proteins. This view was strongly supported by recent purification of yeast snR42 and snR30 box H/ACA snoRNPs (Watkins *et al*., 1998). The isolated snoRNP core particles contained three common snoRNP proteins, the Gar1p, Nhp2p and Cbf5p. Electron microscopy revealed a highly symmetric bipartite structure for these complexes and, intriguingly, predicted a molecular mass that would be consistent with a particle consisting of a snoRNA and two copies of each of the Gar1, Nhp2 and Cbf5 proteins. The detailed architecture of box H/ACA snoRNPs remains to be understood. Since the Cbf5p lacks an apparent RNA-binding motif, it seems unlikely that it would bind directly to the snoRNA. Another H/ACA snoRNP protein, the Nhp2p, would be a more likely candidate to bind to box H/ACA snoRNAs, since it contains an RNA-binding motif also present in ribosomal proteins (Koonin *et al*., 1994; Watkins *et al*., 1998). The

Gar1 snoRNP protein, although it has been reported to interact *in vitro* with snR10 and snR30 snoRNAs (Bagni and Lapeyre, 1998), seems to bind to the snoRNP particle through interaction with the Cbf5p (Henras *et al*., 1998).

The 5′hp and 3′hp domains of box H/ACA snoRNPs act apparently in a highly co-operative manner. Destruction of any of the functionally essential elements—the H or the ACA box and the helical stems bracketing the pseudouridylation pockets either in the 5′ or 3′ hairpin—impeded rRNA pseudouridylation mediated by both the 5′- and 3′-terminal pseudouridylation centres (Figures 6 and 7). We envisage that to construct a functional snoRNP complex, a direct or perhaps an adaptor protein-mediated interaction is required between the two sets of snoRNP proteins which are bound to the 5′ or the 3′ hairpin domain of the snoRNA. Of course, this model would also explain why H/ACA snoRNAs that possess only one functional pseudouridylation pocket still contain two hairpin domains. The hairpin element that lacks a pseudouridylation pocket is required to provide scaffolding for snoRNP proteins to construct the functionally active bipartite structure of the snoRNP.

Similarly to pseudouridylaton guide snoRNAs, many 2′-*O*-methylation guide snoRNAs feature two rRNA methylation centres (Kiss-László *et al*., 1998). The two rRNA methylation domains consist of an rRNA complementary sequence that is followed by either the C/D or C′/D′ box motifs. Interestingly, this bipartite structural organization is preserved even in those methylation guide snoRNAs which do not contain an RNA recognition motif next to the internal C′/D′ boxes (Kiss-Lászó *et al*., 1998). At present, the significance of these intriguing structural and functional parallels drawn between the rRNA methylation and pseudouridylation guide snoRNAs is nebulous. However, it might underscore further the notion that the two major classes of eukaryotic snoRNAs evolved from a common ancestor molecule (Ganot *et al*., 1997b). In the future, an understanding of the molecular mechanisms of the snoRNA-directed rRNA modification reactions will provide us with more insights into the complex world of small nuclear RNAs and may also facilitate the understanding of other RNA-guided processes, such as certain RNA editing mechanisms.

