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Nucleolar localization signals of Box H/ACA small nucleolar RNAs

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The two major families of small nucleolar RNAs (snoRNAs), Box C/D and Box H/ACA, are generated in the nucleoplasm and transported to the nucleolus where they function in rRNA processing and modification. We have investigated the sequences involved in the intranuclear transport of Box H/ACA snoRNAs by assaying the localization of injected fluorescent RNAs in *Xenopus* oocyte nuclear spreads. Our analysis of U17, U64 and U65 has revealed that disruption of either of the conserved sequence elements, Box H or Box ACA, eliminates nucleolar localization. In addition, the stem present at the base of the 3′ hairpin is required for efficient nucleolar localization of U65. Fragments or rearrangements of U65 that consist of Box H and Box ACA flanking either the 5′ or 3′ hairpin are targeted to the nucleolus. The targeting is dependent on the presence of the Box sequences, but not on their orientation. Our results indicate that in each of the two major families of snoRNAs, a motif composed of the signature conserved sequences and an adjacent structural element that tethers the sequence elements directs the nucleolar localization of the RNAs. We demonstrate that telomerase RNA is also targeted to the nucleolus by a Box ACA-dependent mechanism.
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Introduction

In eukaryotes, the nucleolus is the site of assembly of ribosomal proteins and ribosomal RNA (rRNA) into ribosomal subunits. While ribosomal proteins are synthesized in the cytoplasm and transported to nucleoli, rRNAs are synthesized, modified and processed from precursors within the nucleolus (Scheer and Weisenberger, 1994; Shaw and Jordan, 1995). rRNA is heavily modified: >200 of the ~7000 nucleotides of mature human rRNA are modified by isomerization of uridine to pseudouridine or methylation of the 2′-OH group of the ribose (Maden, 1990; Ofengand *et al.*, 1995; Ofengand and Bakin, 1997; Ofengand and Fournier, 1998). The purpose of 2′-*O*-methylation and pseudouridylation of rRNA is not known. While individual modifications have not been found to be essential for cellular viability (Maxwell and Fournier, 1995; Balakin *et al.*, 1996; Qu *et al.*, 1999), the positions of modified rRNA nucleotides are concentrated in the functional core of rRNA and are conserved, implying an important, perhaps collective function (Bakin *et al.*, 1994; Lane *et al.*, 1995). The modified precursor rRNA transcript is processed to produce three mature species (5.8S, 18S and 28S rRNA in vertebrates) that are incorporated into the large and small ribosomal subunits (Hadjiolov, 1985; Eichler and Craig, 1994; Venema and Tollervey, 1995; Sollner-Webb *et al.*, 1996).

Modification and processing of rRNA requires small nucleolar RNAs (snoRNAs). Two major classes of snoRNAs can be identified based on common sequence elements, predicted structures and associated proteins (Maxwell and Fournier, 1995; Smith and Steitz, 1997; Tollervey and Kiss, 1997). Members of the Box C/D family snoRNAs guide the 2′-*O*-methylation of rRNA (Kiss-László *et al.*, 1996; Nicoloso *et al.*, 1996). Box H/ACA snoRNAs guide pseudouridylation of rRNA (Ganot *et al.*, 1997a; Ni *et al.*, 1997). In both cases, it appears that the snoRNAs select the modification sites by base-paired interaction with rRNA, but do not catalyze the modification. In addition, a few Box C/D (U3, U8, U14 and U22) and Box H/ACA (U17/E1, E2, E3, and yeast snR10 and snR30) snoRNAs are required for rRNA processing (Maxwell and Fournier, 1995; Venema and Tollervey, 1995; Enright *et al.*, 1996; Tollervey and Kiss, 1997). However, the vast majority of snoRNAs appear to direct the many modifications of rRNA.

The snoRNAs function in the nucleolus, but precursor snoRNAs are generated by transcription from chromosomes in the nucleoplasm (Suh *et al.*, 1987; Sollner-Webb, 1993; Gao *et al.*, 1997). In this work, we have investigated the role of snoRNA sequences and structures in the transport of three Box H/ACA class snoRNAs (U17, U64 and U65) to the nucleolus. The Box H/ACA RNAs have similar predicted core secondary structures that can be described as hairpin–hinge–hairpin–tail structures (Balakin *et al.*, 1996; Ganot *et al.*, 1997b). The RNAs share two sequence elements, termed Box H (consensus AnAnnA), which is found in the 'hinge', and Box ACA (consensus ACA), located in the 'tail', three nucleotides from the 3′ terminus of each RNA (Balakin *et al.*, 1996; Ganot *et al.*, 1997b). The Box H and Box ACA sequences are found in single-stranded regions of the RNAs flanking the 3′ hairpin. Site selection for pseudouridylation of rRNA is accomplished by base-pairing between sequences in

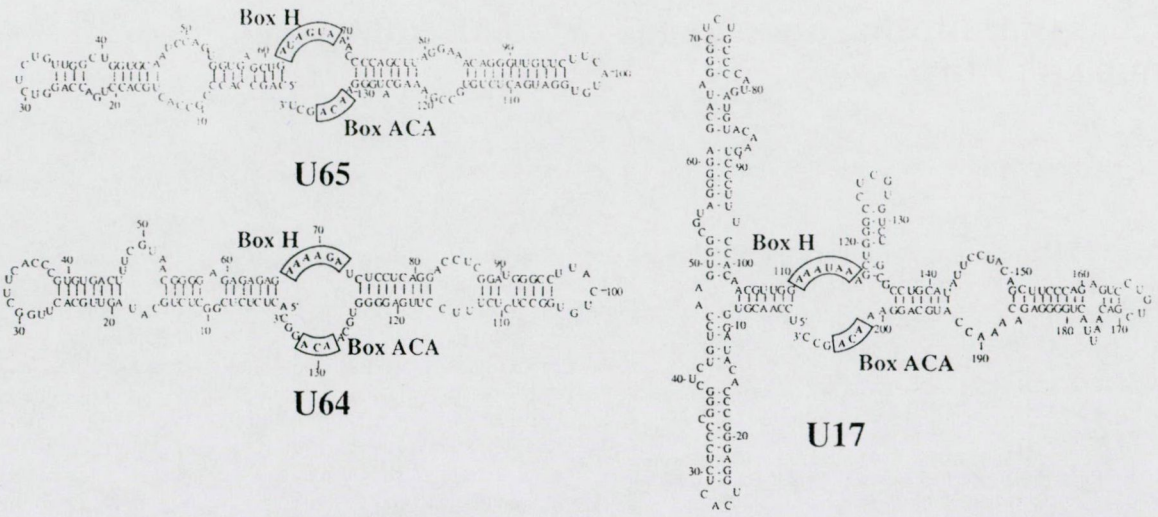

Fig. 1. Sequence and secondary structures of U65, U64 and U17 Box H/ACA snoRNAs (Cecconi *et al.*, 1994; Ganot *et al.*, 1997b; Bortolin *et al.*, 1999). The conserved Box H and Box ACA sequences are indicated.

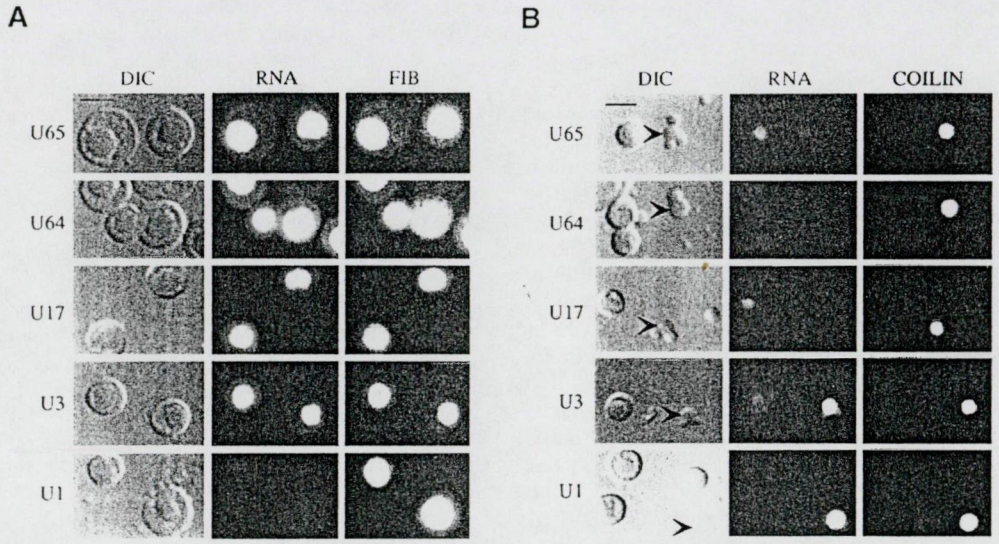

Fig. 2. Microinjected fluorescently labeled RNAs localized in *Xenopus* oocyte nuclear spreads. (**A**) Box H/ACA snoRNAs co-localize with endogenous fibrillarin in nucleoli. Fluorescently labeled U65, U64 and U17, as well as U3 and U1, were transcribed *in vitro* (see Materials and methods) and injected separately into *Xenopus* oocyte nuclei. m^{7}G-capped U3 snoRNA and U1 snRNA served as positive and negative controls, respectively, for nucleolar localization. The nuclear spreads were prepared 1 h after injection. Endogenous fibrillarin was detected by indirect immunofluorescence using anti-fibrillarin antibody, 17C12 (Hultman *et al.*, 1994) and Texas Red-labeled secondary antibodies (FIB). The RNAs were observed by fluorescence microscopy. Differential interference contrast (DIC) and fluorescence images (RNA and FIB) are shown for each sample. The DIC panels show 2–5 nucleoli. Bar, 10 μm. (**B**) Box H/ACA snoRNAs are not observed in coiled bodies. Nuclear spreads were made 15 min after the injection of fluorescently labeled RNAs. Coiled bodies were stained by indirect immunofluorescence using antibody H1 against p80 coilin (Tuma *et al.*, 1993) and Texas Red-labeled secondary antibodies (COILIN). A single coiled body is present in each panel (indicated by an arrow). The control U3 snoRNA and U1 snRNA localize to coiled bodies as reported previously (Narayanan *et al.*, 1999). DIC and fluorescence images (RNA and COILIN) are shown for each sample.

one of the two pseudouridylation loops of a Box H/ACA snoRNA and sequences flanking the modification site in rRNA (Ganot *et al.*, 1997a). The pseudouridylation loops are located within the hairpins of Box H/ACA guide RNAs flanked by the proximal and distal stem regions.

The sequences within Box H/ACA snoRNAs important for their accumulation and function in pseudouridylation have been investigated. These studies have been performed in both yeast and vertebrate systems, where the RNAs are generated by distinct mechanisms. Vertebrate Box H/ACA snoRNAs (including U17, U64 and U65) are generated by processing from introns, whereas most Box H/ACA RNAs are transcribed independently in yeast (Maxwell and Fournier, 1995; Tollervey and Kiss, 1997). Accumulation of all Box H/ACA snoRNAs examined requires Box H and Box ACA, and the 3′-proximal stem has been demonstrated to be essential for stability in yeast (Balakin *et al.*, 1996; Ganot *et al.*, 1997b; Bortolin *et al.*, 1999).

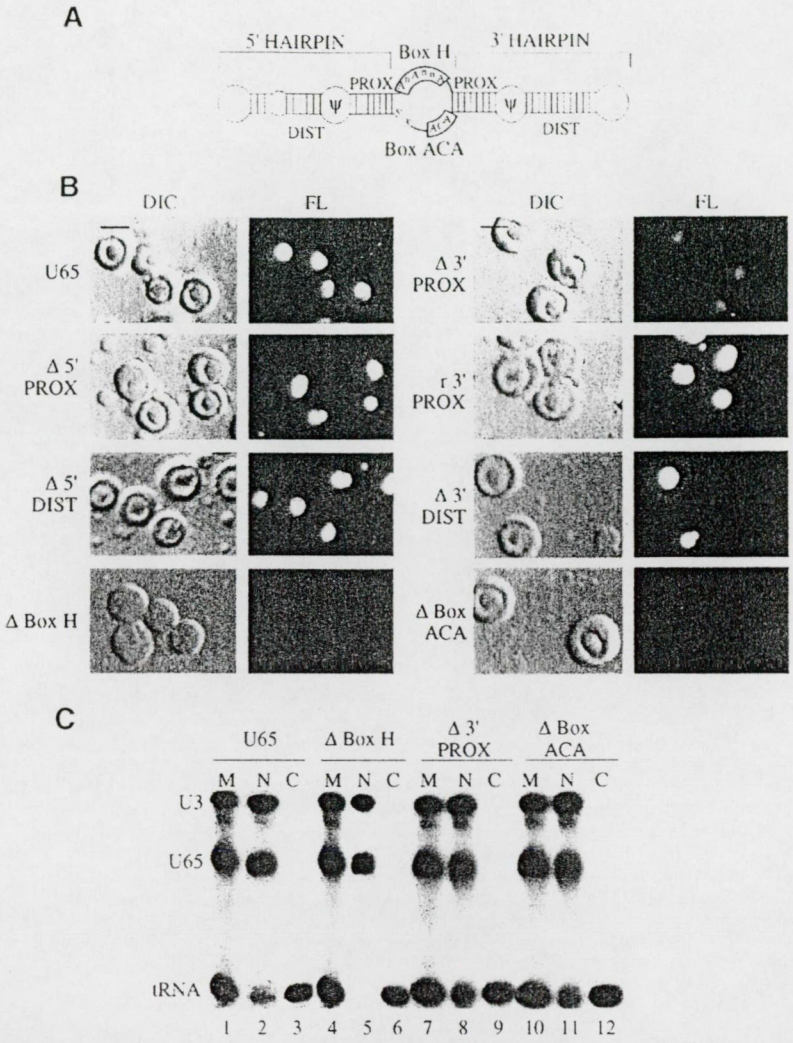

Fig. 3. Identification of *cis*-acting sequences essential for the nucleolar localization of U65 Box H/ACA snoRNA. (**A**) Schematic representation of the secondary structure of U65 snoRNA denoting the positions of Box H, Box ACA, and also the 5′ and 3′ hairpins. In addition, the stem region of each hairpin that is proximal (PROX) and distal (DIST) to the Box H/ACA region, and the pseudouridylation loops (ψ) are indicated. (**B**) Role of Box H, Box ACA and the 3′-proximal stem in the nucleolar localization of U65 snoRNA. Block sequence substitution (indicated as Δ) to disrupt the proximal and distal stems of each of the 5′ and 3′ hairpins, as well as the Box H and Box ACA sequence elements, was performed (Bortolin *et al.*, 1999; see Materials and methods). In addition, compensatory changes were introduced into the Δ 3′ PROX construct to restore a stem structure (r 3′ PROX). Fluorescently labeled RNAs were injected and nuclear spreads were made 1 h after injection. The DIC panels show 2–5 nucleoli, and the fluorescence panels (FL) show RNA signals. Bar, 10 μm. (**C**) Variants of U65 that are not localized to nucleoli are present within the nucleus. Samples from the same set of injected oocytes were analyzed for nucleolar localization [see (B)] and by gel electrophoresis following dissection into nuclear (N) and cytoplasmic (C) fractions. RNAs were co-labeled with ^{32}P (see Materials and methods) and detected by autoradiography. Marker lanes 1, 4, 7 and 10 (M) show RNA samples prior to injection. U3 and tRNA were included as controls for accurate oocyte dissection and RNA stability, retention (U3) and export (tRNA).

On the other hand, the 5′ cap is important for the accumulation of independently transcribed Box H/ACA snoRNAs, but not for intron-derived RNAs, which do not contain 5′ caps (Bortolin *et al.*, 1999). The 5′-proximal stem is essential for accumulation of intronic Box H/ACA snoRNAs, but not for capped RNAs (Bortolin *et al.*, 1999). Box H and Box ACA are also essential for generating correctly processed 5′ and 3′ snoRNA termini (Balakin *et al.*, 1996; Bortolin *et al.*, 1999). Finally, the ability of the snoRNAs (including U64 and U65) to function in pseudouridylation depends on the integrity of Box H, Box ACA and each of the four stem regions that flank the pseudouridylation loops in the 5′ and 3′ hairpins (Bortolin *et al.*, 1999).

Box H/ACA snoRNAs, including U17, U64 and U65, have been found to fractionate with nucleoli (Kiss *et al.*, 1996; Ganot *et al.*, 1997b) or to be localized in nucleoli in cytological preparations (Cecconi *et al.*, 1995; Selvamurugan *et al.*, 1997; Shaw *et al.*, 1998). We are interested in the mechanism of transport of snoRNAs to the nucleolus and have examined the distribution of fluorescently labeled H/ACA RNAs following injection into the nucleus of *Xenopus* oocytes. These RNAs specifically localize to the fibrillar region of the nucleolus.

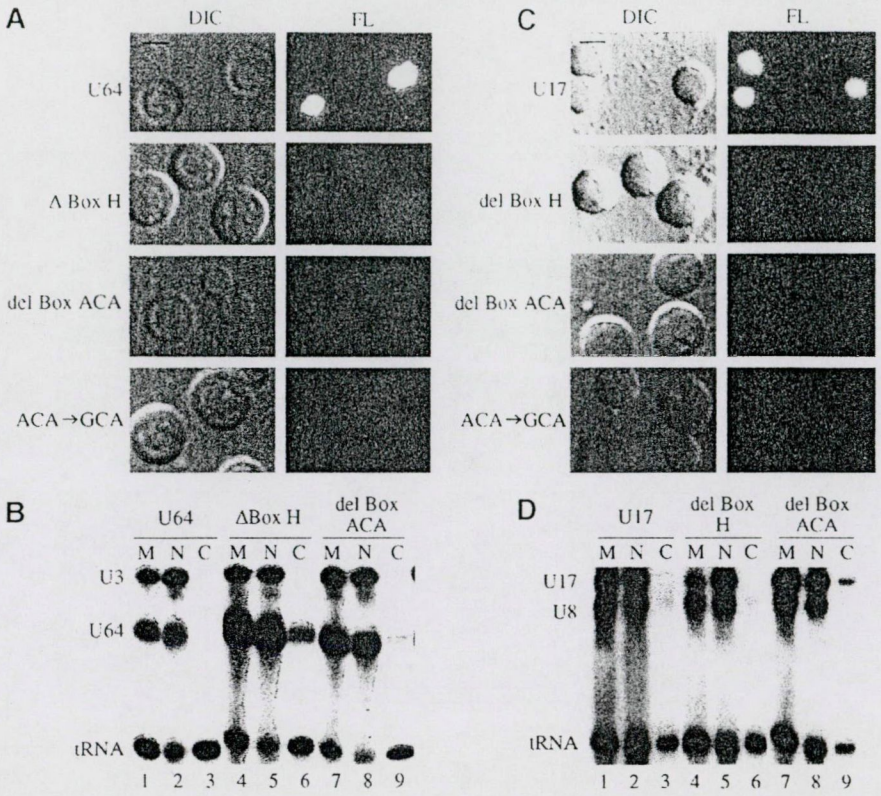

Fig. 4. Box H and Box ACA are required for nucleolar localization of U64 and U17. Variants of U64 and U17 Box H/ACA snoRNAs were injected into *Xenopus* oocyte nuclei. Nuclear spreads were prepared [(**A**) and (**C**)] and RNAs were analyzed by gel electrophoresis [(**B**) and (**D**)] 1 h after injection. Block substitution of Box H (Δ Box H) and deletion of Box ACA (del Box ACA), as well as point mutation of Box ACA (ACA→GCA), disrupted nucleolar localization of U64 (A). Similarly, deletion of Box H or Box ACA (del Box H or del Box ACA), or point mutation of Box ACA (ACA→GCA) in U17, blocked localization of RNA to nucleoli (C). DIC indicates differential interference contrast and FL indicates fluorescence. Bar, 10 μm. The amount of the variant U64 or U17 RNAs present in the nucleus at the time of analysis is shown in (B) and (D), respectively. Nuclear (N) and cytoplasmic (C) fractions were obtained 1 h after injection from the same set of injected oocytes as used for the nuclear spread analysis. Marker lanes 1, 4 and 7 [(B) and (D)] are samples before injection (M). U3 (B) or U8 (D) and tRNA were used as controls for oocyte dissection, and RNA stability, retention and export.

and examination of variant RNAs has allowed us to determine that the conserved Box H and Box ACA sequences along with an adjacent stem structure are necessary for nucleolar localization of RNA. Our results also indicate that telomerase RNA is targeted to nucleoli by the same mechanism.

Results

Specific targeting of Box H/ACA snoRNAs to the nucleolus

Previously, we determined the sequences and structures critical for localization of Box C/D family snoRNAs to the nucleolus by injecting fluorescently labeled wild-type and variant RNAs into the nucleus of *Xenopus* oocytes and assessing the RNA localization in nuclear spreads (Narayanan *et al.*, 1999). Similarly, we have analyzed the nucleolar localization of three Box H/ACA family snoRNAs in this work. Fluorescently labeled human U65, U64 and U17 snoRNAs (Figure 1) are present in nucleoli 1 h following injection into *Xenopus* oocytes (Figure 2A). The RNAs co-localize with the nucleolar protein fibrillarin to the fibrillar region of the nucleolus (Figure 2A). The localization of the Box H/ACA RNAs is similar to that observed with U3 Box C/D snoRNA (Figure 2A). U1 small nuclear RNA was not observed in nucleoli at any time point examined (Figure 2A; 15 min, 4, 8 and 24 h, A.Narayanan, R.Terns and M.Terns, unpublished data). In addition, while we found that Box C/D snoRNAs transiently localize to coiled bodies prior to nucleoli (Narayanan *et al.*, 1999), U65, U64 and U17 do not localize to coiled bodies at an early time point (15 min after injection; Figure 2B), or at any time point examined (including 1, 4 and 8 h after injection; A.Narayanan, R.Terns and M.Terns, unpublished data). Box H/ACA snoRNAs appear to be retained in the nucleus (Figure 3C) like Box C/D snoRNAs (Terns and Dahlberg, 1994; Terns *et al.*, 1995).