Materials and methods

General procedures

All procedures used for manipulating DNA, RNA and oligodeoxynucleotides were done according to standard laboratory techniques (Sambrook *et al*., 1989). The identity of all constructions was verified by sequence analyses. Growth and handling of *Saccharomyces cerevisiae* were done by standard techniques (Sherman, 1991). Construction of yeast strains ΔsnR5, ΔsnR34 and ΔsnR36 has been described (Ganot *et al*., 1997a; Ni *et al*., 1997). Expression plasmids were introduced into yeast cells using the lithium acetate transformation procedure (Ito *et al*., 1983). The following oligodeoxynucleotides were used in this study: (1) TATAAGCTTAATAGGAACTCATGGTG; (2) TATGGTACCTGATGGTTTTCTTATCCTGA; (3) TTTGGTACCTTTCTCGAGCTTCACTTCATTACTCTCTTGTTTAC; (4) TTTAGATCTATAATTGAAGTATATGTACG; (5) TTTGGTACCATCATTCAATAAACTGATC; (6) TTTCTCGAGATATGTACACCTAGAGCG; (7) TATGGTACCTTGCCCTGTGCCTCGCTCG; (8) TTACTCGAGTGATATGAGACGTTCTAATTA; (9) TTTGGTACCTTGAACTGTGCCTCGCTCG; (10) TATGTCGACGGGCTAAAACAATTAGACTTC; (11) AATTGTTTTAGAACGTTGATC; (12) CAACGGGCTAAAGCAATTAGACTTC; (13) CAACGGGCTGAAACAATTAGACTTC; (14) CAACGGGCAAAGCAATTTAGACTTC; (15) GGCTGGAAGTGGGCCAATTTTTTTTGTTCC; (16) TTTGGTACCTCAGCCACCCGCCACTGC; (17) ATTCTCGAGCTGTTCCCATGCTTTCGG; (18) CCGCTCGAGATGCGGCTTACTGTGCAGATGATGTAAAAG; (19) TTCCGCGGCCGCTATGGCCGACGTCGACTGCAGTGCATCAGCGATCTTGG; (20) ATAGACATATGGAGGCGTG; (21) CCAGCTCAAGATCGTAATAT; (22) GTTATTACATCATTTGA; (23) CCGCTCGAGCGGGTCCCATGCTTTCG; (24) ATGCTTTCGGCACAGAGTCATCC.

Construction of plasmids for transformation of yeast cells

Construction of yeast expression vectors pFL45/SNR5 and pFL45/ACT has been described (Ganot *et al*., 1997a). Construction of many plasmids relied on PCR amplification reactions. For each amplification reaction, hereafter, the 5′ end-specific primer is indicated first and it is followed by the 3′ end-specific primer. The pFL45/SNR expression cassette was obtained as follows. The promoter (oligos 1 and 2) and terminator (oligos 3 and 4) regions of the yeast *SNR5* gene were PCR amplified and, after *Hin*dIII–*Kpn*I and *Kpn*I–*Bgl*II digestions, respectively, were ligated and inserted into the *Hin*dIII and *Bam*HI sites of pFL45. To obtain pFL45/ACT/SNR5, pFL45/ACT/SNR36 and pFL45/SNR36, the cDNAs of snR5 and snR36 were PCR amplified using oligos 5 and 6 or oligos 7 and 8, respectively. The amplified fragments were digested by *Kpn*I–*Xho*I and inserted into the same sites of pFL/45/ACT and pFL45/SNR. The same approach was used to construct pFL45/SNR36-*S1*, except that a mutagenic 5′ end-specific primer (oligo 9) that contained appropriate base changes was used. To create pFL45/ACT/SNR36-Δ*IH1*, the 5′ (oligos 7 and 9) and 3′ (oligos 10 and 8) halves of snR36 were amplified, religated using the PCR-introduced *Sal*I site and inserted into the *Kpn*I and *Xho*I sites of pFL45/ACT.

Construction of cDNAs of snR36 or snR5 carrying altered H box motifs or 5′ stem sequences was achieved by the megaprimer amplification approach (Datta, 1995). To obtain snR36-*S2* and snR36-*S1*+*S2* cDNAs, the 5′-terminal region of snR36 was amplified using primers 7 and 11 or 9 and 11, respectively. In the second amplification step, the resulting fragments were used as megaprimers in combination with a common 3′ end-specific primer (oligo 8). The mutant snR36 cDNAs were inserted into the *Kpn*I–*Xho*I sites of pFL45/ACT and pFL45/SNR, resulting in pFL45/ACT/SNR36-*S2*, pFL45/ACT/SNR36-*S1*+*S2*, pFL45/SNR36-*S2* and pFL45/SNR36-*S1*+*S2*. To create snR36-*H1*, snR36-*H2* and snR36-*H0* cDNAs, megaprimers encompassing the 3′ half of snR36 were generated by using oligos 12, 13 and 14 as 5′-specific primers that carried altered H box motifs, respectively, and a common 3′ end-specific primer (oligo 8). In the second amplification reaction, a common 5′-specific primer (oligo 7) was used with the mutant megaprimers. The amplified cDNAs were inserted into the *Kpn*I–*Xho*I sites of pFL45/ACT. To obtain pFL45/SNR5-*H*, using oligos 15 and 4 and the pFL45/SNR5 plasmid as a template, the 3′ half of the *SNR5* gene was amplified and used as a megaprimer with oligo 1 in the second amplification reaction. The resulting snR5-*H* cDNA was digested by *Hin*dIII and *Bgl*II and inserted into the *Hin*dIII and *Bam*HI sites of pFL45.

To generate pFL45/SNR/U65, the human U65 snoRNA was PCR amplified using oligos 16 and 17, cut with *Kpn*I and *Xho*I and inserted into the same sites of pFL45/SNR. The cDNA of human U24 snoRNA was amplified (oligos 18 and 19), digested with *Xho*I and *Sal*I and cloned into the *Xho*I site of pFL45/SNR/U65, resulting in pFL45/SNR/U65/U24.

A series of mutant U65 cDNAs, U65-5′hp, U65-3′hp, U65-*H*, U65-*ACA*, U65-5′Ud, U65-5′Ur, U65-5′Bd, U65-5′Br, U65-3′Ud, U65-3′Ur, U65-3′Bd and U65-3′Br (Figures 5, 6 and 7), were constructed by PCR amplification using appropriate mutagenic oligonucleotides in combination with either a common 5′ end- (oligo 16) or 3′ end-specific primer (oligo 17). Structures of the mutagenic oligonucleotides are available upon request. The amplified cDNAs were digested with *Kpn*I and *Xho*I and were used to replace the wild-type U65 RNA sequences in pFL45/SNR/U65/U24.

RNA analyses

Yeast cellular RNAs were isolated by the guanidine thiocyanate/phenol–chloroform extraction method (Tollervey and Mattaj, 1987). For Northern analyses, if not stated otherwise, 10 μg of cellular RNAs were separated on a 6% sequencing gel, electroblotted onto a Hybond-N nylon membrane (Amersham) and probed with kinase-labelled oligonucleotides complementary to yeast snR5 (oligo 20), snR36 (oligo 21), U24 (oligo 22), and to either the 5′- (oligo 23) or 3′-terminal (oligo 24) sequences of human U65. Primer extension mapping of the 5′ terminus of snR36 RNA was performed using 5′ end-labelled oligo 21 and 10 μg of yeast cellular

RNAs. Mapping of pseudouridine residues at positions 2822 and 2876 in the yeast 25S rRNA was performed by primer extension analyses of CMC-alkali-treated yeast cellular RNAs as described earlier (Bakin and Ofengand, 1993; Ganot *et al*., 1997). Primer extension products were analysed on 6% sequencing gels.

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References

Bagni,C. and Lapeyre,B. (1998) Gar1p binds to the small nucleolar RNAs snR10 and snR30 *in vitro* through a nontypical RNA binding element. *J. Biol. Chem*., **273**, 10868–10873.

Bachellerie,J.-P. and Cavaillé,J. (1997) Guiding ribose methylation of rRNA. *Trends Biochem. Sci*., **22**, 257–261.

Bakin,A. and Ofengand,J. (1993) Four newly located pseudouridine residues in *Escherichia coli* 23S ribosomal RNA are all at the peptidyltransferase center: analysis by the application of a new sequencing technique. *Biochemistry*, **32**, 9754–9762.

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.

Bonneaud,N., Ozier-Kalogeropoulos,O., Li,G., Labouesse,M., Minvielle-Sebastia,L. and Lacroute,F. (1991) A family of low and high copy replicative, integrative and single-stranded *S.cerevisiae*/*E.coli* shuttle vectors. *Yeast*, **7**, 609–615.