Box H, Box ACA and the 3′-proximal stem are critical for localization of U65 to nucleoli

Localization of human H/ACA snoRNAs to nucleoli in *Xenopus* oocytes implied that conserved sequences and structures would be involved in localization. The two conserved sequence elements identified in the Box H/ACA snoRNAs, Box H and Box ACA, are both located in single-stranded regions of the RNAs (Figures 1 and 3A). The Box H/ACA snoRNAs share hairpin–hinge–hairpin–tail predicted secondary structure elements typified

by U65 and U64 RNAs (Figure 3A). Box H is located in the hinge between the 5′ hairpin and the 3′ hairpin (Figure 3A). Box ACA is located three nucleotides from the 3′ terminus of mature RNAs in the single-stranded tail (Figure 3A). Among the RNAs that guide pseudouridylation, each functional hairpin consists essentially of a stem proximal to Box H, an internal loop where base-pairing with rRNA for pseudouridylation site selection occurs, a distal stem region and a terminal loop (Figure 3A). We tested the ability of variants of U65 in which conserved elements were disrupted to localize to nucleoli. Each of the four stem regions was disrupted by substitution of the sequence of one strand of the stem. Disruption of either the proximal or distal stem in the 5′ hairpin (Δ 5′ PROX and Δ 5′ DIST) or the distal stem of the 3′ hairpin (Δ 3′ DIST) did not significantly affect the ability of the RNA to localize to nucleoli (Figure 3B). However, disruption of the proximal stem of the 3′ hairpin nearly eliminated nucleolar localization (Δ 3′ PROX; Figure 3B). Restoration of the 3′-proximal stem by compensatory changes in the sequence of the other strand of the stem re-established the ability of the RNA to localize to nucleoli (r 3′ PROX; Figure 3B), indicating that the 3′-proximal stem structure (but not sequence) is vital for nucleolar localization. In addition, substitution of either the Box H or Box ACA sequence element prevents localization of U65 to nucleoli (ΔH and ΔACA; Figure 3B). Importantly, each of the variant RNAs that is not observed in nucleoli is present in the nucleus at the time of analysis 1 h after injection (Figure 3C). Thus, Box H, Box ACA and the 3′-proximal stem formed by sequences adjacent to Box H and Box ACA are important for localization of U65 to nucleoli.

Box H and Box ACA are also required for localization of U17 and U64 snoRNAs to nucleoli

We tested whether the conserved sequence elements Box H and Box ACA were also involved in the nucleolar localization of two additional Box H/ACA snoRNAs. We found that disruption of either Box element in U64 or in U17 blocked localization of the RNAs to nucleoli (Figure 4A and C). Again, although the RNAs did not localize to nucleoli, they were present in nuclei at the time of analysis (Figure 4B and D). Furthermore, point mutation of one of the three conserved residues of Box ACA (ACA→GCA) also prevented localization of both U64 and U17 (Figure 4A and C). In addition, we analyzed a series of mutations in Box H (consensus sequence AnAnnA) in U64 RNA (Figure 5). The results indicate that all of the three conserved A residues of Box H (positions 1, 3 and 6) are necessary for the function of Box H in localization. In summary, our results indicate that Box H, Box ACA and the 3′-proximal stem that tethers these two sequence elements are required for nucleolar localization of Box H/ACA family snoRNAs.

Minimal elements sufficient for localization of RNA to the nucleolus

We were interested in determining the minimal sequences required for targeting of a Box H/ACA snoRNA to nucleoli. We analyzed the localization of fragments of U65 RNA and found that an RNA composed of the hinge including Box H, the 3′ hairpin and the tail including Box

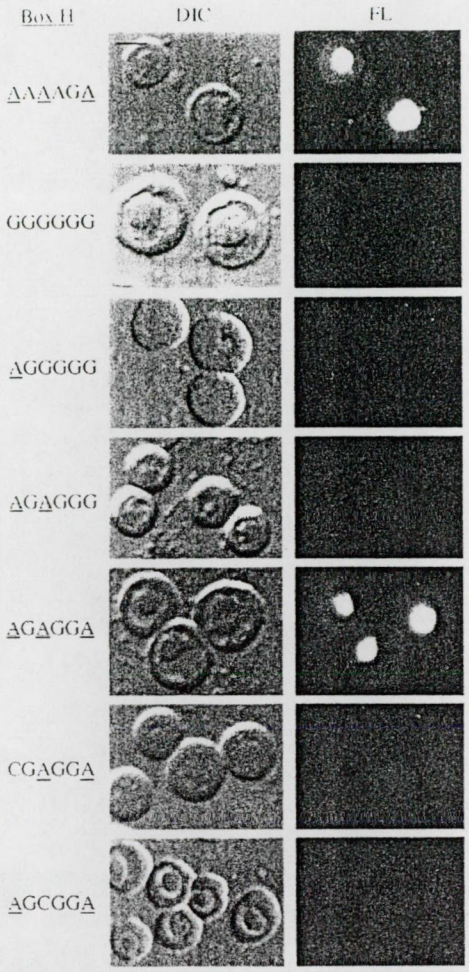

Fig. 5. All three conserved adenine nucleotides of the Box H region of U64 are essential for nucleolar localization. *In vitro* transcribed, fluorescently labeled RNAs were injected into oocytes and nuclear spreads were prepared 1 h after injection and analyzed by fluorescence microscopy. The Box H sequence of wild-type (AAAAGA) and each U64 variant RNA is indicated to the left (Ganot *et al.*, 1997b). Conserved adenine residues are underlined. DIC indicates differential interference contrast panels and FL indicates fluorescence panels. Bar, 10 μm.

ACA (H-3′Hp-ACA) was transported to the nucleolus (Figure 6A). However, a similar fragment consisting of the 3′ hairpin and Box ACA, but lacking Box H (3′Hp-ACA), was not observed in nucleoli (Figure 6A) despite its presence in the nucleus (Figure 6B). In addition, a fragment of U65 consisting of the 5′ hairpin and Box H (5′Hp-H) was present in the nucleus (Figure 6B) but not in nucleoli (Figure 6A). These results indicate that Box H and Box ACA are each essential for localization of the fragments as well as full-length Box H/ACA snoRNAs. Box ACA is not directly up- or downstream of the 5′ hairpin in wild-type Box H/ACA snoRNAs. However, rearrangement of the elements of U65 to generate an RNA comprised of Box H, the 5′ hairpin and Box ACA (H-5′Hp-ACA), in which the 3′ hairpin is essentially replaced by the 5′ hairpin, results in targeting to the nucleolus (Figure 6A). Thus, RNAs composed of either hairpin flanked upstream by Box H and downstream by

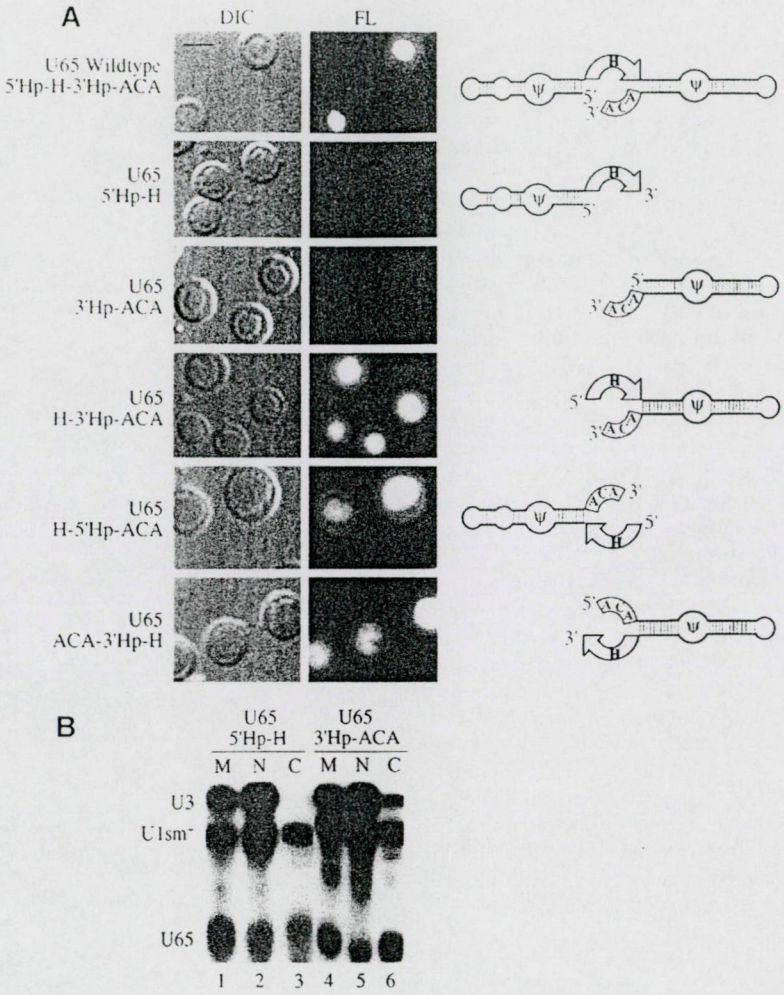

Fig. 6. RNAs consisting of Box H, Box ACA, and either the 5′ or the 3′ hairpin are sufficient for nucleolar localization. (**A**) Fragments of U65 that contain the 5′ hairpin and Box H (5′Hp-H), or the 3′ hairpin and Box ACA (3′Hp-ACA), were analyzed. In addition, a larger fragment of U65 consisting of Box H, the 3′ hairpin and Box ACA (H-3′Hp-ACA) was tested. Finally, two RNAs consisting of rearranged elements of U65 were tested: Box H, the 5′ hairpin and Box ACA (H-5′Hp-ACA), and Box ACA, the 3′ hairpin and Box H (ACA-3′Hp-H). Schematic representations of the injected RNAs are shown to the right. The arrow indicates the orientation of Box H. Nuclear spreads were prepared 1 h after injection of fluorescently labeled RNAs. Differential interference contrast (DIC) and fluorescence panels (FL) are shown. Bar, 10 μm. (**B**) The presence of RNAs that are not localized to nucleoli within the nucleus. RNAs were extracted from the nuclear (N) and cytoplasmic (C) fractions of the same batch of injected oocytes that were analyzed for nucleolar localization (A). The fractionation was performed 1 h after injection. Marker lanes 1 and 4 (M) indicate samples prior to injection. U3 and U1sm⁻ were used to assess oocyte dissections and RNA stability, retention and export.

Box ACA contain signals sufficient for localization to the nucleolus. Furthermore, we found that placement of Box ACA upstream and Box H downstream of the 3′ hairpin (ACA-3′Hp-H) resulted in targeting to the nucleolus (Figure 6A), indicating that Box H and Box ACA function in both orientations relative to a hairpin to target the RNA to the nucleolus.

We further tested the requirement for the proximal stem (PROX), pseudouridylation loop (LOOP) and distal stem region (DIST) of the hairpin in the localization of a minimal RNA to the nucleolus. Substitutions were introduced into the fragment of U65 consisting of Box H, the 3′ hairpin and Box ACA (H-3′Hp-ACA) to disrupt a stem structure (Δ) and compensatory changes were made to restore the stem (r). Disruption of either the proximal (Δ PROX) or distal (Δ DIST) stem resulted in loss of nucleolar signal (Figure 7A) and RNA stability (Figure 7B). However, RNAs in which the stem structures were restored by substitution of the sequence of the opposite strand of the stem are targeted to the nucleolus (r PROX and r DIST; Figure 7A), indicating that the sequences of the stems are not important for targeting of the fragment. In addition, we introduced substitutions into one strand of the pseudouridylation loop which were expected to result in base-pairing with the opposite strand and loss of the loop structure (Δ LOOP). Although the stability of the Δ LOOP RNA was compromised, sufficient RNA remained in the nucleus (based upon experiments in which we titrated the amount of RNA required in the nucleus to produce a significant nucleolar signal; A.Narayanan, R.Terns and M.Terns, unpublished data), but did not localize to nucleoli (Figure 7A and B). When compensatory mutations were introduced into the sequence of the opposite strand to disrupt base-pairing and restore the

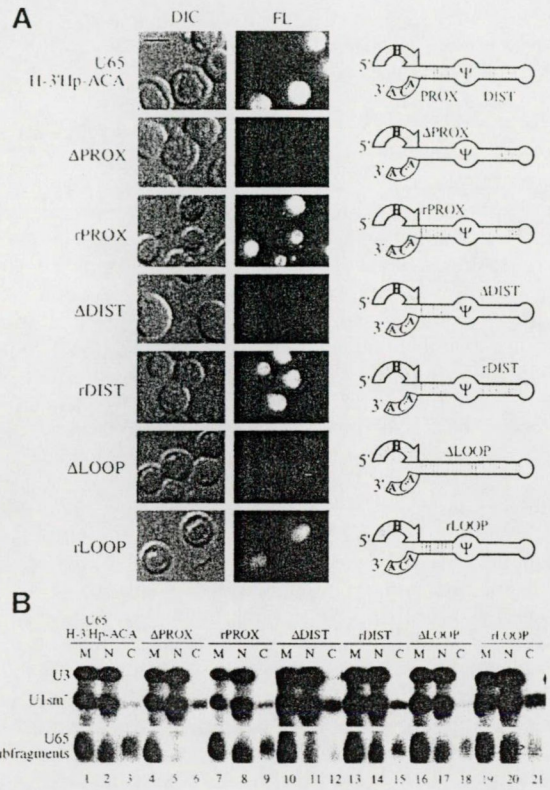

Fig. 7. Essential elements of a fragment of U65 sufficient for nucleolar localization. (**A**) Substitutions were made in the sequence of a fragment of U65 that localizes to the nucleolus (H-3'Hp-ACA) to disrupt (Δ) and restore (r) the primary substructures of the hairpin: the stem proximal to the Box H/ACA region (PROX), the pseudouridylation loop (LOOP) and the distal stem (DIST). Schematic representations of the labeled RNAs are shown to the right of the corresponding set of panels. Nuclear spreads were made 1 h after injection. DIC denotes differential interference contrast and FL denotes fluorescence panels. Bar, 10 μm. (**B**) The injected RNAs were extracted from the nuclear (N) and cytoplasmic (C) fractions of the oocytes 1 h after injection and analyzed by gel electrophoresis and autoradiography. Marker lanes 1, 4, 7, 10, 13, 16 and 19 (M) are samples before injection. U3 and U1sm⁻ were used as controls for oocyte dissection, and RNA stability, retention and export.

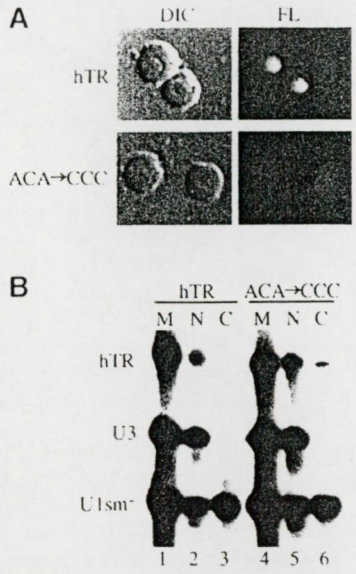

Fig. 8. Localizaton of telomerase RNA to the nucleolus depends on Box ACA. (**A**) Fluorescently labeled human telomerase RNA was microinjected into *Xenopus* oocyte nuclei. Wild-type (hTR) and Box ACA mutant (ACA→CCC) telomerase RNAs were injected. Nuclear spreads were prepared 1 h after injection and the RNAs were observed by fluorescence microscopy. Differential interference contrast (DIC) and fluorescence images (FL) are shown for each sample. (**B**) Both RNAs are present in the nucleus 1 h after injection. The RNAs (co-labeled with ^{32}P) were analyzed by gel electrophoresis following dissection into nuclear (N) and cytoplasmic (C) fractions, and detected by autoradiography. Marker lanes 1 and 4 (M) show RNA samples prior to injection. U3 and U1sm⁻ were included as controls for accurate oocyte dissection, and RNA stability, retention (U3) and export (tRNA).

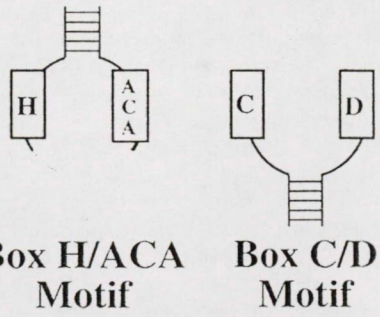

Fig. 9. Sequence/structural elements essential for the nucleolar localization of the two major snoRNA families. In the case of both the Box H/ACA and Box C/D snoRNAs, we have found that a motif comprised of the signature conserved sequence elements (Box C and Box D, or Box H and Box ACA) and an adjacent structural element (typically a stem) is essential for targeting the RNA to the nucleolus. The shaded stem of the Box H/ACA motif indicates that this stem is conserved in this family of snoRNAs but was not found in this study to be essential for nucleoloar localization. The shaded stem of the Box C/D motif indicates that some members of the Box C/D family (e.g. U3) contain two adjacent stems that function to tether the Box C and D elements.

loop, localization of the RNA to the nucleolus was restored substantially (r LOOP; Figure 7A). These results indicate that the presence of an internal loop in the hairpin is important for transport of the snoRNA fragment to the nucleolus, but that the specific sequence of the loop is not critical for localization.

Box ACA also functions in the localization of telomerase RNA to the nucleolus

Telomerase RNA functions to maintain telomeres at the ends of chromosomes (Blackburn and Greider, 1995). Potential sequence and structural similarities of telomerase RNA to the Box H/ACA snoRNAs have recently been identified (Mitchell *et al.*, 1999). In addition, a small fraction of telomerase RNA has been reported in nucleoli in steady-state biochemical fractionation studies (Mitchell *et al.*, 1999). We injected fluorescently labeled human telomerase RNA into *Xenopus* oocytes and observed specific localization of the RNA to the fibrillar regions of nucleoli (Figure 8A), indicating that telomerase RNA does associate with nucleoli. Furthermore, mutation of the putative Box ACA sequence located three nucleotides from the 3′ terminus of the RNA blocked targeting of telomerase RNA to the nucleolus (ACA→CCC; Figure 8A) similar to U65, U64 and U17 Box H/ACA snoRNAs

(Figures 3 and 4). Although the Box ACA mutant telomerase RNA was not observed in nucleoli 1 h after injection, it was present in the nucleus (Figure 8B). These results indicate that the Box ACA sequence element, identified by sequence and structural homology in telomerase RNA, functions similarly in telomerase RNA to target the RNA to the nucleolus.

Discussion

We have found that the two sequence elements conserved among members of the Box H/ACA family of snoRNAs, and a stem structure that brings these two elements adjacent to one another, are required for the localization of these RNAs to the nucleolus (Figures 3 and 4). In previous work, we and others determined that a motif composed of the two sequence elements conserved among members of the other major snoRNA family, Box C and Box D, and a structural element that linked the two sequence elements directed the targeting of Box C/D snoRNAs to the nucleolus (Samarsky *et al.*, 1998; Narayanan *et al.*, 1999). The similarity in the composition of the essential elements of the Box H/ACA and Box C/D motifs is striking (Figure 9). Both the Box H/ACA and Box C/D motifs are also important in the stability and function of the RNAs (Balakin *et al.*, 1996; Ganot *et al.*, 1997b; Bortolin *et al.*, 1999; reviewed in Maxwell and Fournier, 1995; Tollervey and Kiss, 1997). It is likely that both motifs mediate their roles in stability, transport and function through proteins that bind at the motif (Schimmang *et al.*, 1989; Caffarelli *et al.*, 1998; Henras *et al.*, 1998; Lafontaine *et al.*, 1998; Watkins *et al.*, 1998a,b; Wu *et al.*, 1998; Lafontaine and Tollervey, 1999).

While Box H, Box ACA and the 3′-proximal stem are essential for the targeting of Box H/ACA snoRNAs to the nucleolus (Figures 3 and 4), it was more difficult to ascertain the minimal elements sufficient for nucleolar localization. We found that a single hairpin flanked by Box H and Box ACA sequences was sufficient for localization (Figure 6). Furthermore, similar fragments lacking either Box H or Box ACA were not targeted to nucleoli (Figure 6). However, disruption of either the proximal or distal stem structure of the hairpin resulted in loss of RNA stability and prevented assessment of the role of the stems in targeting to the nucleolus (Figure 7). Replacement of the proximal and distal stems with stems of different sequence did not disrupt nucleolar localization, indicating that the sequence of the stems does not play a role in localization (Figure 7). Our experiments also suggest that the pseudouridylation loop structure may be important in targeting the snoRNA fragment to the nucleolus (Figure 7). While the specific sequence of the pseudouridylation loop does not play an essential role in nucleolar localization of the fragment, we did observe a slight decrease in nucleolar signal associated with the RNA in which the sequence of the loop was replaced (r LOOP; Figure 7). This may reflect unforeseen structural consequences of the sequence changes or a small contribution of the pseudouridylation loop sequence to targeting. A smaller fragment of U65 snoRNA comprised of Box H and Box ACA flanking the proximal stem with a terminal tetranucleotide loop was not sufficiently stable for analysis (unpublished data). Although an RNA composed of Box H, Box ACA and a single hairpin still localizes to the nucleolus, it seems unlikely that it could direct rRNA pseudouridylation, because snoRNAs carrying a destroyed distal or proximal 5′ stem are already inactive in pseudouridylation guiding (Bortolin *et al.*, 1999).

Interestingly, we found that the 5′ and 3′ hairpins were interchangeable in their ability to act as an appropriate structural context for Box H and Box ACA (Figure 6). Furthermore, the orientation of the Box elements relative to a given hairpin was not important (Figure 6). Thus, the Box H/ACA sequences can function equivalently toward both the 5′ and 3′ hairpin within a Box H/ACA snoRNA.

We found previously that Box C/D family snoRNAs localize to coiled bodies prior to nucleoli and that disruption of the Box C/D motif appeared to block transfer of the RNAs from coiled bodies to nucleoli, resulting in retention of RNAs in coiled bodies (Narayanan *et al.*, 1999). Interestingly, we did not observe U65, U64 or U17 Box H/ACA snoRNA, or any of the variant Box H/ACA snoRNAs tested, in coiled bodies at any time point that we examined (Figure 2B; unpublished data). On the other hand, proteins associated with all Box H/ACA snoRNAs, including the putative pseudouridine synthase, NAP57 (Meier and Blobel, 1994), and GAR1 (A.Narayanan, R.Terns and M.Terns, unpublished data), have been detected in coiled bodies. Furthermore, there is evidence that precursor Box H/ACA snoRNAs are enriched in coiled bodies in plants (Shaw *et al.*, 1998). The RNAs that we injected in this work were not precursor forms. It is also possible that the Box H/ACA snoRNAs were not detected in coiled bodies because the RNAs rapidly traverse these structures. While we did not observe Box H/ACA snoRNAs in coiled bodies in this work, our results do not preclude an association of Box H/ACA snoRNAs with coiled bodies.

The distinct localization of telomerase RNA to nucleoli that we have reported here raises interesting questions about this RNA. Telomerase RNA functions as a template for the synthesis of telomeric DNA repeats at the termini of chromosomes in eukaryotes, and it was realized only recently that it possesses a 3′ domain that strongly resembles Box H/ACA snoRNAs in vertebrates (Mitchell *et al.*, 1999). Only vertebrate telomerase RNAs appear to have acquired or retained a Box H/ACA snoRNA-like domain (Mitchell *et al.*, 1999). Given its similarity to Box H/ACA snoRNAs, one might hypothesize that telomerase RNA also functions in RNA modification or processing within the nucleolus. Alternatively, telomerase RNA may itself be modified or matured in the nucleolus. It is not clear why telomerase RNA might possess a Box H/ACA domain, but it is clear from our work that this domain, and in particular the Box H/ACA motif, targets human telomerase RNA to nucleoli.

Materials and methods

Generation of U65, U64, U17 and telomerase RNA wild-type and mutant constructs

Oligodeoxynucleotides used in this study are listed in Table I.

Many of the wild-type and mutant snoRNAs used in this study (Table II, left column; see Results) were synthesized by *in vitro* transcription from PCR templates generated by the combination of DNA templates and oligodeoxynucleotide pairs shown in Table II (middle and right columns, respectively).