Caffarelli,E., Fatica,A., Prislei,S., De Gregorio,E., Fragapane,P. and Bozzoni,I. (1996) Processing of the intron-encoded U16 and U18 snoRNAs: the conserved C and D boxes control both the processing reaction and the stability of the mature snoRNA. *EMBO J*., **15**, 1121–1131.

Cavaillé,J. and Bachellerie,J.-P. (1996) Processing of fibrillarin-associated snoRNAs from pre-mRNA introns: an exonucleolytic process exclusively directed by common stem–box terminal structure. *Biochimie*, **78**, 443–456.

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.

Cecconi,F., Mariottini,P. and Amaldi,F. (1995) The *Xenopus* intron-encoded U17 snoRNA is produced by exonucleolytic processing of its precursor in oocytes. *Nucleic Acids Res*., **23**, 4670–4676.

Chanfreau,G., Rotondo,G., Legrain,P. and Jacquier,A. (1998) Processing of a dicistronic small nucleolar RNA precursor by the RNA endonuclease Rnt1. *EMBO J*., **17**, 3726–3737.

Datta,A.K. (1995) Efficient amplification using 'megaprimer' by asymmetric polymerase chain reaction. *Nucleic Acids Res*., **23**, 4530–4531.

Eichler,D.C. and Craig,N. (1994) Processing of eukaryotic ribosomal RNA. *Prog. Nucleic Acids Res. Mol. Biol*., **49**, 179–239.

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.

Green,R. and Noller,H.F. (1997) Ribosomes and translation. *Annu. Rev. Biochem*., **66**, 679–716.

Hadjiolov,A.A. (1985) *The Nucleolus and Ribosome Biogenesis. Cell Biology Monographs*. Springer-Verlag, Wien, Germany.

Henras,A., Henry,Y., Bousquet-Antonelli,C., Noaillac-Depeyre,J., Gélugne,J.-P. and Caizergues-Ferrer,M. (1998) Nhp2p and Nop10p are essential for the function of H/ACA snoRNPs. *EMBO J*., **17**, 7078–7090.

Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol*., **153**, 163–168.

Kiss,T. and Filipowicz,W. (1995) Exonucleolytic processing of small nucleolar RNAs from pre-mRNA introns. *Genes Dev*., **9**, 1411–1424.

Kiss,T., Bortolin,M.-L. and Filipowicz,W. (1996) Characterization of the intron-encoded U19 RNA, a new mammalian small nucleolar RNA that is not associated with fibrillarin. *Mol. Cell. Biol*., **16**, 1391–1400.

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.

Kiss-László,Z., Henry,Y. and Kiss,T. (1998) Sequence and structural elements of methylation guide snoRNAs essential for site-specific ribose methylation of pre-rRNA. *EMBO J*., **17**, 797–807.

Koonin,E.V. (1996) Pseudouridine synthases: four family of enzymes containing a putative uridine-binding motif also conserved in dUTPases and dCTP deaminases. *Nucleic Acids Res*., **24**, 2411–2415.

Koonin,E.V., Bork,P., and Sander,C. (1994) A novel RNA-binding motif in omnipotent suppressors of translation termination, ribosomal proteins and ribosome modification enzyme? *Nucleic Acids Res*., **22**, 2166–2167.

Lafontaine,D.L.J., Bousquet-Antonelli,C., Henry,Y., Caizergues-Ferrer,M. and Tollervey,D. (1998) The box H+ACA snoRNAs carry Cbf5p, the putative rRNA pseudouridine synthase. *Genes Dev*., **12**, 527–537.

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.

Maden,B.E.H. (1990) The numerous modified nucleotides in eukaryotic ribosomal RNA. *Prog. Nucleic Acids Res*., **39**, 241–303.

Maden,B.E.H. (1996) Click here for methylation. *Nature*, **383**, 675–676.