Table I. Oligodeoxynucleotides used in this study[a]

1. 5'TAATACGACTCACTATAGGGTCAGCCACCCGCCACTGC3'
2. 5'TAATACGACTCACTATAGGGTGTCGGTGGCGCCAC3'
3. 5'AGCTGTTCCCATGCTTTCG3'
4. 5'AGCGGGTCCCATGCTTTCG3'
5. 5'AGCTGTTGGGTACGAATCGG3'
6. 5'TAATACGACTCACTATAGGGATAGTAAACCCCAGCTTAGG3'
7. 5'TAATACGACTCACTATAGGGCCCAGCTTAGGAAACAGG3'
8. 5'TAATACGACTCACTATAGGGATAGTAAACCAGCCACCCGCCAC3'
9. 5'TAATACGACTCACTATAGGGACAGCTCCCAGCTTAGGAAACAGG3'
10. 5'TAATACGACTCACTATAGGGATAGTAAACCGGGTCGAAAGGAAACAG3'
11. 5'TAATACGACTCACTATAGGGATAGTAAACCCCAGCTTTCCTTACAGGGTTG3'
12. 5'GTTTACTATCAGCTCACCAC3'
13. 5'AGCTGTTCAGCTCACCACTGG3'
14. 5'GTTTACTATCCCATGCTTTCGGCACAG3'
15. 5'AGCTGTTCCCATGCTTAGGAAACAGAGTC3'
17. 5'GCCTGTTGCACCCCTCAAGG3'
18. 5'GCCTGCACCCCTCAAGG3'
19. 5'TAATACGACTCACTATAGGG3'
20. 5'ATAGAATTCGTAATACGACTCACTAT3'
21. 5'ATATGCGCATGTGTGAGCCGAGTCCTGGGTG3'
22. 5'ATAAAGCTTGTAATACGACTCACTAT3'
23. 5'ATATGCGCAGGGGTGAGCCGAGTCCTGGGTG3'

[a]T7 promoter sequences are underlined.

Table II. snoRNAs used in this study

snoRNA	DNA templates	5' + 3' oligos
U65 wild-type	pFL45/SNR/U65/U24	1 + 3
r 5' PROX	U65-5'Br	2 + 3
r 5' DIST	U65-5'Ur	1 + 3
Δ 5' DIST	U65-5'Ud	1 + 3
Δ 5' PROX	U65-5'Bd	1 + 3
Δ Box H	U65-H	1 + 3
r 3' PROX	U65-3'Br	1 + 3
r 3' DIST	U65-3'Ur	1 + 3
Δ 3' DIST	U65-3'Ud	1 + 3
Δ 3' PROX	U65-3'Bd	1 + 5
Δ Box ACA	U65-ACA	1 + 4
U65-5'Hp-H	pFL45/SNR/U65/U24	1 + 12
U65-3'Hp-ACA	pFL45/SNR/U65/U24	7 + 3
U65-H-3'Hp-ACA	pFL45/SNR/U65/U24	6 + 3
U65-H-5'Hp-ACA	pFL45/SNR/U65/U24	8 + 13
U65-ACA-3'Hp-H	pFL45/SNR/U65/U24	9 + 14
r PROX	U65-3'Br	10 + 3
r DIST	U65-3'Ur	6 + 3
Δ DIST	U65-3'Ud	6 + 3
Δ PROX	U65-3'Bd	6 + 5
Δ LOOP	pFL45/SNR/U65/U24	6 + 15
r LOOP	Δ LOOP	11 + 15
U64 wild-type	pU64	19 + 17
U64 Δ Box H (all mutants)	pU64H series	19 + 17
U64 del Box ACA	pU64ACA	19 + 18

Plasmids encoding wild-type human U65 (pFL45/SNR/U65/U24) and mutated derivatives of U65 (previously called U65-5'Br, U65-5'Ur, U65-5'Ud, U65-5'Bd, U65-H, U65-3'Br, U65-3'Ur, U65-3'Ud, U65-3'Bd and U65-ACA) have been described previously (Bortolin *et al.*, 1999). Likewise, plasmids encoding wild-type human U64 (pU64) and mutant forms of human U64 (pU64H series and pU64ACA) were reported previously (Ganot *et al.*, 1997b). A plasmid encoding human U17a (pHU17; F.Dragon and W.Filipowicz, unpublished) was used to generate Hinge and ACA mutant constructs. In all cases, block substitution mutations (Δ) indicate that each nucleotide of a conserved Box element or one side of a stem structure was replaced with the complementary nucleotide. To restore (r) the stem structures, the sequences of the opposite side of the mutated stem were changed to their Watson–Crick complement. In a few cases, deletion mutants (del) were analyzed. A recombinant pUC19 plasmid carrying a full-length cDNA of the human telomerase RNA (hTR) (kindly provided by Professor W.Filipowicz, Friedrich-Miescher Institut, Basel, Switzerland) was used as a template for PCR amplification of cDNAs of the wild-type (oligos 20 and 21) and ACA→CCC mutant (oligos 22 and 23) hTR RNAs. The wild-type and mutant hTR cDNAs were inserted into the *Eco*RI–*Sma*I or *Hin*dIII–*Sma*I sites of pUC19, respectively. After *Esp*I digestion, the linear DNAs were used as templates for transcription with T7 RNA polymerase.

In vitro RNA synthesis

PCR products (100 ng) or linearized plasmids (1 μg) were used as templates for *in vitro* transcription. Wild-type U65, stem mutants and Box H and ACA mutants of U65, subfragments of U65, U64, and Box H and ACA mutants of U64 were transcribed from PCR-derived DNA fragments (details about the oligos and plasmids used are provided in Tables I and II). Linearized plasmids were used for the transcription of the ACA→GCA point mutant of U64 (*Bsa*HI), the 5' and 3' subfragments (5'-H and 3'-ACA) of U64 (*Pst*I), and wild-type and mutant U17 constructs (*Nae*I). All transcriptions of Box H/ACA snoRNAs were driven by a T7 promoter. *Xenopus* U8, U1 and U1sm$^-$, U3 and tRNA^{i}Met RNAs were transcribed *in vitro* as described previously (Narayanan *et al.*, 1999). The RNAs were labeled both with [^{32}P]GTP (800 Ci/mmol; ICN Radiochemicals) and fluorescein-12-UTP (Boehringer Mannheim) to allow simultaneous detection of the microinjected RNA both by autoradiography after purification and gel electrophoresis, and by fluorescence microscopy after nuclear spread preparation.

Injection of RNAs into Xenopus oocytes

Detailed protocols for microinjection and micromanipulation of *Xenopus* oocytes have been described previously (Terns and Goldfarb, 1998). Briefly, oocyte clusters were subjected to collagenase treatment (2 mg/ml collagenase in 1× MBSH minus calcium) for 90–120 min and washed well with 1× MBSH buffer. Model PL1-100 picoinjector microinjector (Medical Systems Corporation) and a glass needle with a 10 μm outer-diameter tip were used for microinjections. RNA samples for injection were prepared by drying using a Savant speed vacuum unit and resuspended in a filter-sterilized solution of blue dextran (20 mg/ml, 2×10^6 mol. wt; Sigma) in microinjection buffer (10 mM NaH_2PO_4 pH 7.2, 70 mM KCl, 1 mM $MgCl_2$). Stage V/VI oocytes were injected with 10 nl of solution containing 1 fmol of each of the test RNAs (fluorescently and ^{32}P labeled) and ~1 fmol each of the control RNAs (^{32}P labeled). Injected oocytes were maintained at 18°C in 1× MBSH buffer. Nuclear injections were monitored using the blue dextran in the injected solution, which turns nuclei blue (Jarmolowski *et al.*, 1994). The injected oocytes were then subjected to two different assays: nucleolar localization (described below) and gel electrophoresis (to determine the stability and nucleocytoplasmic distribution of the RNA). Gel electrophoresis of the injected RNA was carried out as described previously (Narayanan *et al.*, 1999). Briefly, RNA was prepared from nuclear (N) and cytoplasmic (C) fractions of injected oocytes, and one

oocyte equivalent of RNA was subjected to gel electrophoresis on an 8% denaturing gel (7 M urea) followed by autoradiography.

Nucleolar localization assay, indirect immunofluorescence and microscopy

Nuclear spreads were prepared as described previously (Gall *et al.*, 1991; Wu *et al.*, 1996; Narayanan *et al.*, 1999). Indirect immunofluorescence was performed on fixed nuclear spreads as described previously (Wu and Gall, 1997; Narayanan *et al.*, 1999). For the detection of endogenous fibrillarin, monoclonal antibody mAb 17C12 (Hultman *et al.*, 1994) was used at 1:1000 dilution [in 1× phosphate-buffered saline (PBS)]. For the detection of endogenous p80 coilin (coiled body marker protein), monoclonal antibody H1 directed against the *Xenopus* P80 coilin homologue (Tuma *et al.*, 1993) was used at 1:10 dilution (in 1× PBS). Texas Red-conjugated anti-mouse secondary antibodies (Jackson Immuno Research Laboratories, Inc.) at 1:150 dilution (in 1× PBS) were used for the detection of the primary antibodies.

A Zeiss Axiovert 100 inverted fluorescence microscope equipped with differential interference contrast optics was used for all observations. Images were acquired using a cooled-CCD camera (Quantix-Photometrics) and IP Laboratory Spectrum software.

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Nucleolar Factors Direct the 2′-O-Ribose Methylation and Pseudouridylation of U6 Spliceosomal RNA

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The nucleolus has long been known as a functionally highly specialized subnuclear compartment where synthesis, posttranscriptional modification, and processing of cytoplasmic rRNAs take place. In this study, we demonstrate that the nucleolus contains all the *trans*-acting factors that are responsible for the accurate and efficient synthesis of the eight 2′-O-methylated nucleotides and three pseudouridine residues carried by the mammalian U6 spliceosomal small nuclear RNA. Factors mediating the formation of pseudouridine residues in the U3 small nucleolar RNA are also present and functionally active in the nucleolus. For selection of the correct target nucleotides in the U6 and U3 RNAs, the nucleolar 2′-O-methylation and pseudouridylation factors rely on short sequences located around the target nucleotide to be modified. This observation further underscores a recently proposed role for small nucleolar guide RNAs in the 2′-O-methylation of the U6 spliceosomal RNA (K. T. Tycowski, Z.-H. You, P. J. Graham, and J. A. Steitz, Mol. Cell 2:629–638, 1998). We demonstrate that a novel 2′-O-methylated nucleotide can be generated in the yeast U6 RNA by use of an artificial 2′-O-methylation small nucleolar guide RNA. We also show that a short fragment of the 5.8S rRNA, when expressed as part of the human U6 RNA, is faithfully 2′-O-methylated and pseudouridylated. These results are most consistent with a trafficking pathway in which the U6 spliceosomal RNA cycles through the nucleolus to undergo nucleolar RNA-directed modifications.

In eukaryotes, most stable cellular RNAs undergo extensive posttranscriptional nucleoside modifications (5, 41, 62). For tRNAs, rRNAs, and small nuclear RNAs (snRNAs), about 90 different modified nucleotides have been identified (39). Despite the great structural diversity of modified residues, methylation of the backbone ribose at the 2′-hydroxyl position and conversion of uridine residues into pseudouridine residues represent the most abundant RNA modifications. The versatile hydrogen-bonding capacity of pseudouridines and the hydrophobic nature of 2′-*O*-methyl groups can modulate the three-dimensional structure of the RNA or fine-tune its interactions with other RNAs or proteins (36, 41, 55).

In tRNAs, modified nucleosides are important determinants of the specificity and efficiency of both aminoacylation and codon recognition (5, 81). The actual role of the large number of 2′-*O*-methyl groups and pseudouridines found in rRNAs and snRNAs is unknown. However, the fact that these modifications cluster around the functionally essential regions of these RNAs suggests that they contribute to RNA function (1, 41, 62). Consistent with this notion, a 2′-*O*-methyl group at G2251 in yeast mitochondrial 21S RNA has been found to be essential for the production of functional 50S ribosomal subunits (71). The importance of modifications in spliceosomal snRNAs has recently been underscored by the findings that in vitro-synthesized U2 snRNAs failed to reconstitute pre-mRNA splicing both in a U2-depleted HeLa cell splicing extract (66) and in *Xenopus* oocytes (57, 82).

A cell follows different strategies to accomplish the accurate synthesis of modified nucleosides in tRNAs, rRNAs, and snRNAs. The formation of pseudouridines and 2′-O-methylated nucleosides in tRNAs is catalyzed by protein enzymes which recognize the sequence and/or structure of the target site (3, 10, 38, 70). In 18S, 5.8S, and 28S rRNAs, the selection of more than 200 2′-O-methylation and pseudouridylation sites that occupy diverse sequence and structural environments is mediated by small nucleolar guide RNAs (snoRNAs). For each modification site, transient base-pairing interactions between a specific snoRNA and the target rRNA sequence occur. Methylation snoRNAs form 10- to 21-bp perfect double helices with rRNAs that are immediately followed by the conserved D or D′ box sequence motifs of the snoRNAs. In the snoRNA-rRNA double helix, the fifth ribosomal nucleotide upstream of the D or D′ box represents the target nucleotide for the methyltransfer reaction (11, 31, 32). Pseudouridylation snoRNAs take part in two short interactions with rRNA sequences that precede and follow the target uridine residue. Normally, in the pseudouridylation snoRNA-rRNA interaction, the substrate uridine is located 14 nucleotides (nt) upstream of the ACA or H box motif of the snoRNA (8, 16, 52). A pseudouridine synthase and a methyltransferase enzyme, most probably the Nap57p/Cbf5p (34) and fibrillarin/Nop1 (53, 75) proteins, that are directly or indirectly bound to the H/ACA or C/D box elements of the snoRNAs catalyze the nucleoside modification reaction. Therefore, the formation of numerous ribosomal pseudouridines and 2′-O-methylated nucleotides most likely is catalyzed by a single pseudouridine synthase and a methyltransferase enzyme.

Less is known about the generation of pseudouridines and 2′-O-methylated residues in spliceosomal snRNAs. In vitro pseudouridylation experiments suggest that multiple pseudouridine synthase activities direct the pseudouridylation of the polymerase II-synthesized U1, U2, and U5 snRNAs (59, 60). Since maturation of these snRNAs proceeds via a cytoplasmic phase (20, 47, 58), it remains unclear whether their modification occurs in the nucleoplasm or the cytoplasm. The U6

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snRNA, an RNA polymerase III product (12, 21), represents the most conserved and most extensively modified spliceosomal RNA (62). Microinjection experiments indicated that U6 does not leave the nucleus of *Xenopus* oocytes, suggesting that its maturation occurs in the nucleus (80). Unexpectedly, it has recently been found that 2′-O-methylation of U6 snRNA at C-77 and perhaps at A-47 is directed by C and D box-containing methylation snoRNAs (79).

The nucleolus has long been considered a subnuclear compartment that is devoted to the maturation of cytoplasmic rRNAs (18). The formation of mature rRNAs, including 2′-O-ribose methylation, pseudouridylation, and nucleolytic processing of the newly synthesized precursor rRNA is assisted by many snoRNAs (48, 72, 73, 77). Here, we demonstrate that factors directing the correct posttranscriptional modification of mammalian U6 snRNA at the eight known 2′-O-methylation and three pseudouridylation sites are present and functionally active in the nucleolus.

MATERIALS AND METHODS

General procedures and oligos. Unless otherwise noted, all cloning and nucleic acid manipulation procedures were performed according to standard laboratory protocols (65). The identity of all constructs was confirmed by sequence analyses. The oligodeoxynucleotides (oligos) used in this study and their sequences are as follows: 1, CTAGTACTAAAATTGGAACGATACAGAGAA; 2, TCGATTCTCTGTATCGTTCCAATTTTAGTA; 3, CTAGAGAAGATTAG CATGGCCC; 4, TCGAGGGCCATGCTAATCTTCT; 5, CTAGATGGCCCC TGCGCAAGGATGACA; 6, TCGATGTCATCCTTGCGCAGGGGCCAT; 7, CTAGAGATGACACGCAAATTCGTGAAGCGC; 8, TCGAGCGCTTCACG AATTTGCGTGTCATCT; 9, CTAGAGTGTAGTATCTGTTCTTATCAGC; 10, TCGAGCTGATAAGAACAGATACTACACT; 11, CTAGAAAGACTAT ACTTTCAGGGATCAC; 12, TCGAGTGATCCCTGAAAGTATAGTCTTT; 13, CTAGATTAATGTGAATTGCAGGACACATGACTAGTC; 14, TCGAG ACTAGTCATGTGTCCTGCAATTCACATTAAT; 15_{XhoI}, TTTCTCGAGCC CCAGTGGAAAGACGCGCAG; 16_{SspI}, CCCAATATTGGAACGCTTCACG AATTTGCG; 17_{SpeI}, TTTACTAGTAATATTTTTACATCAGGTTG; 18_{SacI}, TTTGAGCTCTGGTAAACCGTGCACCGGCG; 19 ATTAATGTGAATTGC AGGACACAGA; 20, CTAGTCTGTGTCCTGCAATTCACATTAAT; 21_{SacI}, CTCAATATTATGTGCTGCCGAAGCG; 22_{SpeI}, CTCACTAGTCATACTAA AATTGGAACGATACA; 23_{XhoI}, TTCTCGAGTAGCTGGGACTACAGAC GG; 24_{BclI}, TTTTGATCACTATAGAAATGATCCCTG; 25_{SpeI}, TTTACTAG TGTTACTAGAGAAGTTTCTC; 26_{KpnI}, TTTGGTACCTTTCTCGCGACAT TGCCAAGC; 27, GATCATTAATGTGAATTGCAGGACACATGA; 28, CTA GTCATGTGTCCTGCAATTCACATTAAT; 29, GATCCTTAATGTGAATT GCAGGACACATGACTAGTG; 30, GATCCACTAGTCATGTGTCCTGCA ATTCACATTAAG; 31_{KpnI}, TTTGGTACCTGGTGCATCAGTTTGGTCAAT TTGATTAAAATGTCATCA; 32_{XhoI}, ATACTCGAGTGTGCAGATGATGT AAAAG; 33, CCAGCTCAAGATCGTAATAT; 34, GTTATTACATCATTT GA; 35_{XbaI}, TGCTCTAGAGTGCTCGCTTCGGCAGC; 36_{XhoI}, CCGCTCGA GAAAATATGGAACGCTTCAC; 37_{XbaI}, TGCTCTAGATCCCAATGATGA GTTGCCATGC; 38_{XhoI}, CCGCTCGAGACCCCTCAGATCTTCATGTGAG; 39, AAAATATTACTAGTCTGTG; 40, ATTTTAGTATGACTAGTCTGTG; 41, GTGGACGGAGCAACTAGTCATG; 42, TTCTCTAGTAACACTAGTC ATG; 43, TTCTCAGGATCCACTAGTCATG; 44, CCAGTGATTTTTTTCTC CATTTTAGC; 45, GTCTTCAAAGTTCTCATTTG; and 46, ACTGCTGATC ATCTCTGTATTG.

Construction of plasmids and transfection of mammalian cells. To generate the pW-*U6-1* expression construct, appropriate oligos (oligos 1 and 2) were annealed and inserted into the *Xba*I and *Xho*I sites of recombinant plasmid pW(Xb/Xh) carrying the mouse ribosomal minigene (16, 19). The same strategy was used to obtain pW-*U6-2* (oligos 3 and 4), pW-*U6-3* (oligos 5 and 6), pW-*U6-4* (oligos 7 and 8), pW-*U2* (oligos 9 and 10), pW-*U3* (oligos 11 and 12), and pW-*5.8S* (oligos 13 and 14). Transfection of mouse L929 (American Type Culture Collection [ATCC] CCL1) cells was achieved by use of the DEAE-dextran method (67).

To construct pGL/U6-*5.8S*(3′), two contiguous fragments of the human U6 gene (33) (GenBank accession no. M14486) from positions −328 to +100 and from positions +101 to +199 were PCR amplified with *Vent* DNA polymerase (New England BioLabs), human genomic DNA as a template, and oligo primers 15_{XhoI}/16_{SspI} and 17_{SpeI}/18_{SacI}, respectively. The amplified fragments were digested with appropriate endonucleases and joined in a quadrimolecular ligation reaction in the presence of the *Xho*I- and *Sac*I-digested pGL2 promoter vector (Promega) and annealed oligos 19 and 20, which represented a fragment of the human 5.8S rRNA gene from positions 62 to 84 and formed *Ssp*I- and *Spe*I-compatible termini. The same approach was used to construct pGL/U6-*5.8S*(5′) and pGL/U3-*5.8S*. For amplification of the 5′ and 3′ halves of the human U6 gene from positions −328 to +122 and from positions +123 to +199, oligos 15_{XhoI}/21_{SacI} and 22_{SpeI}/18_{KpnI} were used as specific primers, respectively. The amplified fragments were digested and connected via ligation of annealed oligos 19 and 20 to the PCR-introduced *Ssp*I and *Spe*I sites. The 5′ and 3′ halves of the human U3 gene (83) (GenBank accession no. X14945) from positions −478 to +33 and from positions +34 to +313 were PCR amplified with oligos 23_{XhoI}/24_{BclI} and 25_{SpeI}/26_{KpnI}, respectively. Annealed oligos 27 and 28 carrying 5.8S-specific tag sequences were inserted between the PCR-introduced *Bcl*I and *Spe*I sites of the U3 gene fragments. The resulting 5.8S-tagged U6 and U3 genes were cloned into the *Xho*I/*Sac*I and *Xho*I/*Kpn*I sites of the pGL2 promoter vector, respectively. To generate pG-*5.8S*, annealed oligos 29 and 30 were inserted into the *Bam*HI site of the pG expression construct (29). Transfection of COS-7 (ATCC CRL 1651) cells was performed with DOTAP [*N*-[1-(2,3-dioleoyloxy) propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate] transfection reagent (Boehringer) according to the manufacturer's instructions.

Cell fractionation. Fractionation of transfected simian COS-7 and mouse cells as well as human HeLa cells was performed as described earlier (27, 78).

Construction of plasmids for transformation of yeast cells. The pFL45/ACT/U24 yeast expression construct has already been described (32). To generate pFL45/ACT/U24-6, the coding region of the human U24 snoRNA gene was PCR amplified with oligos 31_{KpnI} and 32_{XhoI} as 3′ and 5′ end-specific primers, respectively. The resulting U24-6 fragment was inserted into the *Xho*I and *Kpn*I sites of the pFL45/ACT expression construct (32). Transformation of the yeast Δ*U24* strain (a *trp1* Δ *his3* Δ *ura3,52 lys2,801 ade2,101 URA3*::*U24*) (31) was performed by the lithium acetate transformation procedure (22).

RNA analysis. Total RNAs from human HeLa, mouse L929, and simian COS-7 cells (17) and yeast cells (76) were isolated by guanidinium thiocyanate-phenol-chloroform extraction. For Northern analysis, 10 μg of yeast cellular RNAs was separated on a 6% sequencing gel, electroblotted onto a Hybond-N nylon membrane, and probed with a mixture of 5′-end-labeled oligos complementary to the yeast snR36 (oligo 33) and human U24 (oligo 34) snoRNAs. RNase A and T_1 mapping was performed as described previously (17). Generation of a sequence-specific antisense RNA probe for human U3 snoRNA has been reported elsewhere (15, 27). To obtain RNA probes for the U6-*5.8S*(5′) and U6-*5.8S*(3′) RNAs, the *Xho*I/*Sac*I fragments of the pGL/U6-*5.8S*(5′) and pGL/U6-*5.8S*(3′) constructs were inserted into the same sites of pBluescript KS (Stratagene). The resulting pU6-*5.8S*(5′) and pU6-*5.8S*(3′) plasmids were linearized with *Xho*I and used as templates for the synthesis of antisense RNA probes by use of T7 RNA polymerase. To generate probes for the human U6 and mgU6-53 RNAs, the coding regions of the U6 (oligos 35_{XbaI} and 36_{XhoI}) and mgU5-53 (oligos 37_{XbaI} and 38_{XhoI}) snRNAs were PCR amplified with human genomic DNA as a template. The resulting DNA fragments were inserted into the *Xba*I/*Xho*I sites of pBluescript KS, linearized with *Xho*I, and transcribed by use of T3 RNA polymerase. To generate an antisense probe for the W-*U6-1* mouse minigene transcript, the *Pst*I/*Eco*RI fragment of pW-*U6-1* encompassing the full-length ribosomal minigene was subcloned into the same sites of pBluescript KS, linearized with *Hin*dIII, and transcribed by use of T7 RNA polymerase.

Mapping of 2′-O-methylated nucleotides and pseudouridine residues. Detection of 2′-*O*-ribose-methylated nucleotides and pseudouridine residues was performed by primer extension analyses as described by Maden et al. (42) and Bakin and Ofengand (2), respectively. Terminally labeled oligos 39 [for U6-*5.8S*(5′) RNA], 40 [for U6-*5.8S*(3′) RNA], 41 [for human U2 snRNA], 42 (for U3-5.8S RNA), 43 (for G-*5.8S* RNA), 44 (for mouse ribosomal minigene transcripts), 45 (for yeast 25S rRNA), and 46 (for yeast U6 snRNA) complementary to the appropriate target RNAs were used as primers for reverse transcription. The extended DNA products were analyzed on 6% sequencing gels.

RESULTS

A putative guide snoRNA for 2′-O-methylation of human U6 snRNA at Am53. During characterization of a cDNA library of human snoRNAs (31), we identified a 109-nt novel small RNA (Fig. 1A). Cell fractionation experiments demonstrated that the newly identified RNA, like the authentic U3 snoRNA (78), copurifies with the nucleolar fraction of human HeLa cells (Fig. 1B, upper panel, lane 5) and is absent from the nucleoplasmic fraction, where the U6 spliceosomal snRNA accumulates (lane 4). The new RNA features all of the structural elements, the box C, C′, D, and D′ motifs and a short 5′-, 3′-terminal helix, that are essential for the expression, nucleolar localization, and function of 2′-O-methylation snoRNAs (31, 32, 37, 64). However, the novel putative 2′-O-methylation snoRNA lacks a significant sequence complementarity to rRNA sequences, indicating that it cannot function in rRNA methylation.

Recently, it was shown that 2′-O-methylation of the Cm77 residue of human U6 spliceosomal snRNA, upon injection into

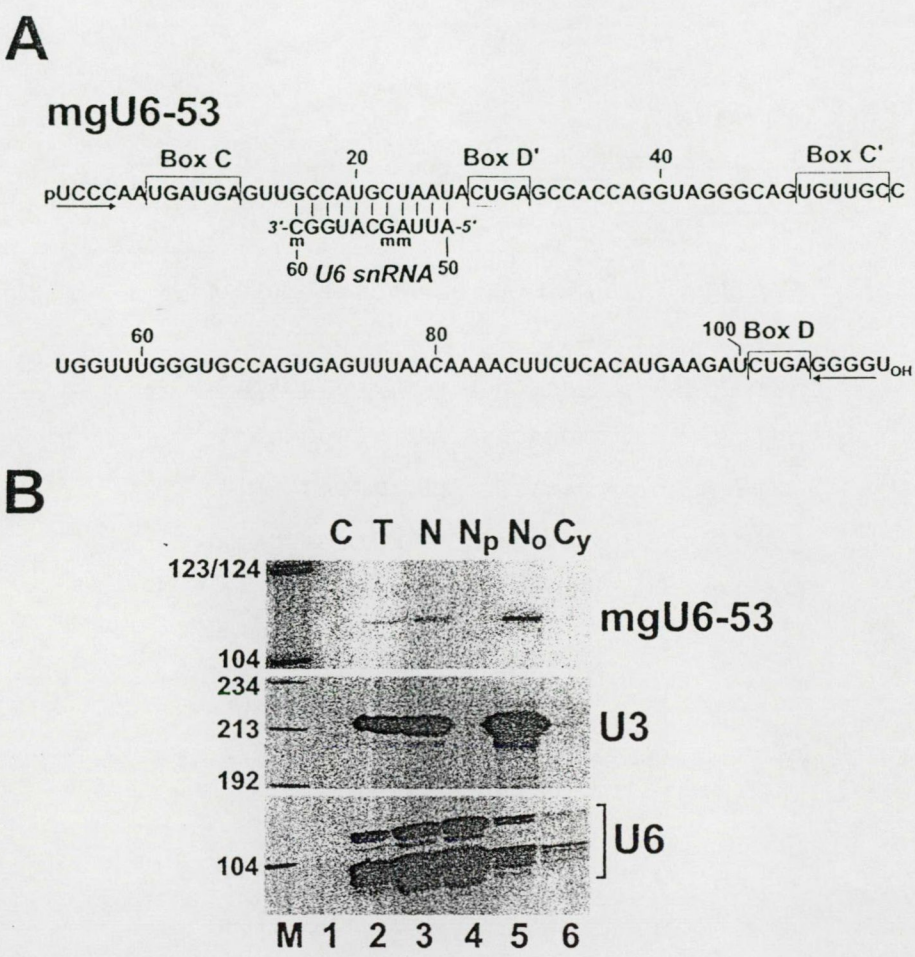
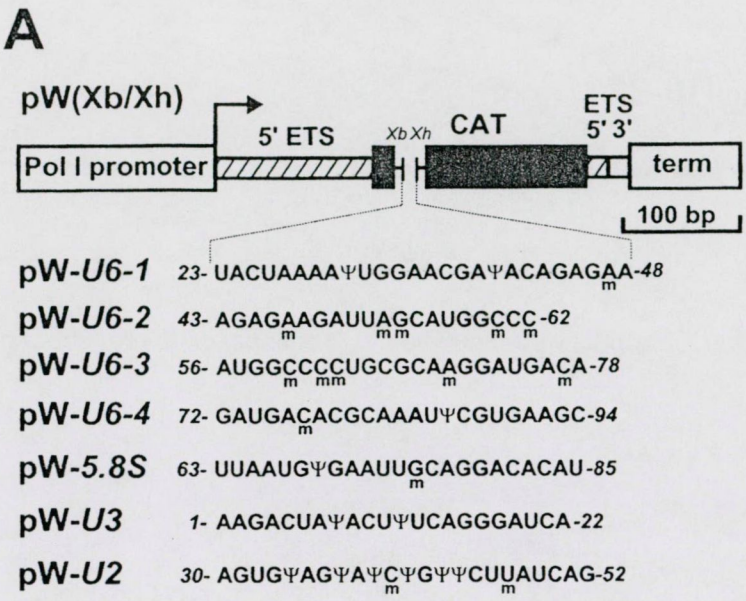


FIG. 1. Human mgU6-53 is a novel C and D box-containing snoRNA that possesses sequence complementarity to U6 spliceosomal RNA. (A) Nucleotide sequence of mgU6-53 snoRNA. The conserved sequence box motifs of 2′-O-methylation snoRNAs are indicated. Sequences potentially involved in the formation of a 5′-, 3′-terminal helix are indicated by inverted arrows. Sequences of the human U6 snRNA that are complementary to the mgU6-53 snoRNA sequence are shown. Nucleotides carrying 2′-O-methyl groups are indicated (m). The nucleotide sequence of mgU6-53 has been deposited in the EMBL database under accession no. AJ243222. (B) Subcellular localization of mgU6-53 snoRNA. RNA samples (200 ng) extracted either from human HeLa cells (T) or from nuclear (N), nucleoplasmic (Np), nucleolar (No), and cytoplasmic (Cy) fractions of HeLa cells were mapped by RNase A and T_1 protection by use of sequence-specific antisense RNA probes as indicated on the right. Lane C represents a control mapping with *Escherichia coli* tRNA. Lane M, size markers in nucleotides (a mixture of *Hae*III- and *Taq*I-digested pBR322).

Xenopus oocytes, is dependent on a C and D box-containing methylation snoRNA called mgU6-77 (methylation guide for U6 RNA at position 77) (79). Another C and D box-containing snoRNA (mgU6-47) was implicated in the synthesis of the 2′-O-methylated Am47 residue in U6 snRNA (79). A closer examination of our new C and D box-containing snoRNA revealed that it carries an 11-nt sequence that is perfectly complementary to the human U6 snRNA from positions 50 to 60 (Fig. 1A). A putative base-pairing interaction between the snoRNA and the U6 snRNA places the D′ box of the snoRNA 5 bp upstream of the A53 residue that is known to be 2′-O-methylated in mammalian U6 snRNAs (62). We therefore concluded that the novel RNA, now termed the mgU6-53 snoRNA, represents a new member of the group of C and D box-containing snoRNAs and is likely to direct the 2′-O-methylation of U6 snRNA.

Factors directing 2′-O-methylation and pseudouridylation of U6 and U3 snRNAs are functional in the nucleolus. Implication of the mgU6-47, mgU6-77, and mgU6-53 snoRNAs in 2′-O-methylation of the U6 spliceosomal snRNA raises the possibility that posttranscriptional modification of the U6 RNA takes place in the nucleolus. We therefore tested whether factors supporting the 2′-O-methylation and pseudouridylation of U6 snRNA are present in the nucleolus. Short overlapping fragments of the rat U6 snRNA, *U6-1* to *U6-4* (Fig. 2A), encompassing its eight 2′-O-methylation (m) and three pseudouridylation (Ψ) sites (62), were inserted into the pW(Xb/Xh) mouse ribosomal minigene (19). As controls, fragments of the human 5.8S rRNA (from U63 to U85), U2 snRNA (from A30 to G52), and U3 snoRNA (from A1 to A22) were inserted into the pW(Xb/Xh) minigene. Each fragment contained residues that are 2′-O-methylated and/or pseudouridylated in wild-type RNAs (Fig. 2A) (41, 62). Upon transfection into mouse cells, the polymerase I-directed transcription of the ribosomal minigene occurs in the nucleolus (18, 50), and the resulting RNA transcript accumulates in the nucleolus (16, 19). Indeed, cell fractionation experiments followed by RNase A and T_1 mapping and phosphorimager quantification showed



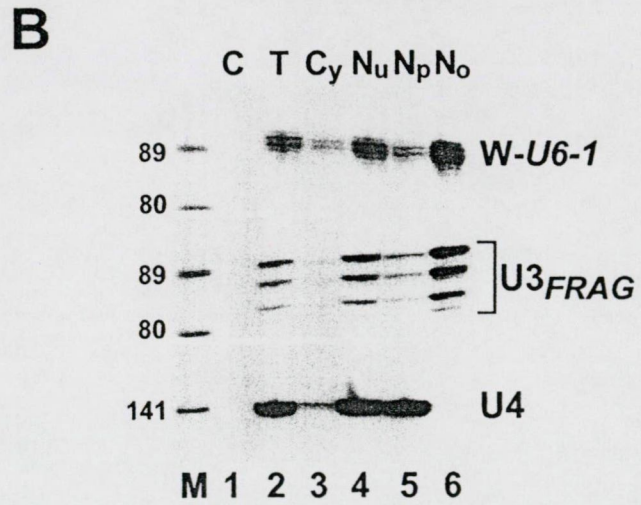


FIG. 2. Nucleolar expression of the mouse ribosomal minigene tagged with human U6, U2, U3, and 5.8S RNA-specific sequences. (A) Schematic structure of the pW(Xb/Xh) mouse ribosomal minigene construct. pW(Xb/Xh) contains the mouse polymerase I (Pol I) promoter and terminator (term), some 5′ (hatched boxes) and 3′ (open box) external transcribed spacer (ETS) sequences, and a fragment of the chloramphenicol acetyltransferase (CAT) gene that carries the *Xba*I (Xb) and *Xho*I (Xh) restriction sites. The pW-*U6-1*, pW-*U6-2*, pW-*U6-3*, pW-*U6-4*, pW-*U5.8S*, pW-*U3*, and pW-*U2* constructs were created by insertion of appropriate synthetic DNA fragments into the *Xba*I and *Xho*I sites of pW(Xb/Xh). Nucleotides which are 2′-O-methylated (m) or pseudouridylated (Ψ) in rat U6, U3, and U2 snRNAs and human 5.8S rRNA are indicated. (B) Subcellular localization of the W-*U6-1* ribosomal minigene transcript. Upon transfection of the pW-*U6-1* minigene into mouse cells, RNA samples (200 ng) extracted either from total cells (T) or from cytoplasmic (C_y), nuclear (N_u), nucleoplasmic (N_p), and nucleolar (N_o) fractions were analyzed by RNase A and T_1 protection by use of antisense RNA probes specific to the W-*U6-1* transcript (upper panel), U3 snoRNA (middle panel), or U4 snRNA (lower panel). Lane C represents a control mapping with *E. coli* tRNA. Lane M, size markers in nucleotides.

that between 90 and 96% of the minigene transcripts studied in our experiments localized to the nucleolus of transfected mouse cells. As a representative example, the intracellular distribution of the W-*U6-1* transcript is shown in Fig. 2B. The small amount of W-*U6-1* RNA detected in the nucleoplasmic fraction derived most likely from cross-contaminating nucleoli, since the nucleolar U3 RNA was also detectable in this fraction (Fig. 2B, lane 5).

The state of 2′-O-methylation and pseudouridylation of the minigene transcripts was observed by primer extension analyses with a ^{32}P-labeled deoxyoligonucleotide primer complementary to the minigene RNA downstream of the insertion site. In the presence of a low concentration of deoxynucleoside triphosphates, (0.004 mM), reverse transcriptase stops 1 nt before and/or at the 2′-O-methylated nucleotide (31, 32, 42). Pseudouridine residues were visualized by primer extension

analysis with CMC [N-cyclohexyl-*N'*-β-(4-methyl-morpholinium) ethylcarbodiimide *p*-tosylate]-treated RNAs (2). CMC reacts irreversibly with N3 of pseudouridine and stops reverse transcriptase 1 nt before the pseudouridylation site.

As expected (16), the 5.8S rRNA sequences in the nucleolar W-*5.8S* transcript were faithfully 2'-O-methylated at G75 (Fig. 3A, lane 10) and pseudouridylated at U69 (Fig. 3B, lane 9). More interestingly, the U6-specific sequences expressed in the nucleolus were accurately 2'-O-methylated at A47, A53, G54 (Fig. 3B, lane 2), C60, C62 (lanes 2 and 4), C63, A70 (lane 4), and C77 (lane 6) as well as pseudouridylated at U31, U40 (lane 1), and U85 (lane 3). The same results were obtained when 2'-O-methylation of the W-*U6-3* RNA and pseudouridylation of the W-*U6-1* RNA were monitored with RNA samples obtained from the nucleolar fraction of transfected mouse cells (data not shown). Both pseudouridine residues of the U3 snoRNA (Ψ8 and Ψ12) (62) were accurately formed in the nucleolar W-*U3* transcript (Fig. 3B, lane 5). However, neither ribose methylation nor pseudouridine formation was observed in the U2-specific tag sequences (Fig. 3A, upper panel, lane 8, and Fig. 3B, lane 7). To ensure that the lack of stop signals in the primer extension mapping of 2'-O-methyl groups in the W-*U2* transcript was not due to the special sequence context of the U2 RNA, we performed control mapping with the human U2 snRNA (Fig. 3A, lower panel, lanes 7 and 8). No RNA modification was detected when another fragment of the U2 RNA (from A48 to U70) carrying two known pseudouridylation sites (Ψ54 and Ψ58) and one 2'-O-methylation site (Am61) was tested (data not shown). Likewise, primer extension analyses of the W-*U6-1* and W-*U6-3* transcripts revealed no stop signals that could have indicated the formation of the base-methylated m^6A43 and m^2G72 nucleotides that are present in the wild-type U6 snRNA (62) (Fig. 3A, lanes 3 and 4, and Fig. 3B, lane 1).

These results demonstrate that *trans*-acting factors directing the site-specific 2'-O-methylation and pseudouridylation of the U6 spliceosomal snRNA and pseudouridylation of the U3 snoRNA are present and are functionally active within the nucleolus. The fact that short fragments of the U6 snRNA and the U3 snoRNA are faithfully methylated and/or pseudouridylated in the nucleolus lends further support to the assumption that modification of these snRNAs is directed by snoRNAs. The snoRNA-directed synthesis of 2'-O-methylated nucleotides requires a 10-bp interaction between the snoRNA and the substrate RNA (11, 31). Since the target nucleotide is located in the middle of this interaction, the synthesis of Cm60 and Cm62 residues lacking U6-specific 3'-flanking sequences in the W-*U6-2* transcript (Fig. 3A, lane 2) was unexpected. However, we noticed that minigene sequences following the *U6-2* sequences are capable of extending a base-pairing interaction with a putative snoRNA up to 12 bp with two mismatches (GGCCCctcgaga; the authentic U6 sequences are shown in uppercase letters).

5.8S rRNA-specific tag sequences carried by U6 RNA are faithfully modified. In view of the above results, it seems possible that the U6 snRNA cycles through the nucleolus for snoRNA-mediated nucleotide modification. The snoRNA-directed synthesis of ribose-methylated nucleotides and pseudouridine residues in the 18S, 5.8S, and 28S rRNAs occurs within the nucleolus shortly after or during the synthesis of precursor rRNA (13, 41). In accordance with this fact, rRNA methylation and pseudouridylation snoRNAs show an exclusive nucleolar localization (48). To assess whether the U6 snRNA can establish an interaction with the rRNA modification snoRNAs, a short fragment of the human 5.8S rRNA containing the pseudouridylated U69 and 2'-O-methylated G75 residues (41) was inserted into the 5'- or 3'-terminal part of the coding region of the human U6 snRNA gene (33) (Fig. 4A). The same 5.8S tag was inserted into the human U3 snoRNA gene (83) as well as into the second exon of the human β-globin gene (29). Upon transfection of the 5.8S-tagged genes into simian COS-7 cells, RNase A and T_1 mapping performed with sequence-specific antisense probes revealed that the chimeric U6-*5.8S(5')* and U6-*5.8S(3')* RNAs (Fig. 4B, lanes 2 and 4) and U3-*5.8S* and GL-*5.8S* RNAs (data not shown) were correctly and efficiently expressed. The observed heterogeneity of the U6-*5.8S(5')*- and U6-*5.8S(3')*-specific protected fragments likely represents RNase mapping artifacts, since fragments protected by the 3'-terminal part of the endogenous U6 snRNA also appeared as a doublet (Fig. 4B, lane 3) and primer extension revealed a unique 5' terminus for both U6-*5.8S(5')* and U6-*5.8S(3')* (Fig. 5B, lanes 1 and 3).

Primer extension mapping of 2'-O-methylated nucleotides in the U6-*5.8S(5')* and U6-*5.8S(3')* RNAs in the presence of 0.004 mM deoxynucleoside triphosphates (Fig. 5A, lanes 2 and 4) resulted in stop signals at the G75 residue, indicating that the 5.8S tag sequence is correctly methylated in both chimeric U6-*5.8S* RNAs. Mapping of pseudouridines revealed that the U69 residue was converted into pseudouridine in both U6-*5.8S(5')* and U6-*5.8S(3')* (Fig. 5B, lanes 1 and 3). Moreover, mapping of the 5'-terminal region of the U6-*5.8S(3')* RNA showed that 2'-O-methylated nucleotides and pseudouridines found in the rat U6 snRNA (62) were also present in the chimeric U6-*5.8S(3')* RNA (Fig. 5A, lanes 5 and 6, Fig. 5B, lane 4, and data not shown), indicating that not only the 5.8S but also the U6-specific sequences were correctly modified in the chimeric U6-*5.8S(3')* RNA. Likewise, when expressed as part of the U3-*5.8S* RNA, the 5.8S tag sequences were correctly 2'-O-methylated at G75 (Fig. 5A, lane 8) and pseudouridylated at U69 (Fig. 5B, lane 5). Pseudouridines found in the rat U3 snoRNA (Ψ8 and Ψ12) were also readily detectable in the chimeric U3-*5.8S* RNA (Fig. 5B, lane 5). However, in marked contrast to the findings for the U6-*5.8S* and U3-*5.8S* RNAs, no 2'-*O*-methyl group and no pseudouridine residue were detected in the 5.8S tag sequences carried by the cytoplasmic β-globin mRNA (Fig. 5A, lane 10, and Fig. 5B, lane 7).

These results demonstrate that the chimeric U6-*5.8S* and U3-*5.8S* RNAs, when expressed in simian cells, undergo correct posttranscriptional modifications. This finding indicates that these RNAs are able to establish physical contacts with rRNA methylation and pseudouridylation snoRNAs as well as with all the factors that are responsible for the site-specific 2'-O-methylation and pseudouridylation of U6 and U3 RNAs.

Targeted 2'-O-methylation of yeast U6 snRNA is directed by an artificial snoRNA. By use of artificial 2'-O-methylation snoRNAs that carry properly designed rRNA recognition motifs, novel 2'-O-methylation sites can be generated in eukaryotic rRNAs (11, 14, 30, 31). We tested whether site-specific 2'-O-methylation of the yeast *Saccharomyces cerevisiae* U6 snRNA can be achieved by an artificial snoRNA that carries an antisense element complementary to the U6 snRNA.