Maden,B.E.H. (1997) Guides to 95 new angles. *Nature*, **389**, 129–131.

Maxwell,E.S. and Fournier,M.J. (1995) The small nucleolar RNAs. *Annu. Rev. Biochem*., **35**, 897–934.

Ni,J., Tien,A.L. and Fournier,M.J. (1997) Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA. *Cell*, **89**, 565–573.

Nitta,I., Kamada,Y., Noda,H., Ueda,T. and Watanabe,K. (1998) Reconstitution of peptide bond formation with *Echerichia coli* 23S ribosomal RNA domains. *Science*, **281**, 666–668.

Ofengand,J. and Bakin,A. (1997) Mapping to nucleotide resolution of pseudouridine residues in large subunit ribosomal RNAs from representative eukaryotes, prokaryotes, archaebacteria, mitochondria and chloroplasts. *J. Mol. Biol*., **266**, 246–268.

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.

Ooi,S.L., Samarsky,D.A., Fournier,M.J. and Boeke,J.D. (1998) Intronic snoRNA biosynthesis in *Saccharomyces cerevisiae* depends on the lariat-debranching enzyme: intron length effects and activity of precursor snoRNA. *RNA*, **4**, 1096–1110.

Peculis,B.A. (1997) RNA processing: pocket guides to ribosomal RNA. *Curr. Biol*., **7**, R480–R482.

Peculis,B.A. and Mount,S.M. (1996) Ribosomal RNA: small nucleolar RNAs make their mark. *Curr. Biol*., **6**, 1413–1415.

Petfalski,E., Dandekar,T., Henry,Y. and Tollervey,D. (1998) Processing of the precursors to small nucleolar RNAs and rRNAs requires common components. *Mol. Cell. Biol*., **18**, 1181–1189.

Qu,L.-H., Henras,A., Lu,Y.-J., Zhou,H., Zhou,W., Zhu,Y.-Q., Zhao,J., Henry,Y., Caizergues-Ferrer,M. and Bachellerie,J.-P. (1999) Seven novel methylation-guide snoRNAs are processed from a common polycistronic transcript by Rat1 and RNase III in yeast. *Mol. Cell. Biol.*, in press.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schimmel,P. and Alexander,R. (1998) All you need is RNA. *Science*, **281**, 658–659.

Sherman,F. (1991) Getting started with yeast. *Methods Enzymol*., **194**, 3–23.

Smith,C.M. and Steitz,J.A. (1997) Sno storm in the nucleolus: new roles for myriad small RNPs. *Cell*, **89**, 669–672.

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. (1996) Small nucleolar RNAs guide ribosomal RNA methylation. *Science*, **273**, 1056–1057.

Tollervey,D. and Kiss,T. (1997) Function and synthesis of small nucleolar RNAs. *Curr. Opin. Cell Biol*., **9**, 337–342.

Tollervey,D. and Mattaj,I.W. (1987) Fungal small nuclear ribonucleoproteins share properties with plant and vertebrate U-snRNPs. *EMBO J.*, **6**, 469–476.

Tycowski,K.T., Smith,C.M., Shu,M.-D. and Steitz,J.A. (1996) 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 preribosomal RNA in *Saccharomyces cerevisiae*. *Yeast*, **11**, 1629–1650.

Watkins,N.J., Gottschalk,A., Neubauer,G., Kastner,B., Fabrizio,P., Mann,M. and Lührmann,R. (1998) Cbf5p, a potential pseudouridine synthase, and Nhp2p, a putative RNA binding protein, are present together with Gar1p in all H box/ACA motif snoRNPs and constitute a common bi-partite structure. *RNA*, in press.

Watkins,N.J., Leverette,R.D., Xia,L., Andrew,M.T. and Maxwell,E.S. (1996) Elements essential for processing intronic U14 snoRNA are located at the termini of the mature snoRNA sequence and include conserved nucleotide boxes C and D. *RNA*, **2**, 118–133.

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