When expressed in yeast cells, the human U24 snoRNA can direct 2'-O-methylation of the 25S rRNA at two positions (32). The upstream antisense element (UAE) directs the methylation of C1450, and the downstream antisense element (DAE) selects the C1436 residue (Fig. 6A). The DAE of human U24 was replaced with sequences complementary to the yeast U6 snRNA from U27 to A40. The resulting U24-6 snoRNA is predicted to direct the 2'-O-methylation of residue G31 in yeast U6 RNA in addition to residue C1450 in 25S rRNA. DNA fragments encoding the mutant U24-6 and wild-type U24 snoRNAs were inserted into the intron region of the yeast

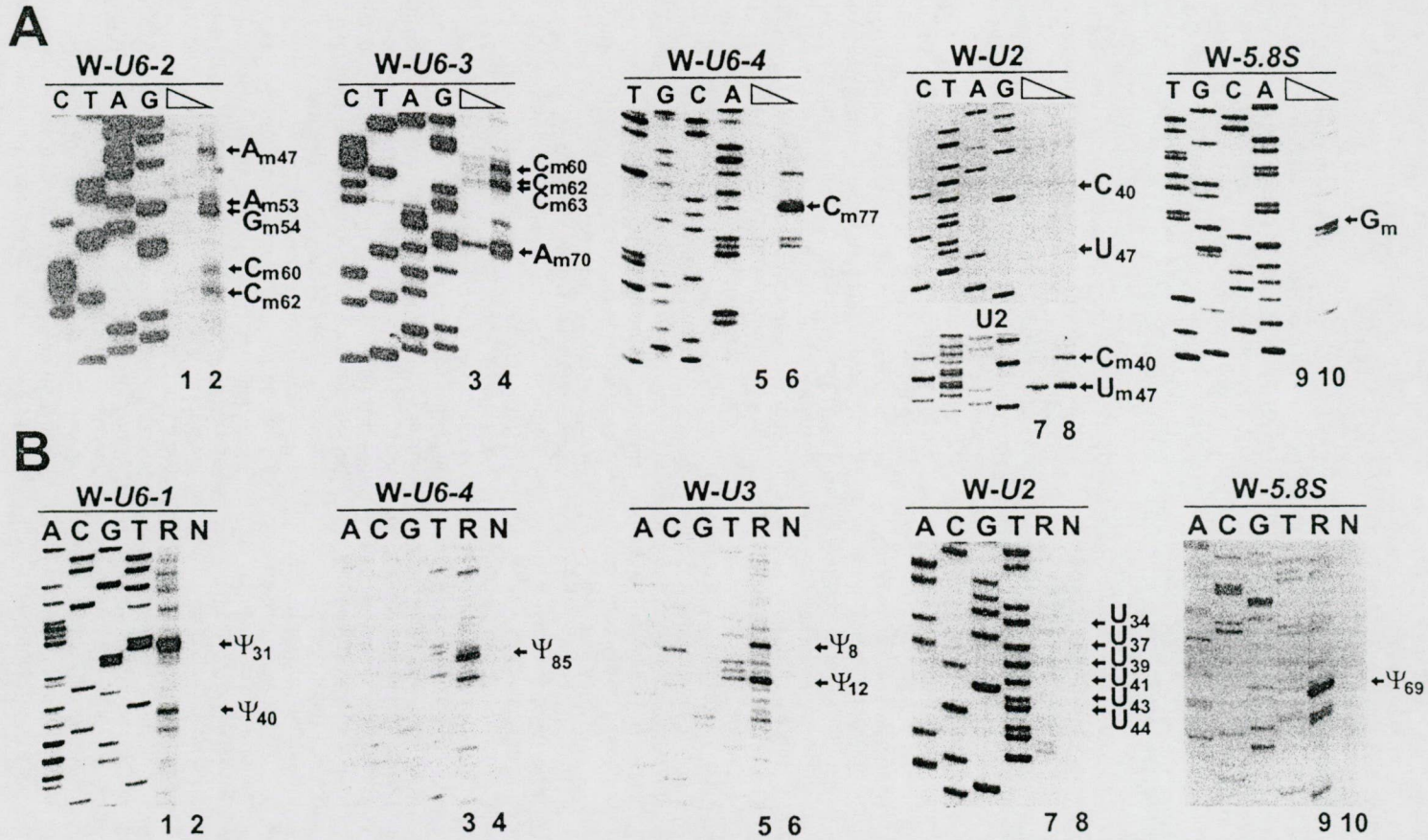


FIG. 3. Primer extension mapping of modified nucleotides in mouse ribosomal minigene transcripts. (A) Mapping of 2′-O-methylated nucleotides. RNAs isolated from mouse cells transfected with the pW-*U6-2*, pW-*U6-3*, pW-*U6-4*, pW-*U2*, or pW-*U5.8S* construct were analyzed by primer extension in the presence of a high (1 mM) or a low (0.004 mM) concentration of deoxynucleoside triphosphates. Panel U2 shows mapping of 2′-O-methyl groups in the wild-type human U2 snRNA. Lanes C, T, A, and G represent sequencing ladders. The extension products were fractionated on 6% sequencing gels. (B) Mapping of pseudouridines. RNAs obtained from mouse cells transfected (R) or not transfected (N) with the pW-*U6-1*, pW-*U6-4*, pW-*U3*, pW-*U2*, or pW-*U5.8S* expression construct were subjected to CMC-alkali treatment. The modified pseudouridine-CMC residues were visualized by primer extension.

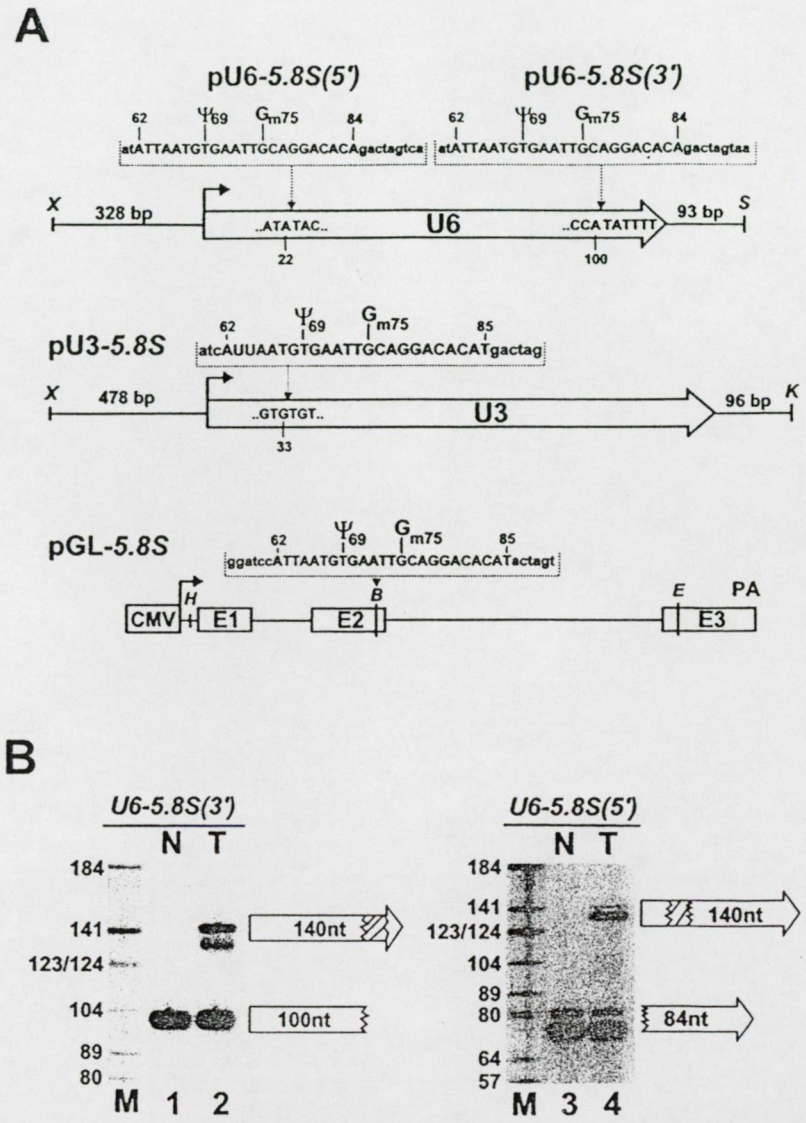


FIG. 4. Expression of human U6 snRNA, U3 snoRNA, and β-globin mRNA tagged with 5.8S-specific sequences. (A) Schematic structures of the expression constructs used for the transfection of COS-7 cells. The coding regions of human U6 and U3 snRNA genes are represented by open arrows. The cytomegalovirus promoter (CMV), three exons (E1 to E3), and the polyadenylation region (PA) of the human β-globin gene are indicated for pGL-*5.8S*. The inserted tag sequences and their positions as well as relevant restriction sites (*B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*dIII; *K*, *Kpn*I; *S*, *Sac*I; *X*, *Xho*I) are indicated above the constructs. Nucleotides representing authentic 5.8S rRNA sequences are in uppercase letters. Nucleotides introduced to facilitate cloning are in lowercase letters. The U69 and G75 residues that are pseudouridylated or 2′-O-methylated in human 5.8S rRNA are marked. (B) Accumulation of U6-*5.8S*(5′) and U6-*5.8S*(3′) RNAs in COS-7 cells. RNAs extracted from transfected (T) and nontransfected (N) cells were analyzed by RNase A and T_1 mapping by use of antisense RNA probes specific for either the U6-*5.8S*(5′) or the U6-*5.8S*(3′) RNA. The protected fragments were separated on a 6% sequencing gel. Lane M, size markers.

alcohol dehydrogenase gene and transformed into a yeast strain that lacks a functional U24 locus (ΔU24) (31, 32). Northern analysis showed that the U24 and U24 snoRNAs were correctly expressed in yeast (Fig. 6B).

The 2′-O-methylation pattern of the 25S rRNA (Fig. 6C) and the U6 snRNA (Fig. 6D) was tested by primer extension analyses. The U24-6 snoRNA (Fig. 6C, upper panel, lane 6), in contrast to the wild-type U24 snoRNA (lane 4), did not support the DAE-dependent methylation of 25S rRNA at C1436. The U24-6 snoRNA, like the wild-type U24 snoRNA, directed the UAE-dependent methylation of the C1450 residue (Fig. 6C, lower panel). More interestingly, in the ΔU24 yeast strain expressing the U24-6 snoRNA, the U6 RNA was 2′-O-methylated at the G31 position (Fig. 6D, lane 4), demonstrating that 2′-O-methylation snoRNAs can direct the site-specific methylation of the U6 spliceosomal snRNA. These results also show that the UAE and DAE of a methylation snoRNA can independently direct 2′-O-methylation of an snRNA and an rRNA (see also reference 79).

DISCUSSION

The rat U6 spliceosomal snRNA carries eight ribose- and two base-methylated nucleosides and three pseudouridines

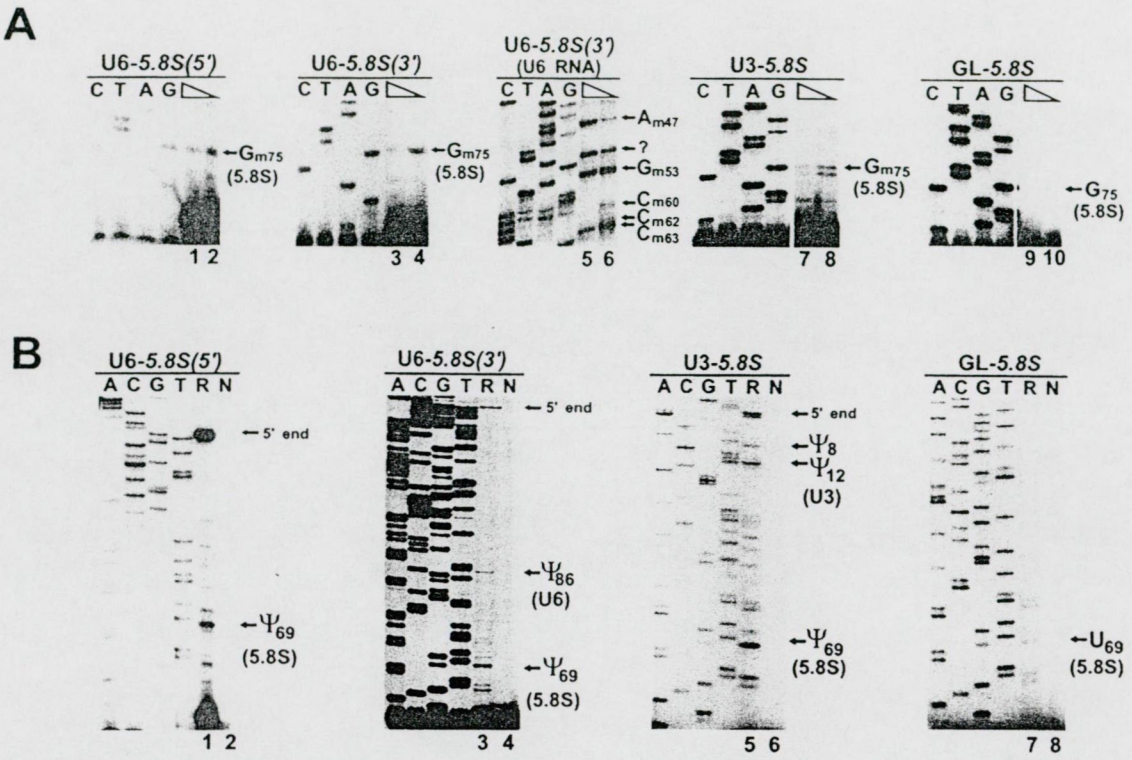


FIG. 5. Primer extension mapping of modified nucleotides. (A) Mapping of 2′-O-methyl groups. RNAs obtained from COS-7 cells transfected with the pU6-5.8S(5′), pU6-5.8S(3′), pU3-5.8S, or pGL-5.8S expression construct were annealed with specific ^{32}P-labeled primers and extended with avian myeloblastosis virus reverse transcriptase in the presence of 1 mM (lanes 1, 3, 5, 7, and 9) or 0.004 mM (lanes 2, 4, 6, 8, and 10) deoxynucleoside triphosphates. The origin of the stop signal at A51 in the U6 snRNA is unknown (lanes 5 and 6). (B) Mapping of pseudouridine residues. RNAs isolated from COS-7 cells transfected (R) or not transfected (N) with the pU6-5.8S(5′), pU6-5.8S(3′), pU3-5.8S, or pGL-5.8S construct were treated with CMC and analyzed by primer extension. For other details, see the legend to Fig. 3.

(62). Many of these modified nucleosides are present in plant U6 snRNA as well (28), indicating that they play an important and phylogenetically conserved role in the assembly and/or function of the U6 snRNP. This study has focused on the molecular mechanism that is responsible for the generation of modified nucleosides in U6 snRNA.

Factors directing site-specific modification of U6 snRNA and U3 snoRNA. We have demonstrated that short fragments of the U6 snRNA embedded in mouse ribosomal minigene transcripts are efficiently and accurately 2′-O-methylated and pseudouridylated within the nucleolus of mouse cells (Fig. 3). Two major conclusions can be drawn from these observations. First, recognition of the correct 2′-O-methylation and pseudouridylation sites in U6 snRNA relies on short nucleotide sequences located around the target site. Second, *trans*-acting factors directing the modification of U6 snRNA at all known 2′-O-methylation and pseudouridylation sites are present and are functionally active in the nucleolus.

After injection of in vitro-transcribed human U6 snRNA into *Xenopus* oocytes, the synthesis of the Cm77 2′-O-methylated nucleotide in human U6 snRNA depends on the presence of a C and D box-containing methylation snoRNA (79). Mammalian cells contain at least two additional C and D box-containing snoRNAs with the potential to direct the 2′-O-methylation of U6 snRNA at Am47 (79) and Am53 (Fig. 1). These observations, coupled with the conclusion that the recognition of all known 2′-O-methylation sites of U6 RNA depends on nucleotide sequences located around the actual target site, rather than the secondary structure of the RNA, strongly support the notion that the 2′-O-methylation of U6 snRNA is directed exclusively by snoRNAs. We have demonstrated that *trans*-acting factors directing the synthesis of all pseudouridine residues in the U6 (Ψ31, Ψ40, and Ψ85) and U3 (Ψ8 and Ψ12) RNAs are also present in the nucleolus (Fig. 3B). Since these pseudouridylation factors recognize the nucleotide sequences around the substrate uridines of the U6 and U3 RNAs, we can anticipate that the pseudouridylation of these snRNAs may turn out to be a guide RNA-mediated process.

Mammalian U6 snRNAs also contain two base-methylated nucleotides, an *N*-6-methyladenosine (m^6A43) and a 2-methylguanosine (m^2G72) (62). No base methylation was detected at A43 or G72 in short fragments of the U6 snRNA that were expressed in the nucleolus (Fig. 3). This finding lends further support to the idea that base methylation of the U6 snRNA is dependent on the three-dimensional structure of the RNA (69) and may take place in the nucleoplasm. Similarly, short U2 snRNA-specific sequences are neither 2′-O-methylated nor pseudouridylated in the nucleolus (Fig. 3), suggesting that factors directing the modification of U2 snRNA are not present in the nucleolus and/or they recognize the three-dimensional structure of the U2 snRNA. Indeed, a yeast tRNA pseudouridine synthase enzyme, Pus1p, is also responsible for the synthesis of the Ψ44 residue in yeast U2 snRNA (44). The nucleoplasmic localization of Pus1p (70) and pseudouridylation analyses of in vitro-synthesized U2 RNAs that were microin-

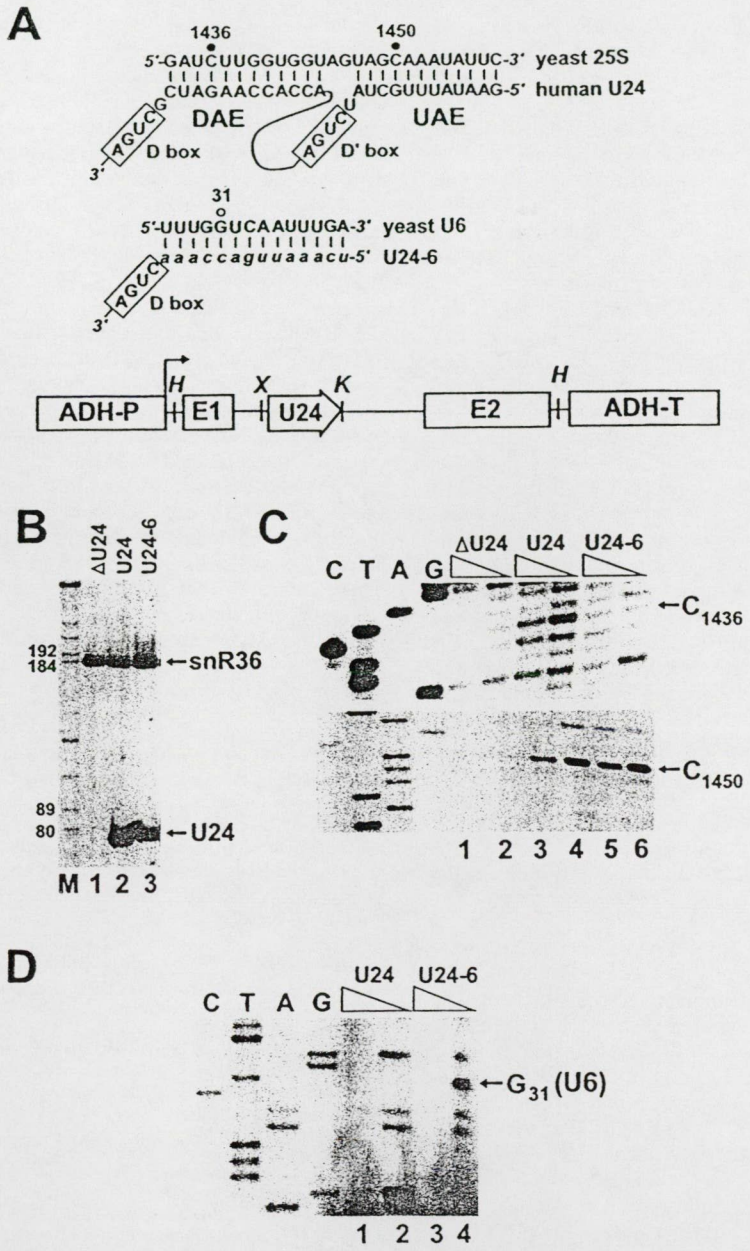


FIG. 6. Site-specific 2'-*O*-ribose methylation of yeast U6 snRNA directed by an artificial snoRNA. (A) Potential base-pairing interactions formed between human U24 snoRNA and yeast 25S rRNA or U24-6 snoRNA and yeast U6 snRNA. Nucleotides known or expected to be 2'-O-methylated are indicated by closed or open circles, respectively. DNA fragments encoding U24 or U24-6 snoRNAs were inserted into the intron of the yeast actin gene that had been placed under the control of the promoter (ADH-P) and terminator (ADH-T) of the yeast alcohol dehydrogenase gene. The relevant restriction sites are shown (*H*, *Hind*III; *X*, *Xho*I; *K*, *Kpn*I). (B) Northern blot analysis of human U24 and U24-6 snoRNAs. RNAs isolated from control ΔU24 yeast cells (lane 1) and ΔU24 cells transformed with the pFL45/ACT expression construct carrying either the U24 (lane 2) or the U24-6 (lane 3) snoRNA gene were fractionated on a 6% sequencing gel and probed with a mixture of labeled oligos specific for U24 and snR36 snoRNAs. Lane M, size markers. (C) Mapping of ribose-methylated nucleotides in yeast 25S rRNA. RNAs obtained from the ΔU24 strain (lanes 1 and 2) and from cells expressing either the U24 (lanes 3 and 4) or the U24-6 (lanes 5 and 6) snoRNA were analyzed by primer extension in the presence of 1 or 0.004 mM deoxynucleoside triphosphates. Lanes C, T, A, and G represent sequencing ladders. (D) Primer extension mapping of ribose-methylated nucleotides in yeast U6 snRNA. Oligo primers specific for the yeast U6 RNA were annealed to RNAs obtained from the yeast ΔU24 strain expressing the human U24 (lanes 1 and 2) or U24-6 (lanes 3 and 4) snoRNA. Primer extension was performed in the presence of 1 mM (lanes 1 and 3) or 0.004 mM (lanes 2 and 4) deoxynucleoside triphosphates.

jected into the nucleoplasm or cytoplasm of *Xenopus* oocytes (82) suggest that the pseudouridylation of U2 snRNA takes place in the nucleoplasm.

Does modification of U6 snRNA occur in the nucleolus? The nucleus is highly compartmentalized, and most nuclear processes can be linked to distinct subnuclear structure (35, 74). The nucleolus, the most extensively studied subnuclear organelle, has long been known as the site of the biogenesis of cytoplasmic ribosomes (18). Recently, several lines of evidence suggest that the nucleolus has more diverse functions than

earlier anticipated. In yeast, the early processing of some precursor tRNAs has been found to occur within the nucleolus (4). A fraction of mammalian telomerase (49), signal recognition particle (63), and RNase P (24) RNAs has been shown to be present in the nucleolus. Microinjection of in vitro-synthesized signal recognition particle (23), RNase MRP (25), and RNase P (24) RNAs into the nucleoplasm of mammalian cells is followed by transient localization of these RNAs to the nucleolus. These findings suggest that in addition to the biogenesis of cytoplasmic ribosomes, the nucleolus also functions in the processing and/or export of some stable small RNAs (61).

Based on biochemical criteria, the mgU6-47, mgU6-53, and mgU6-77 putative 2′-O-methylation guide RNAs for U6 RNA have been localized to the nucleolus (79) (Fig. 1B). These results, coupled with the observation that *trans*-acting factors directing the 2′-O-methylation and pseudouridylation of U6 and U3 snRNAs are functionally active in the nucleolus (Fig. 3), suggest that the nucleolus may function in the posttranscriptional modification of the U6 spliceosomal snRNA and the U3 snoRNA. Since mature U6 snRNA shows a steady-state nucleoplasmic localization, the notion that its posttranscriptional modification occurs in the nucleolus presupposes that U6 snRNA cycles through the nucleolus during its maturation. Demonstration that the wild-type U6 snRNA in yeast (Fig. 6) and the chimeric U6-*5.8S* RNA in mammalian cells (Fig. 5) can undergo snoRNA-directed 2′-O-methylation and pseudouridylation further supports this notion. Recently, it was shown that an in vitro-transcribed U6 snRNA, upon injection into the nucleoplasm of *Xenopus* oocytes, transiently accumulates in the dense fibrillar compartment of the nucleolus (51). The fibrillar compartment of the nucleolus also harbors the fibrillarin snoRNP protein that is associated with all 2′-O-methylation snoRNAs. This protein is most likely the methyltransferase enzyme that catalyzes the ribose methylation of the target nucleotide specified by the RNA component of the snoRNP particles (53, 75).

The above-mentioned study (51) also revealed that microinjected U6 snRNA localizes to other conserved subnuclear organelles, the coiled bodies, in addition to the nucleolus. The nucleolus and coiled bodies have several common antigens and appear to be remarkably related in both structure and function (6, 45, 74). Coiled bodies are attached to the nucleolar periphery and, under certain physiological conditions, are found within the nucleolus (40, 43, 54), suggesting that they either emerge from or fuse to the nucleolar structures (6, 45). An exciting feature of coiled bodies is that they contain all the major spliceosomal snRNPs, including the U6 snRNP (9, 46). Hence, coiled bodies have been implicated in the processing, modification, or export of spliceosomal snRNAs (6, 40, 45).

Although no H or ACA box pseudouridylation guide snoRNP has yet been detected in coiled bodies, the presence of some C and D box-containing snoRNPs has been well documented (26, 56, 64, 68). Since the C and D boxes have been identified as the key transport elements which target these RNAs to the nucleolus and coiled bodies (37, 64), all snoRNAs carrying these box motifs may be present in coiled bodies. Thus, snoRNA-directed 2′-O-methylation of U6 snRNA may take place in coiled bodies (6, 79). Coiled bodies also contain the putative 2′-O-methyltransferase (fibrillarin) and pseudouridine synthase (Nap57/Cbf5) enzymes that likely catalyze the U6 modification reactions. However, the putative guide RNAs directing U6 methylation are localized to the nucleolus and are practically absent from the nucleoplasm (79) (Fig. 1B). In marked contrast, a coiled body-specific nuclear protein, p80 coilin, was detected mainly, if not exclusively, in the nucleoplasmic fraction (data not shown). Therefore, a potential copurification of coiled bodies with nucleoli cannot account for the observed nucleolar localization of the U6 methylation snoRNAs. We cannot, however, exclude the possibility that coiled bodies contain a small but still sufficient amount of guide snoRNPs to conduct the modification of U6 snRNA. Likewise, the available data cannot unambiguously rule out the formal possibility that the modification of U6 snRNA occurs in the nucleoplasm. In this context, however, it is noteworthy that, despite our repeated efforts, artificial snoRNAs failed to direct the 2′-O-methylation of the human β-globin mRNA and precursor mRNA as well as the nucleoplasmic U19H RNA (7, 27).

In summary, the data presented in this paper are most consistent with the idea that the posttranscriptional modification of U6 spliceosomal snRNA and U3 snoRNA takes place in the nucleolus, although we acknowledge that other subnuclear compartments, such as coiled bodies, may also contribute to this process. Nevertheless, the fact that *trans*-acting factors directing the site-specific 2′-O-methylation and pseudouridylation of the U6 spliceosomal RNA and pseudouridylation of the U3 snoRNA are present and are functionally active in the nucleolus documents that the nucleolus, directly or indirectly, is involved in the biogenesis of some snRNAs and snoRNAs. These results further substantiate the emerging idea that the nucleolus is a multifunctional organelle that functions in the maturation and/or intracellular trafficking of different classes of cellular RNAs.

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Characterisation of the U83 and U84 small nucleolar RNAs: two novel 2'-*O*-ribose methylation guide RNAs that lack complementarities to ribosomal RNAs.

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Characterisation of the U83 and U84 small nucleolar RNAs: two novel 2′-*O*-ribose methylation guide RNAs that lack complementarities to ribosomal RNAs

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ABSTRACT

In eukaryotic cells, the site-specific 2′-*O*-ribose methylation of ribosomal RNAs (rRNAs) and the U6 spliceosomal small nuclear RNA (snRNA) is directed by small nucleolar RNAs (snoRNAs). The C and D box-containing 2′-*O*-methylation guide snoRNAs select the correct substrate nucleotide through formation of a long 10–21 bp interaction with the target rRNA and U6 snRNA sequences. Here, we report on the characterisation of two novel mammalian C/D box snoRNAs, called U83 and U84, that contain all the elements that are essential for accumulation and function of 2′-*O*-methylation guide snoRNAs. However, in contrast to all of the known 2′-*O*-methylation guide RNAs, the human, mouse and pig U83 and U84 snoRNAs feature no antisense elements complementary to rRNA or U6 snRNA sequences. The human U83 and U84 snoRNAs are not associated with maturing nucleolar pre-ribosomal particles, suggesting that they do not function in rRNA biogenesis. Since artificial substrate RNAs complementary to the evolutionarily conserved putative substrate recognition motifs of the U83 and U84 snoRNAs were correctly 2′-*O*-methylated in the nucleolus of mouse cells, we suggest that the new snoRNAs act as 2′-*O*-methylation guides for cellular RNAs other then rRNAs and the U6 snRNA.

INTRODUCTION

The eukaryotic nucleolus is a specialised subnuclear organelle devoted to the biogenesis of cytoplasmic ribosomes (1). This complex process includes the RNA polymerase I-directed synthesis of the long precursor ribosomal RNA (pre-rRNA), its nucleolytic processing into mature sized 18S, 5.8S and 25/28S rRNAs and packaging of the mature rRNAs with more than 80 ribosomal proteins. Formation of functional 18S, 5.8S and 25/28S rRNAs is assisted by many small nucleolar RNAs (snoRNAs) that occur in the form of ribonucleoprotein particles (snoRNPs) (reviewed in 2–5). A few snoRNAs are required for the nucleolytic processing of pre-rRNA, but the majority of them function as guide RNAs in the post-transcriptional modification of rRNAs (for reviews see 4,6). Many snoRNAs that contain the conserved H box and ACA sequence elements direct the site-specific pseudouridylation of rRNAs (7–9). Another large group of snoRNAs which share the C and D box motifs guide the synthesis of 2′-*O*-methylated nucleotides (10–13).

Both classes of rRNA modification guide snoRNAs select the substrate nucleotides through formation of direct base pairing interactions with rRNA sequences. The C and D box-containing 2′-*O*-methylation guide snoRNAs carry one or, less frequently, two 10–21 nt long rRNA recognition motifs, also known as antisense elements (14). The antisense elements are located either close to the 3′-end of the snoRNA (downstream antisense element) or on the 5′-half of the snoRNA (upstream antisense element) (Fig. 1). The downstream antisense element is followed by the D box and the upstream antisense element is flanked by the D′ box motif, which usually represents a slightly altered version of the D box (10,15). A Watson–Crick helix formed by the antisense element of the snoRNA and the complementary rRNA sequences places the D or D′ box of the snoRNA exactly 5 bp from the 2′-*O*-methylated nucleotide in the rRNA (Fig. 1). A putative methyltransferase enzyme, most likely the fibrillarin snoRNP protein (16,17), which binds directly or indirectly to the D and D′ box of the snoRNA selects the target nucleotide to be methylated. The C′ box and probably also the C box play a crucial role in the methytransfer reaction directed by the upstream or downstream antisense element, respectively (13).

Recently, snoRNAs have also been implicated in site-specific modification of the U6 spliceosomal small nuclear RNA (snRNA). Ribose methylation of the C77 residue in an *in vitro* synthesised human U6 snRNA, after injection into *Xenopus* oocytes, is dependent on the presence of a C/D box snoRNA, termed mgU6-77 (18). The antisense elements of two other C/D box snoRNAs, mgU6-47 and mgU6-53, can position the mA47 and mA53 residues of the U6 snRNA for 2′-*O*-methylation (18,19). Moreover, the nucleolus contains all the factors that are required for the site-specific synthesis of the remaining five ribose-methylated nucleotides of mammalian U6 snRNA (19). The correct recognition of these methylation sites depends on

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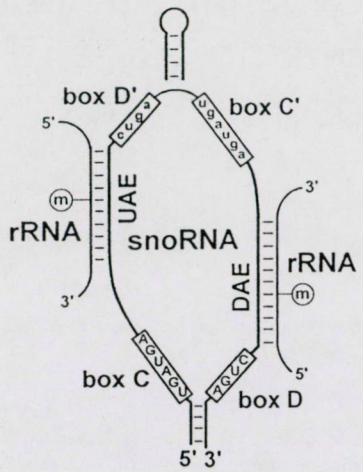

Figure 1. Selection of 2′-*O*-methylated nucleotides by box C/D snoRNAs. The consensus sequences of the conserved C, C′, D and D′ boxes are indicated. The upstream (UAE) and/or downstream (DAE) antisense element of the snoRNA forms a double helix with complementary rRNA sequences. A nucleotide in the rRNA that base pairs with the fifth nucleotide 5′ to the D or D′ box of the snoRNA is 2′-*O*-methylated. The position of an internal hairpin that frequently folds together the D′ and C′ boxes is indicated (13).

short snRNA sequences located around the target nucleotide. These findings strongly support the idea that 2′-*O*-ribose methylation of the U6 snRNA is mediated exclusively by C/D box snoRNAs.

In this study, identification and characterisation of two novel C and D box-containing snoRNAs, termed U83 and U84, suggest that C/D box snoRNAs may also function in 2′-*O*-methylation of cellular RNAs other than the 18S, 5.8S and 25/28S rRNAs or the U6 spliceosomal RNA.

MATERIALS AND METHODS

Oligodeoxynucleotides

Oligonucleotides used for PCR amplification, DNA sequencing, primer extension or cloning were synthesised by the standard phosphoramidite method and purified on a 20% polyacrylamide–8 M urea gel. The following oligonucleotides were used in this study: 1, CTAGTCAAGGGTGATAGA; 2, TCGATCTATCACCCTTGA; 3, CTAGTCATGGGTGATAGA; 4, TCGATCTATCACCCATGA; 5, GCGCAAAGCGCTCACCTTTCG; 6, GCACTGAGGTGCTCCTGTTT; 7, GGAGCACCTCATGTGCA; 8, GCGCTTTGCGCAGTGAT; 9, NNNNTATCACCCATG.

Expression constructs for transfection of mouse cells

Construction of the pW(*Xb/Xh*) mouse ribosomal minigene expression vector has been described (20,21). To obtain pW-*U84t*, oligodeoxynucleotides 1 and 2 were annealed and inserted into the *Xba*I and *Xho*I sites of pW(*Xb/Xh*). The same approach was used to generate pW-*U83t* (oligos 3 and 4). Transfection of plated mouse L929 cells (ATCC CCL1) was performed by the DEAE–dextran method (22).

RNA analyses

RNAs from human HeLa and mouse L929 cells or from the nuclear, nucleolar, nucleoplasmic and cytoplasmic fractions of HeLa cells were extracted by the guanidine thiocyanate/phenol-chloroform extraction method (23). Primer extension analysis of the 5′-termini of human U83 and U84 snoRNAs was performed using 5′-end-labelled oligonucleotides 5 and 6 as primers, respectively, and the samples were electrophoresed on denaturing 8% polyacrylamide gels. The 3′-terminal sequences of U83 and U84 were determined by the T4 RNA ligase–PCR approach as described (24), except that oligos 7 and 8 were used as U83- and U84-specific primers for the PCR amplification reaction.

RNase A/T1 mappings were performed as described (23). Antisense RNA probes for the human U3 snoRNA and the U4 snRNA were synthesised *in vitro* (24). To generate sequence-specific probes for the human U83 and U84 snoRNAs, the pU83 and pU84 recombinant plasmids carrying the full-length cDNAs of U83 and U84 were linearised with *Eco*RI and used as templates for *in vitro* transcription with T3 RNA polymerase. The probes were purified on a 6% sequencing gel.

Ribose-methylated nucleotides were mapped by primer extension analyses (25). To monitor 2′-*O*-methylation of the mouse ribosomal minigene transcripts, the 5′-end-labelled 3-oCAT2 oligonucleotide (20), complementary to the pW transcript downstream from the inserted target sequences, was used as primer. To detect a potential target RNA for the U83 2′-*O*-methylation guide snoRNA, 2 pmol of terminally labelled oligo 9 was annealed to 10 µg of HeLa total cellular or nuclear RNA and extended by AMV reverse transcriptase. The extended DNA products were analysed on a 6% sequencing gel.

Cell fractionation and glycerol gradient analyses

Human HeLa cells were fractionated into nuclear, nucleolar, nucleoplasmic and cytoplasmic fractions as described (26). Preparation and fractionation of HeLa cell extracts on 10–30% glycerol gradients were performed as described (26,27).

RESULTS

Mammalian U83 and U84 C/D box snoRNAs lack sequences complementary to rRNAs

Partial sequences of two novel putative snoRNAs were obtained during characterisation of a cDNA library of human snoRNAs (10). Northern assays of human HeLa and mouse cellular RNAs revealed that the new RNAs contain about 75–80 nt, whose sequences are conserved in mammalian cells (data not shown). The 3′-terminal sequences of the two human RNAs were determined by the oligoribonucleotide ligation–PCR amplification procedure (24; Fig. 2b) and the correct 5′-ends were defined by primer extension analyses (Fig. 2a). The novel human RNAs, hereafter named U83 and U84, consist of 76 and 78 nt, respectively (Fig. 3). Database searches revealed that sequences 80–85% homologous to the human U83 and U84 RNAs are present in the fifth and first introns, respectively, of the pig and mouse *BAT1* gene that encodes for a putative RNA helicase (GenBank accession nos Z34846 and AC007080). Moreover, a perfect copy of the U83 RNA was found in the fifth intron of the human *BAT1* gene (GenBank accession no. AF029062). Unfortunately, the 5′-terminal part of the

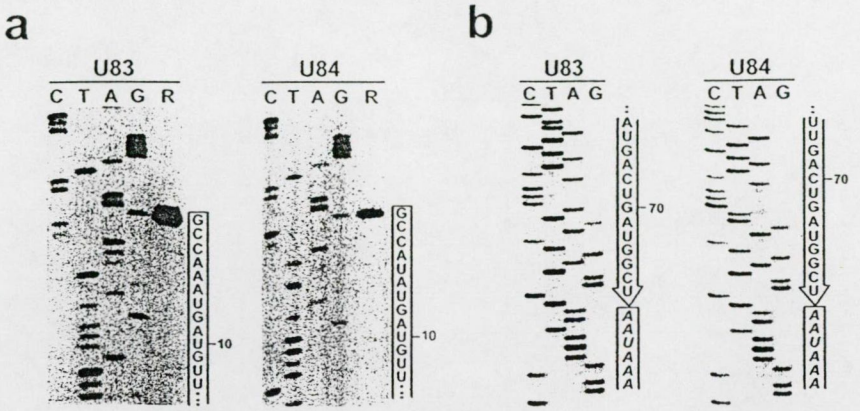

Figure 2. Characterisation of human U83 and U84 snoRNAs. (**a**) Primer extension analyses of the 5′-termini of U83 and U84 RNAs. Terminally labelled oligonucleotides specific for U83 and U84 were annealed to human nuclear RNAs and extended by AMV reverse transcriptase (lanes R). Lanes C, T, A and G represent dideoxy sequencing reactions using the same oligonucleotides as primers and recombinant plasmids carrying the full-length cDNAs of U83 and U84 as templates. The extended products were separated on an 8% sequencing gel. The 5′-terminal sequences of the U83 and U84 RNAs are shown. (**b**) Determination of the 3′-terminal sequences of human U83 and U84 snoRNAs by the T4 RNA ligase/PCR procedure. The 3′-terminal sequences of the two snoRNAs and the 5′-terminal sequence of the oligoribonucleotide ligated to the U83 and U84 RNAs are shown.

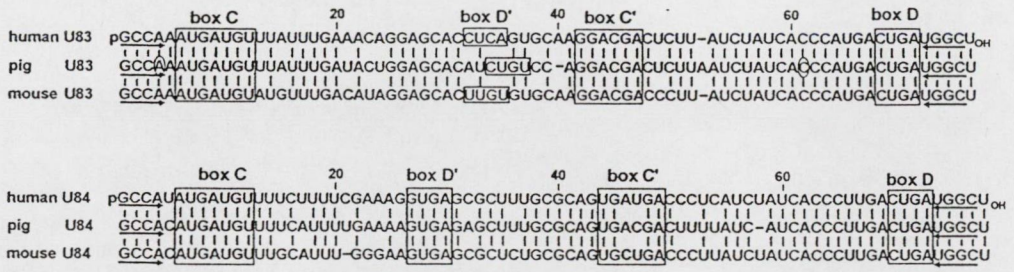

Figure 3. Alignments of human, pig and mouse U83 and U84 snoRNAs. The C and D boxes and the potential C′ and D′ box motifs are indicated. The vertical lines highlight nucleotides conserved in the aligned snoRNAs and dashes stand for gaps. Inverted arrows indicate sequences capable of forming base pairing interactions. The presence of an additional A and C residue in the pig U83 RNA at positions 4 and 61 (circled), as compared to the published sequence (GenBank accession no. Z34846), has been verified by sequence analysis of the appropriate region of the pig *BAT1* locus (data not shown). Sequences of the human U83 and U84 snoRNAs have been deposited in the EMBL database (accession nos AJ243200 and AJ243199, respectively).

human *BAT1* gene that may encompass the human U84 RNA gene has not yet been characterised. Nevertheless, we concluded that the U83 and U84 RNAs represent novel intron-encoded RNAs that are processed from the fifth and first introns of the mammalian *BAT1* gene.

Alignments of the human, pig and mouse U83 and U84 RNAs are shown in Figure 3. These RNAs feature all of the elements, the C, C′, D and D′ box sequence motifs and a short 5′-, 3′-terminal helix structure, that are essential for nucleolar accumulation and function of intron-encoded 2′-*O*-methylation guide snoRNAs directing the modification of rRNAs and U6 snRNA (18,19,28–30). However, neither U83 nor U84 RNA possesses sequences that could form a long base pairing interaction with rRNA or U6 snRNA sequences. The best interaction, potentially formed by U83 (positions 58–66) or U84 (positions 60–68) snoRNA with the 18S rRNA sequence between positions 404 and 413, would consist of eight canonical base pairs interrupted by one non-canonical C:A or C:U base pair, respectively. Based on a recent functional analysis of the guide RNA and rRNA duplex (31), such a poor interaction would be insufficient to support the 2′-*O*-methylation reaction. Consistent with this conclusion, no 2′-*O*-methylated nucleotide was reported in this region of the human 18S rRNA (32).

U83 and U84 RNAs are nucleolar but are not associated with precursor ribosomal particles

We assayed the intracellular distribution of the U83 and U84 RNAs, whose C and D box elements predict a nucleolar localisation.

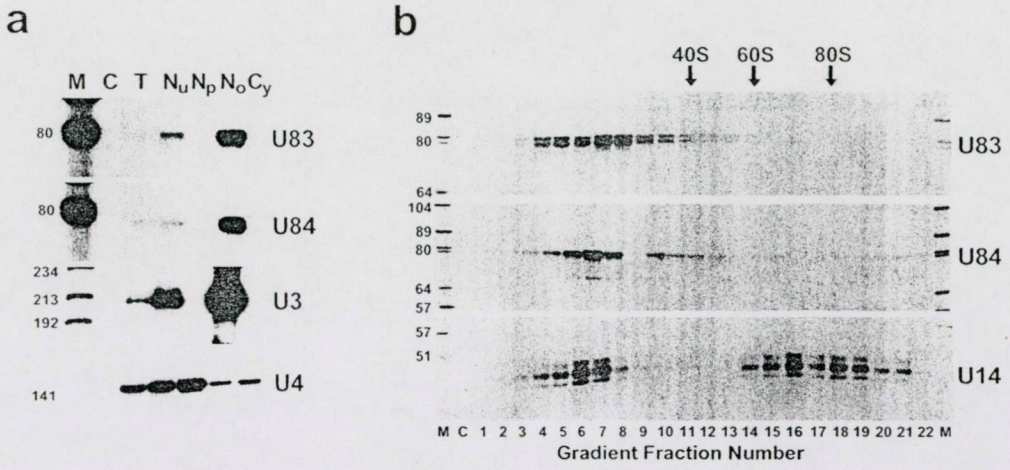

Figure 4. Human U83 and U84 RNAs are not associated with higher order nucleolar structures. (**a**) Intracellular localisation of human U83 and U84 snoRNAs. RNAs extracted either from human HeLa cells (T) or from nuclear (N), nucleoplasmic (Np), nucleolar (No) and cytoplasmic (Cy) fractions of HeLa cells were mapped by RNase A/T1 protection using sequence-specific antisense RNA probes as indicated. Lane C, control mapping with *Escherichia coli* tRNA. Lane M, size markers (a mixture of *Hae*III- and *Taq*I-digested pBR322). (**b**) Sedimentation analyses of U83, U84 and U14 snoRNP particles. HeLa cell extract was fractionated on a 10–30% glycerol gradient. RNAs were isolated from each fraction and subjected to RNase A/T1 mapping with antisense RNA probes specific for the U83, U84 or U14 snoRNA. Positions of HeLa ribosome markers are indicated. Lanes C and M represent control mappings and size markers, respectively.

Human HeLa cells were fractionated into nuclear, nucleoplasmic, nucleolar and cytoplasmic fractions. RNAs obtained from each fraction as well as from whole HeLa cells were mapped by RNase A/T1 protection using antisense RNA probes specific for the U83 and U84 RNAs (Fig. 4a). As controls, we also measured the U3 snoRNA and the U4 spliceosomal RNA content of each fraction. The U83 and U84 RNAs, like the U3 snoRNA, were highly enriched in the nucleolar fraction of HeLa cells and were hardly detectable in the nucleoplasmic fraction where U4 snRNA accumulated, demonstrating that the U83 and U84 RNAs represent authentic snoRNAs.

The nucleolar localisation of the U83 and U84 snoRNAs suggests that they may function in the nucleolar maturation of rRNAs. snoRNAs involved in the nucleolytic processing or nucleotide modification of rRNAs are associated with higher order structures that likely represent ribosomal particles undergoing maturation in the nucleolus (11,21,26,27,33,34). To assess whether the U83 and U84 snoRNAs are associated with large nucleolar structures, a HeLa cell extract was fractionated in a non-denaturing glycerol gradient (Fig. 4b). Distribution of the U83 and U84 snoRNAs as well as U14 snoRNA, which functions both in the nucleolytic processing and 2′-*O*-methylation of 18S rRNA (35,36), was investigated by RNase mapping. About half of the cellular U14 snoRNA sedimented together with large particles that showed sedimentation properties similar to the 60S and 80S cytoplasmic ribosomal particles. Very similar results were obtained with another snoRNA, U3 snoRNA (data not shown), which functions in the early processing of pre-rRNA (37–40). In contrast to the U14 and U3 snoRNAs, the U83 and U84 snoRNAs sedimented exclusively in 10–20S structures that likely represent snoRNP monoparticles. These findings indicate that the U83 and U84 snoRNPs are not associated with pre-ribosomal particles in the nucleolus.

U83 and U84 snoRNAs direct 2′-*O*-methylation of artificial substrate RNAs

A few C/D box snoRNPs, such as the U3 (37–40), U8 (41,42), U22 (43) and U14 (35,44) RNPs, function in the nucleolytic processing of rRNAs. These snoRNPs, similarly to the authentic rRNA 2′-*O*-methylation particles, also contain the fibrillarin protein that likely catalyses the 2′-*O*-methyltransfer reaction (16,17). This is contrary to the fact that the above mentioned snoRNPs, with the exception of U14, apparently do not function in rRNA methylation. We therefore examined whether U83 and U84 snoRNP particles possess the potential to direct RNA ribose methylation.

Sequences immediately preceding the D box motifs of the U83 and U84 snoRNAs are perfectly conserved in the human, mouse and pig RNAs (Fig. 2). We examined whether the mouse intracellular U83 and U84 snoRNAs are capable of directing site-specific 2′-*O*-methylation of properly designed artificial substrate RNAs in the nucleolus. To this end, two short DNA fragments (*U83t* and *U84t*) were inserted into a mouse ribosomal minigene (W) that possesses the RNA polymerase I promoter and terminator regions but lacks rRNA sequences (20; Fig. 5a). The RNA transcripts generated from the W-*U83t* and W-*U84t* constructs carry 15 nt long sequences that are perfectly complementary to the putative downstream antisense elements of the U83 or U84 snoRNAs. Since the RNA polymerase I-directed transcription of the ribosomal minigenes occurs in the nucleolus, the W-*U83t* and W-*U84t* RNAs are expected to accumulate in the nucleolus (19–21).

The pW-*U83t* and pW-*U84t* constructs were transfected into mouse cells and the state of ribose methylation of the expressed minigene transcripts was monitored by primer extension (Fig. 5b). In the presence of low concentration of

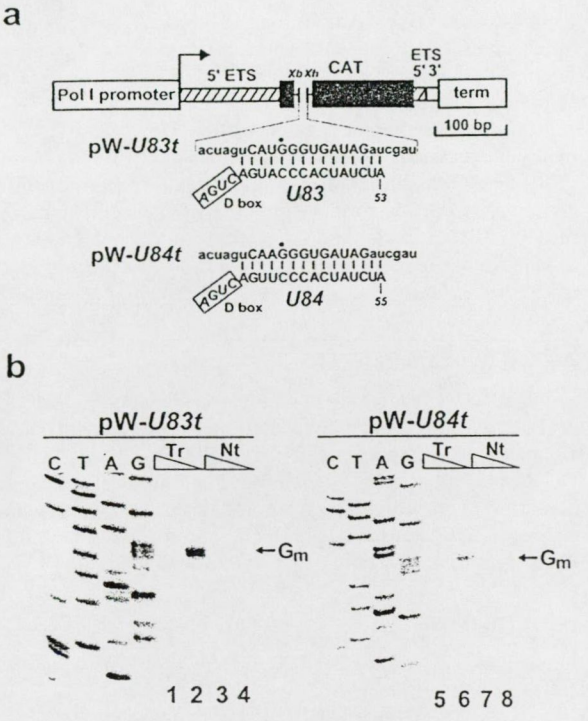

Figure 5. Ribose methylation of artificial substrate RNAs in mouse cells. (**a**) Schematic structure of the pW-*U83t* and pW-*U84t* expression constructs used for transfection of mouse cells. The RNA polymerase I promoter and terminator, the terminal regions of the 5′ (hatched box) and 3′ (open box) external transcribed sequences (ETS) and a fragment of the chloramphenicol acetyltransferase (CAT) gene are indicated. To generate pW-*U83t* and pW-*U84t*, appropriate synthetic DNA fragments were inserted into the *Xba*I (*Xb*) and *Xho*I (*Xh*) sites of pW(*Xb*/*Xh*). Nucleotides facilitating the cloning are in lower case letters. The potential base pairing interactions formed between the expressed artificial substrate RNAs and the putative antisense elements of the U83 and U84 snoRNA are shown. Nucleotides predicted to be 2′-*O*-methylated are indicated by closed circles. (**b**) Primer extension mapping of 2′-*O*-methylated nucleotides. A 5′-end-labelled deoxyoligonucleotide was annealed with RNAs extracted from mouse cells non-transfected (Nt) or transfected (Tr) with the indicated expression constructs and extended with AMV reverse transcriptase in the presence of 1 or 0.004 mM dNTPs (as indicated above the lanes). Lanes C, T, A and G are dideoxy sequencing reactions performed on the pW-*U83t* or pW-*U84t* expression constructs.

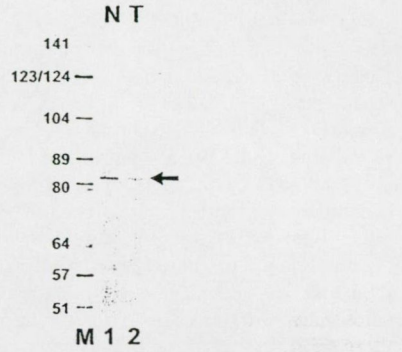

Figure 6. Primer extension analysis. RNAs obtained from human HeLa cells (T) or from a nuclear fraction of HeLa cells (N) were annealed with 5′-end-labelled oligo 9 and extended with AMV reverse transcriptase. The extended DNA products were size-fractionated on a 6% sequencing gel. Lane M, size marker.

dNTPs, ribose-methylated nucleotides interfere with the passage of reverse transcriptase and result in stops 1 nt before or at the modified nucleotide (25). When RNAs obtained from cells transfected with the pW-*U83t* or pW-*U84t* expression construct were analysed in the presence of 0.004 mM dNTPs, strong stop signals were obtained 1 nt before the G residues that are predicted to be 2′-*O*-methylated by the endogenous U83 and U84 snoRNPs (Fig. 5, lanes 2 and 6). In contrast, no reverse transcriptase stops were observed in the presence of 1 mM dNTPs (lanes 1 and 5) or during mapping of control RNAs derived from non-transfected mouse cells (lanes 3, 4, 7 and 8). These results show that the U83 and U84 snoRNAs are capable of directing site-specific 2′-*O*-methylation of artificial substrate RNAs and suggest that these snoRNAs likely function in the nucleolus as 2′-*O*-methylation guide RNAs.

In an attempt to detect a potential target RNA for the U83 methylation guide RNA, a terminally labelled oligodeoxynucleotide, identical to the U83 snoRNA sequences from position 56 to 66 and extended by four randomly synthesised 5′-terminal nucleotides, was annealed to human HeLa total cellular or nuclear RNAs. After incubation with AMV reverse transcriptase, an extended DNA product of ~82–83 nt was reproducibly detected in total cellular RNAs (Fig. 6, lane 2) and, more efficiently, in RNAs extracted from the nuclear fraction of HeLa cells (lane 1). To identify this RNA molecule and to investigate whether it really represents the natural substrate of the U83 2′-*O*-methylation guide snoRNP remains a challenge for the future.

DISCUSSION

We have identified two novel mammalian snoRNAs that represent new members of the family of C and D box-containing intron-encoded snoRNAs (Fig. 2). The mouse, pig and, most likely, human U83 and U84 snoRNAs are encoded in the fifth and first introns of the *BAT1* putative RNA helicase gene, respectively.

More than 50 C/D box snoRNAs have been identified in vertebrates and their final number is predicted to exceed 100 (3–4). The vast majority of C/D box snoRNAs function as guide RNAs in the site-specific 2′-*O*-methylation of rRNAs. Selection of the ribosomal 2′-*O*-methylation sites is mediated by transient base pairing interactions formed between the target ribosomal sequences and the antisense elements of the guide snoRNAs (Fig. 1). The efficiency of the methyltransfer reaction greatly depends on the length and regularity of the snoRNA–rRNA duplex formed (31). The natural rRNA methylation guide snoRNAs possess at least 10 nt long rRNA antisense elements. A snoRNA–rRNA interaction composed of eight canonical and one non-canonical base pairs—the best interaction that could be drawn between the U83 or U84 snoRNA and mammalian 18S rRNA (see Results)—would not be expected to support rRNA methylation (31). In accordance

with this conclusion, no 2′-*O*-methyl group was encountered in this particular region of the mammalian 18S rRNA (32). Hence, we concluded that neither the U83 nor the U84 snoRNA can direct ribose methylation of rRNAs in mammalian cells. A few C/D box snoRNAs, such as U3, U8, U14 and U22, function in the nucleolytic processing of pre-rRNAs (reviewed in 3,4). At the moment, we cannot unambiguously exclude the formal possibility that the U83 and U84 snoRNAs may function in the nucleolytic processing of rRNAs. However, in marked contrast to snoRNAs involved in rRNA modification or processing, the U83 and U84 snoRNAs are not associated with precursor ribosomal particles. This strongly argues against a role for these snoRNAs in rRNA processing.

A comparison of mammalian U83 and U84 snoRNAs revealed that, beside the C and D box motifs, the 3′-terminal regions preceding the D boxes show the highest degree of conservation (Fig. 2). In fact, these sequences are perfectly conserved in human, mouse and pig RNAs, underlining their functional importance. The demonstration that artificial substrate RNAs complementary to the conserved putative downstream antisense regions of the U83 and U84 snoRNAs are accurately 2′-*O*-methylated in mouse cells strongly supports the idea that the U83 and U84 snoRNAs function as 2′-*O*-ribose methylation guide RNAs in the cell (Fig. 5).

The notion that the U83 and U84 snoRNAs function as methylation guide RNAs raises questions about the nature of their target RNAs. The potential target recognition sequences of U83 and U84 show a striking similarity to each other (Fig. 2), indicating that these snoRNAs might direct the 2′-*O*-methylation of two sequence variants of the same cellular RNA. Primer extension analyses of human cellular RNAs revealed an RNA molecule that carries a sequence motif complementary to the putative RNA recognition motif of the U83 snoRNA (Fig. 6). Interestingly, the potential target RNA for the U83 guide snoRNA was detected mainly in the nuclear fraction of human cellular RNAs.

Recently, C/D box snoRNAs have been demonstrated to function in the 2′-*O*-methylation of U6 spliceosomal snRNA (18,19). Most likely, synthesis of the eight 2′-*O*-methylated nucleotides of mammalian U6 snRNA is directed exclusively by C/D box snoRNAs (19). Moreover, guide RNAs, most probably H/ACA box snoRNAs, have been implicated in the synthesis of the pseudouridine residues present in the U6 snRNA and the U3 snoRNA (19). These results show that snoRNAs can function in the post-transcriptional modification of various classes of cellular RNAs. Besides maturing rRNAs and authentic snoRNAs, several small RNAs are believed to appear, at least transiently, in the nucleolus (reviewed in 45). Some yeast precursor tRNAs cycle through the nucleolus to undergo nucleolytic processing (46). A fraction of mammalian telomerase (47), signal recognition particle (48) and RNase P (49) RNAs has been found in the nucleolus. Upon microinjection into the nucleoplasm, *in vitro* synthesised signal recognition particle (50), RNase MRP (51), RNase P (49) and U6 (A.Narayanan, R.Terns and M.Terns, personal communication) RNAs transiently localise to the nucleolus, suggesting that the nucleolus may function in the biogenesis of these RNAs. Unfortunately, computer-aided inspection of vertebrate small RNA sequences, including U snRNAs, snoRNAs, the telomerase, RNase P and MRP RNAs, as well as the cytoplasmic signal recognition particle RNA and the known tRNAs, failed to identify a potential substrate for the U83 or U84 2′-*O*-methylation guide snoRNAs. Of course, it is also possible that the U83 and U84 snoRNAs function in the 2′-*O*-methylation of some as yet unidentified snRNAs (52).

In summary, characterisation of the U83 and U84 2′-*O*-methylation guide snoRNAs that most likely function in the post-transcriptional modification of an as yet unidentified cellular RNA lends further support to the recently emerging idea that the nucleolus is a multifunctional subnuclear organelle that functions in the maturation of different classes of cellular RNAs.

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PUBLICATION IV

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A small nucleolar guide RNA functions both in 2'-*O*-ribose methylation and pseudouridylation of the U5 spliceosomal RNA.

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A small nucleolar guide RNA functions both in 2′-*O*-ribose methylation and pseudouridylation of the U5 spliceosomal RNA

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In eukaryotes, two distinct classes of small nucleolar RNAs (snoRNAs), namely the fibrillarin-associated box C/D snoRNAs and the Garlp-associated box H/ACA snoRNAs, direct the site-specific 2′-*O*-ribose methylation and pseudouridylation of ribosomal RNAs (rRNAs), respectively. We have identified a novel evolutionarily conserved snoRNA, called U85, which possesses the box elements of both classes of snoRNAs and associates with both fibrillarin and Garlp. *In vitro* and *in vivo* pseudouridylation and 2′-*O*-methylation experiments provide evidence that the U85 snoRNA directs 2′-*O*-methylation of the C45 and pseudouridylation of the U46 residues in the invariant loop 1 of the human U5 spliceosomal RNA. The U85 is the first example of a snoRNA that directs modification of an RNA polymerase II-transcribed spliceosomal RNA and that functions both in RNA pseudouridylation and 2′-*O*-methylation.
Keywords: 2′-*O*-methylation/pseudouridylation/RNA modification/snoRNA/snRNA

Introduction

In stable cellular RNAs, many nucleotides undergo site-specific post-transcriptional covalent modifications. In ribosomal RNAs (rRNAs) and small nuclear RNAs (snRNAs), conversion of uridine into pseudouridine (Ψ) and 2′-*O*-methylation of the backbone ribose represent the most common modifications. In mammals, the 18, 5.8 and 28S rRNAs together carry >100 2′-*O*-methyl groups and ~95 pseudouridines (Maden, 1990; Ofengand *et al.*, 1995). In the major spliceosomal snRNAs (U1, U2, U4, U5 and U6), 23 pseudouridines and 30 2′-*O*-methylated nucleotides have been detected (Reddy and Busch, 1988).

The actual function of the modified nucleotides in rRNAs and snRNAs is largely unknown. Most ribosomal pseudouridines and 2′-*O*-methyl groups are dispensable for cell growth (Ni *et al.*, 1997; Lowe and Eddy, 1999). Unmodified U1, U4, U5 and U6 snRNAs can support pre-mRNA splicing *in vitro*, but modification of the 5′-terminal region of the U2 snRNA is essential for the splicing reaction (Yu *et al.*, 1998 and references therein). Generally, the pseudouridines and 2′-*O*-methyl groups cluster around the functionally important regions of stable cellular RNAs (Maden, 1990; Szkukalek *et al.*, 1995; Gu *et al.*, 1996) and they are thought to fine-tune the function of the RNAs by facilitating specific RNA–RNA and RNA–protein interactions.

Site-specific pseudouridylation and 2′-*O*-methylation of rRNAs is directed by a large population of snoRNAs within the nucleolus. The 2′-*O*-methylation guide snoRNAs possess the conserved C (consensus RUGAUGA) and D (CUGA) boxes, which are frequently folded together with an adjacent 5′,3′-terminal stem. Internal regions of box C/D snoRNAs carry imperfect copies of the C and D boxes, called C′ and D′ boxes (Kiss-László *et al.*, 1996, 1998; Tycowski *et al.*, 1996). The D and/or D′ boxes are preceded by long (10–21 nt) recognition sequences that, through formation of double helices with rRNA sequences, position the D or D′ box of the snoRNA exactly 5 nt from the 2′-*O*-methylated ribosomal nucleotide (Cavaillé *et al.*, 1996; Kiss-László *et al.*, 1996). The pseudouridylation guide snoRNAs consist of 5′ and 3′ hairpin domains, connected and followed by single-stranded hinge and tail regions that carry the conserved H (AnAnnA) and ACA boxes, respectively (Balakin *et al.*, 1996; Ganot *et al.*, 1997b). Two short rRNA recognition motifs of the snoRNA base pair with rRNA sequences flanking the uridine to be converted into pseudouridine. In the resulting 'pseudouridylation pocket', the substrate uridine is positioned ~14 nt upstream of the H or ACA box of the snoRNA (Ganot *et al.*, 1997a; Ni *et al.*, 1997).

The 2′-*O*-methylation and pseudouridylation guide snoRNAs are present in the nucleolus as ribonucleoprotein (snoRNP) particles. While the snoRNAs select the correct nucleotides for 2′-*O*-methylation and pseudouridylation, the associated snoRNP proteins accomplish the modification reaction. The box C/D snoRNAs are packaged with at least three proteins, Nop56p, Nop58p and fibrillarin. Since fibrillarin shares common sequence and structural motifs with known methyltransferases, it is likely to be the 2′-*O*-methyltransferase enzyme (Wang *et al.*, 2000). Thus far, four box H/ACA snoRNP proteins have been identified. Nap57p (Cbf5p in yeast) has been demonstrated to function as pseudouridine synthase (Lafontaine *et al.*, 1998; Zebarjadian *et al.*, 1999), but the role of the three remaining proteins, Garlp, Nhp2p and Nop10p, remains conjectural (Henras *et al.*, 1998; Watkins *et al.*, 1998).

The cellular machinery directing modification of spliceosomal snRNAs is poorly understood. Unexpectedly, three box C/D snoRNAs have been implicated in 2′-*O*-methylation of the RNA polymerase (pol) III-synthesized U6 snRNA (Tycowski *et al.*, 1998; Ganot *et al.*, 1999). Factors directing 2′-*O*-methylation and pseudouridylation of the pol II-transcribed U1, U2, U4 and U5 snRNAs remain largely unknown. In contrast to the U6 snRNA, maturation of the pol II-specific snRNAs possesses a cytoplasmic phase (Mattaj, 1986). Following

synthesis, the U1, U2, U4 and U5 snRNAs are transported into the cytoplasm where binding of seven Sm proteins precedes the hypermethylation of their primary cap structures to 2,2,7-methylguanosine (TMG) and the trimming of their 3′-terminal trailer sequences. The mature snRNAs are re-imported into the nucleus as snRNPs. For synthesis of the Ψ44 residue in the yeast U2 snRNA, a tRNA modification enzyme, the Pus1p pseudouridine synthase, is responsible (Massenet *et al.*, 1999). *In vitro* RNA modification studies suggested that multiple activities accomplish pseudouridylation of mammalian U1, U2 and U5 snRNAs (Patton, 1993, 1994). Thus far, however, no *trans*-acting factor functioning in modification of mammalian pol II-specific snRNAs has been identified. Here, we describe a novel snoRNA, called U85, which can direct 2′-*O*-methylation of the C45 and pseudouridylation of the neighbouring U46 residue in the invariant loop 1 of the U5 spliceosomal snRNA.

Results

Identification of an evolutionarily conserved box C/D+H/ACA snoRNA

RNAs immunoprecipitated from a human HeLa cell extract with anti-fibrillarin antibodies were pCp-labelled and analysed on a 6% sequencing gel (Figure 1A). Besides many previously identified box C/D snoRNAs, a novel slowly migrating RNA species, referred to hereafter as U85, was detected. The U85 RNA was recovered, purified further on a 12% native polyacrylamide gel and its 3′-terminal nucleotides were determined by chemical sequencing. This sequence information was used for synthesis and cloning of a cDNA of U85 (see Materials and methods). The correct 5′ end of the new RNA was determined by primer extension (data not shown). Sequence analysis showed that the human U85 RNA consists of 330 nt (Figure 2) and, apart from the conserved C and D boxes, shows no similarity to any known RNA. Northern blot analysis of human, mouse and fruit fly cellular RNAs with a human U85-specific antisense RNA probe revealed a hybridizing RNA of 315–330 nt long in each of these species (Figure 1B). Although probing of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* cellular RNAs failed to unveil a U85-like RNA in yeast RNA (data not shown), we concluded that the U85 RNA represents a novel C and D box-containing fibrillarin-associated snoRNA that shows significant sequence conservation during evolution.

Nucleotide sequence of the *Drosophila* U85 snoRNA was determined by cloning and characterization of its cDNA (see Materials and methods). The fruit fly U85 snoRNA is 316 nt long and similarly to the human U85 snoRNA, it features a 5′-terminal box C and a 3′-terminal box D motif as well as internal C′ and D′ boxes (Figure 2). A computer-aided modelling of the human and *Drosophila* U85 snoRNAs revealed that the two RNAs fold into similar two-dimensional structures. In both snoRNAs, the 5′- and 3′-terminal regions containing the C and D boxes are predicted to form a long hairpin-like structure. In the middle of the human (from position 73 to 229) and *Drosophila* (from position 84 to 209) U85 snoRNA, a large region folds into a hairpin–hinge–hairpin structure highly reminiscent of the consensus structure of box H/ACA snoRNAs (Ganot *et al.*, 1997b). In the H/ACA-like domain of the two snoRNAs, the single-stranded hinge region carries a perfect H box and the 3′-terminal hairpin is followed by an ACA motif (Figure 2). In the human U85 snoRNA, the 3′ hairpin of the H/ACA-like domain contains an additional short hairpin (*IH*, inserted hairpin), which is frequently found in vertebrate and yeast box H/ACA snoRNAs (Ganot *et al.*, 1997b).

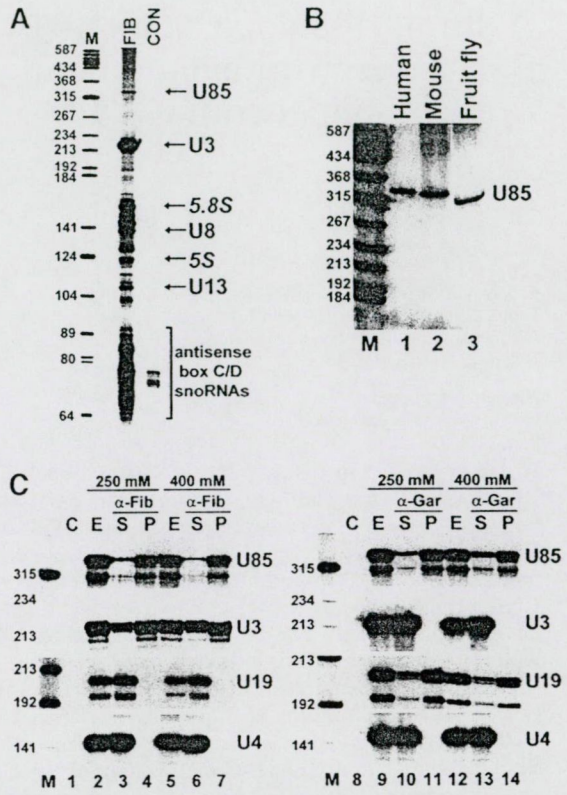

Fig. 1. Characterization of U85 snoRNA. (**A**) Human fibrillarin-associated snoRNAs. RNAs immunopreciptated with monoclonal anti-fibrillarin antibody (72B9) were 3′ end-labelled and fractionated on a 6% sequencing gel (lane FIB). Lane CON, control precipitation with monoclonal anti-Sp1 antibody. Lane M, size markers. (**B**) Northern analysis. RNAs from human, mouse and fruit fly cells were fractionated on a 6% denaturing gel and probed with an internally labelled antisense human U85 RNA. (**C**) Anti-fibrillarin and anti-hGAR1 antibodies recognize the human U85 snoRNP. Extracts prepared from HeLa cells in the presence of 250 or 400 mM NaCl were reacted with anti-fibrillarin (α-Fib) or anti-hGAR1 (α-Gar) antibodies. Distribution of the U85, U3 and U19 snoRNA and the U4 snRNA in the extracts (lanes E) and supernatants (lanes S) or pellets (lanes P) of the immunoprecipitation reactions were determined by RNase A/T1 protections. Lanes C represent control mappings with *Escherichia coli* tRNA. Lanes M, size markers.

We tested whether the human U85 snoRNA is complexed with Gar1p, which is a common component of all box H/ACA snoRNPs (Balakin *et al.*, 1996; Ganot *et al.*, 1997b). Extracts prepared from HeLa cells in the presence of 250 or 400 mM NaCl were immunoprecipitated with anti-hGAR1 and as a control, with anti-fibrillarin antibodies (Figure 1C). Distribution of the U85 snoRNA, the U3 box C/D snoRNA, the U19 box H/ACA snoRNA and the U4 spliceosomal snRNA was measured

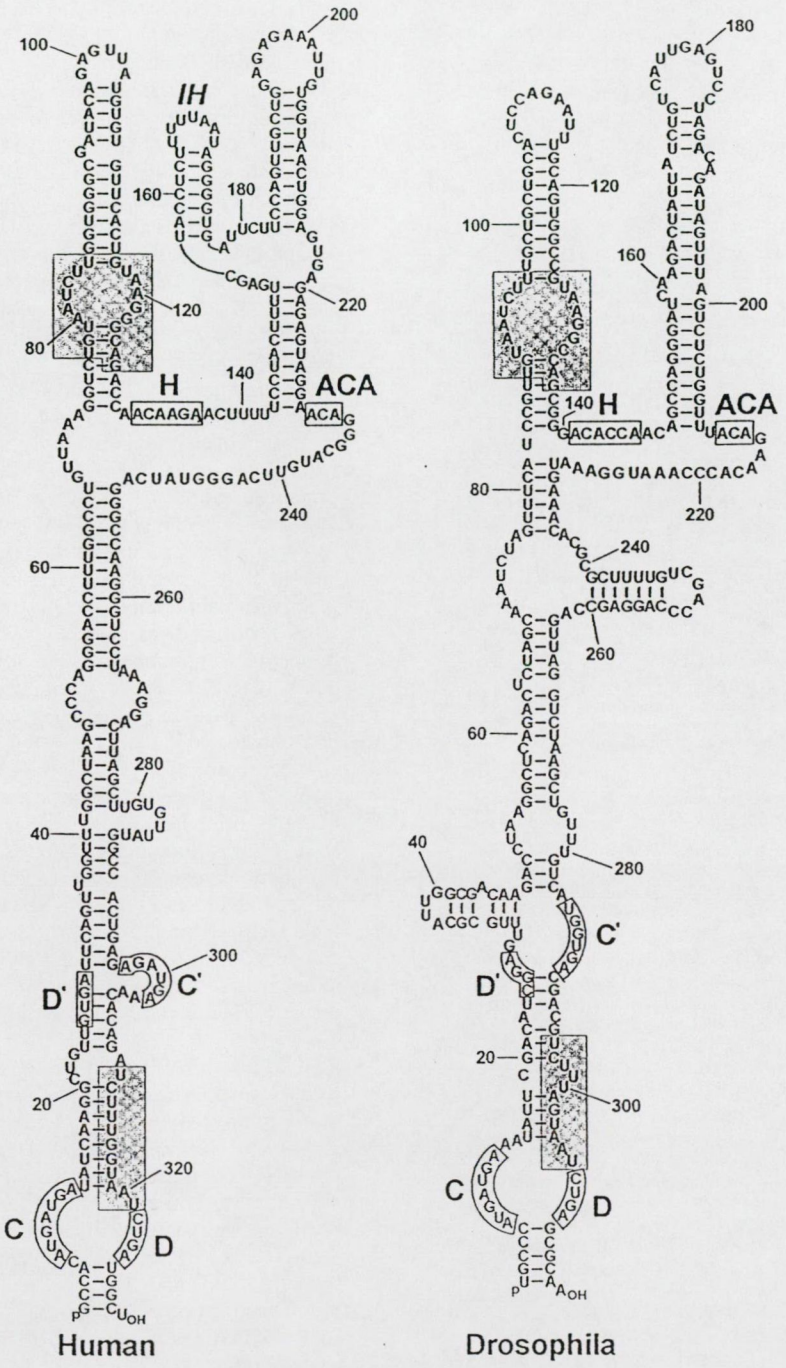

Fig. 2. Proposed secondary structures of the human and fruit fly U85 snoRNAs. The box C, C′, H, ACA, D and D′ motifs are boxed. Other sequences conserved between the two snoRNAs are shaded.

by RNase A/T1 mapping. In each immunoprecipitation reaction, the U85 RNA was found mainly in the pellet (Figure 1C, lanes 4, 7, 11 and 14), demonstrating that the human U85 snoRNA is specifically associated with fibrillarin and Gar1p. As expected, the anti-fibrillarin and anti-hGAR1 antibodies precipitated the U3 (lanes 4 and 7) and U19 snoRNPs (lanes 11 and 14), respectively, but neither of them recognized the U4 snRNP (lanes 4, 7, 11 and 14).

The C and D boxes direct accumulation of the intron-encoded U85 snoRNA

In vertebrates, most snoRNAs are processed from pre-mRNA introns. Although organization of the human U85

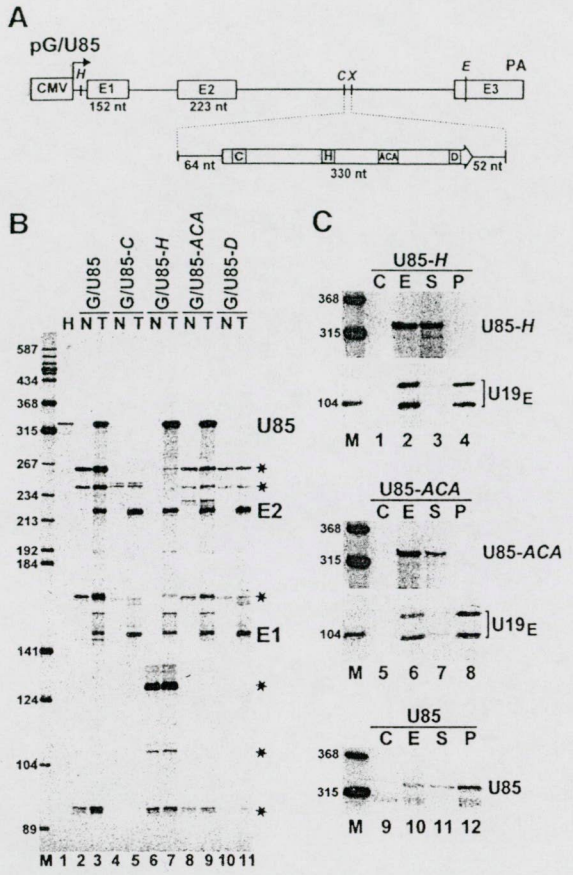

Fig. 3. Processing of the human U85 intronic snoRNA. (**A**) Structure of the expression constructs used for transfection of COS7 cells. The exons (E1, E2 and E3) and the polyadenylation site (PA) of the human β-globin gene and the promoter region of the cytomegalovirus (CMV) are indicated. Relevant restriction sites are shown (*H*, *Hin*dIII; *C*, *Cla*I; *X*, *Xho*I; *E*, *Eco*RI). The U85 snoRNA gene inserted into the second intron of the β-globin gene is indicated by open arrow. In the pG/U85-*C*, pG/U85-*H*, pG/U85-*ACA* and pG/U85-*D* expression constructs, the C, H, ACA or D boxes of U85 were replaced with C residues. (**B**) RNase A/T1 protection. Simian COS7 cells were transfected with the pG/U85, pG/U85-*C*, pG/U85-*H*, pG/U85-*ACA* or pG/U85-*D* expression construct. RNAs extracted from transfected (lanes T) or non-transfected (lanes N) cells were mapped with appropriate antisense RNA probes as indicated above the lanes. Lane H, control mapping with HeLa RNA. RNAs protected by the human U85 snoRNA, the first (E1) and second (E2) exon of the globin mRNA are indicated. (**C**) The U85-*H* and U85-*ACA* snoRNAs are not associated with Gar1p. Extracts of COS7 cells transfected with the pG/U85-*H*, pG/U85-*ACA* or the pG/U85 expression construct were subjected to immunoprecipitation with anti-hGAR1 antibodies. RNAs recovered from the extracts (E), the supernatant (S) or the pellet (P) of the immunoprecipitation reactions were mapped by RNase A/T1 protection by using RNA probes specific for the U85-*H*, U85-*ACA*, U85 and U19 snoRNAs. Lanes C, control mappings with *E.coli* tRNA. Lanes M, size markers.

snoRNA gene remains unclear, we found a perfect copy of the *Drosophila* U85 snoRNA within the first intron of the CG1142 gene (DDBJ/EMBL/GenBank accession No. AE003671), indicating that the *Drosophila* and presumably the human U85 snoRNAs are intron encoded. For processing and accumulation of the box C/D and H/ACA intronic snoRNAs, the conserved box elements are absolutely essential. Since the U85 snoRNA features boxes of both classes of snoRNAs, we decided to determine elements supporting its accumulation. The human U85 gene with short flanking sequences was inserted into the second intron of the human β-globin gene, which had been placed under the control of the cytomegalovirus (CMV) promoter (Figure 3A). The resulting G/U85 construct as well as the G/U85-*C*, G/U85-*H*, G/U85-*ACA* and G/U85-*D* constructs, in which the corresponding box element of U85 was replaced with a short C stretch, were transfected into simian COS7 cells. Accumulation of the U85 RNA and the spliced β-globin mRNA was measured by RNase protection with sequence-specific antisense RNA probes (Figure 3B). As indicated by the spliced exons (E1 and E2), β-globin mRNA was expressed in each transfected cell line (lanes 3, 5, 7, 9 and 11). Wild-type U85 snoRNA (lane 3) and U85 snoRNAs with altered H (lane 7) or ACA (lane 9) boxes were efficiently and, compared with the authentic human U85 snoRNA (lane 1), accurately processed from the β-globin pre-mRNA. In contrast, U85 snoRNAs carrying an altered C (lane 5) or D (lane 11) box showed no accumulation, demonstrating that the C and D boxes, but not the H and ACA boxes, provide metabolic stability for the U85 snoRNA. Other protected RNA bands, indicated by asterisks, resulted from partial protection of human U85-specific probes by the endogenous simian U85 snoRNA and therefore, they were also present in mapping reactions performed with RNAs from non-transfected cells.

When extracts of COS7 cells transfected with the G/U85-*H* and G/U85-*ACA* constructs were reacted with anti-hGAR1 antibodies, neither the U85-*H* nor the U85-*ACA* snoRNA was immunoprecipitated (Figure 3C, lanes 4 and 8). In comparison, the endogenous U19 H/ACA snoRNP (lanes 4 and 8) and the wild-type U85 snoRNA processed from the β-globin pre-mRNA (lane 12) were depleted. We concluded that the H and ACA boxes of the U85 snoRNA, although not required for RNA accumulation, play an essential role in snoRNP assembly.

The U85 snoRNA is predicted to function in pseudouridylation and 2′-O-methylation of the U5 spliceosomal RNA

Alignment of the human and fruit fly U85 snoRNAs revealed three highly conserved and presumably functionally important sequence motifs that occupy identical positions on the two-dimensional structures of the two snoRNAs (Figure 2, shaded nucleotides). The conserved sequences precede the D box and form a putative pseudouridylation pocket, suggesting that the U85 snoRNA may function as a 2′-*O*-methylation and pseudouridylation guide RNA.

To identify potential substrate RNAs for the U85 putative guide snoRNA, sequences of all known stable cellular RNAs, such as rRNAs, tRNAs, snRNAs and snoRNAs, have been carefully scrutinized. This search revealed that the alleged 2′-*O*-ribose recognition motif and pseudouridylation pocket of the human U85 snoRNA could position the C45 and the neighbouring U46 residues of the U5 spliceosomal snRNA for 2′-*O*-methylation and pseudouridylation, respectively (Figure 4A). Similar interactions could be drawn for the *Drosophila* U85 and U5 RNAs. Indeed, the C45 and U46 residues in the human and the corresponding C46 and U47 residues in the *Drosophila*

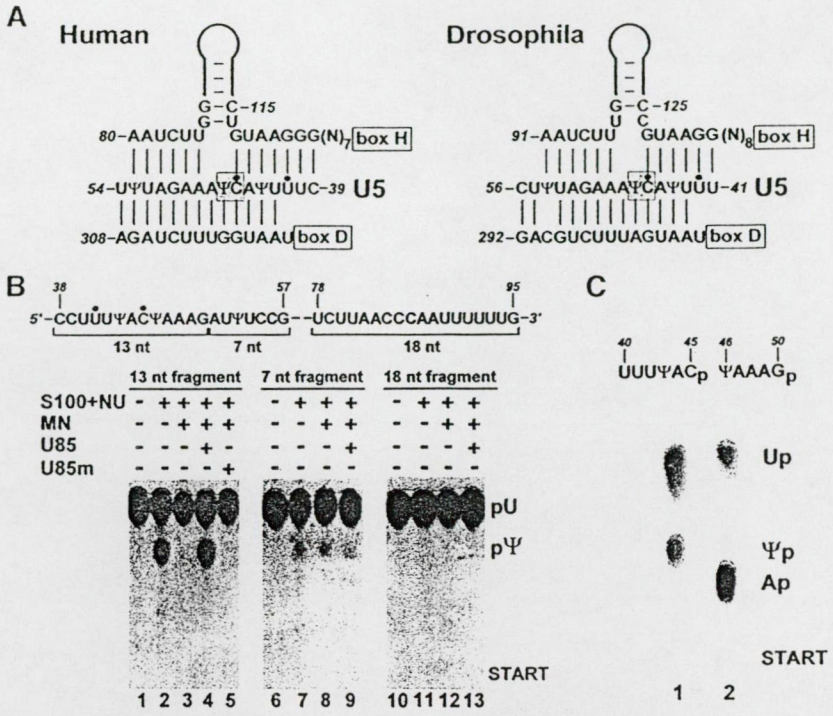

Fig. 4. The U85 snoRNA can direct modification of the U5 snRNA. (**A**) Potential hydrogen bonding between U85 and U5 RNAs in human and *Drosophila* cells. The 5′ hairpins in the H/ACA domains of the U85 snoRNAs are schematically represented. Pseudouridines (Ψ) and 2′-*O*-methyl groups (closed circles) in the U5 snRNA are indicated. Nucleotides potentially selected by the U85 snoRNAs are shaded. (**B**) *In vitro* pseudouridylation of U5 RNA. In the presence or absence of *in vitro* synthesized wild-type (U85) or mutant (U85m) human U85 snoRNA, [α-^{32}P]UTP-labelled U5 snRNA was incubated in a HeLa extract (S100+NU) that had been either not treated or treated with micrococcal nuclease (MN). Three RNase T1 fragments of the U5 substrate RNAs were digested with nuclease P1 and analysed by one-dimensional TLC. PhosphorImager quantification revealed that the pseudouridine content of the 13 nucleotide fragment was ~35% of the theoretical yield. (**C**) *In vitro* transcribed U85 and [^{32}P]ATP-labelled U5 RNA were incubated with an MN-treated extract. The 6- (lane 1) and 5-nt (lane 2) RNase CL-3 subfragments of the 13-nt RNase T1 fragment of substrate U5 RNA were digested with RNase T2 and analysed by TLC.

U5 snRNA are known to be 2′-*O*-methylated and pseudouridylated, respectively (Myslinski *et al.*, 1984; Reddy and Busch, 1988). Neither the human nor the *Drosophila* U85 snoRNA showed significant complementarity to any other stable RNA, supporting the intriguing hypothesis that the U85 snoRNA directs 2′-*O*-ribose methylation and pseudouridylation of two consecutive nucleotides in the U5 snRNA.

The U85 snoRNA directs in vitro pseudouridylation of the U5 snRNA

Previously, an *in vitro* modification system that employs a HeLa S100 extract in combination with a nuclear extract has been successfully used for site-specific synthesis of all three pseudouridines present in the human U5 snRNA at positions 43, 46 and 53 (Patton, 1991, 1993, 1994). To identify *trans*-acting factors directing pseudouridylation of the U5 snRNA, *in vitro* synthesized uridylate-labelled U5 snRNA was incubated with the U5 modification system. The modified U5 RNA was recovered and fragmented by RNase T1. After polyacrylamide gel fractionation, each uridine-containing T1 fragment was digested with nuclease P1 and its pseudouridine content was determined by TLC (Figure 4B and data not shown). Apart from the 13- and 7-nt fragments, which carry the U43, U46 and U53 residues (Figure 4B, lanes 2 and 7), none of the T1 fragments contained pseudouridines. As an example, TLC analysis of the 18-nt U78–G95 fragment is shown (lane 11). Similarly, no pseudouridine was detected in a control experiment when the U5 RNA was not incubated with HeLa extracts (lanes 1, 6 and 10). After pre-treatment of the extract with micrococcal nuclease (MN), formation of pseudouridines in the 13-nt fragment was abolished (lane 3), indicating that some nucleic acid component(s) of the extract play(s) an essential role in pseudouridylation of the U43 and U46 residues. In contrast, MN treatment did not impair pseudouridine formation in the 7-nt fragment (lane 8), suggesting that formation of Ψ53 is supported exclusively by a protein factor(s).

When an MN-treated modification extract was supplemented with *in vitro* transcribed U85 snoRNA, pseudouridine synthesis in the 13-nt fragment was restored (Figure 4B, lane 4). Inclusion of U85 snoRNA, however, did not facilitate pseudouridylation of the 7- and 18-nt fragments (lanes 9 and 13). Upon reconstitution of pseudouridylation of a [^{32}P]ATP-labelled U5 RNA by adding *in vitro* synthesized U85 to an MN-treated extract, RNase T2 digestion liberated ^{32}P-labelled pseudouridine 3′-monophosphates only from the 6-nt RNase CL-3 subfragment (40-UUUUAC-45) of the 13-nt T1 fragment (Figure 4C, lane 1), further supporting that U85 specifically restores pseudouridylation of the U46 residue. Even

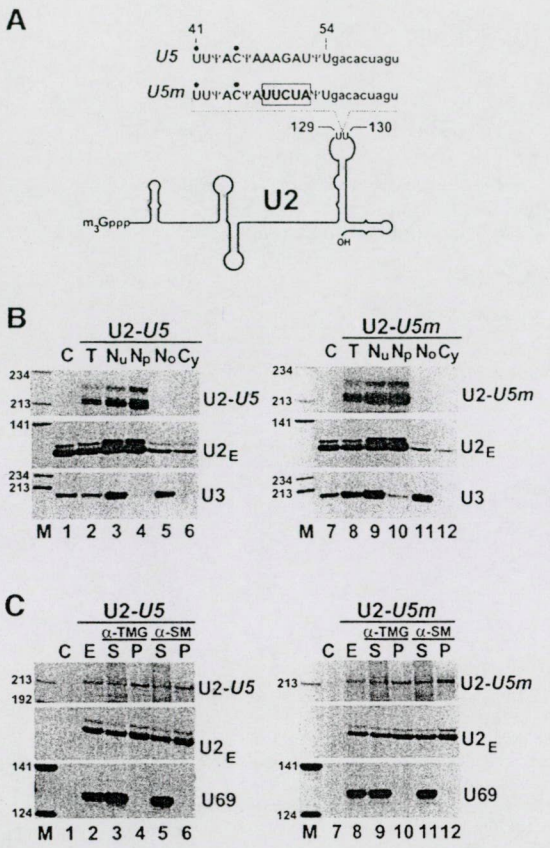

Fig. 5. Expression of chimeric U2 snRNAs in COS7 cells. (**A**) Schematic structure of the U2–*U5* and U2–*U5m* snRNAs. Sequences of the *U5* and *U5m* tags and positions of pseudouridines (Ψ) and 2′-*O*-methylated nucleotides (closed circles) are shown. The authentic U5 sequences are in upper case. Altered nucleotides in the *U5m* tag are shaded. (**B**) Subcellular localization of U2–*U5* and U2–*U5m* RNAs. RNAs extracted either from whole cells (T) or from the nuclear (Nu), nucleoplasmic (Np), nucleolar (No) and cytoplasmic (Cy) fractions of COS7 cells transfected with the pGL/U2-*U5* or pGL/U2-*U5m* construct were mapped by RNase A/T1 protection using antisense RNA probes specific for the U2–*U5* and U2–*U5m* snRNAs, and the U3 snoRNA. Lanes C, control mappings with RNAs from non-transfected cells. Lanes M, size markers. (**C**) Immunoprecipitation. Extracts obtained from COS7 cells transfected with the pGL/U2-*U5* or pGL/U2-*U5m* construct were precipitated with anti-trimethylguanosine (α-TMG) and anti-SM (α-SM) antibodies. RNAs extracted from cell extracts (E) or from the supernatant (S) and pellet (P) of the immunoprecipitation reactions were mapped by RNase protection by using RNA probes specific for the U2–*U5* and U2–*U5m* snRNAs, and the U69 snoRNA. Lanes C, control mappings with *E.coli* tRNA.

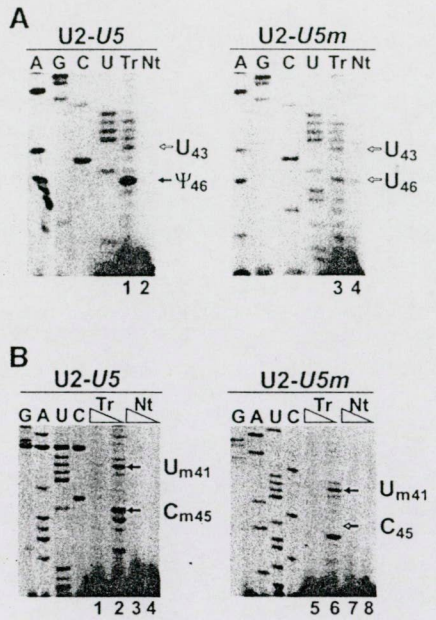

Fig. 6. Primer extension mapping of pseudouridines and 2′-*O*-methylated nucleotides in the U2–*U5* and U2–*U5m* snRNAs. (**A**) Mapping of pseudouridines. CMC-alkali-treated cellular RNAs extracted from COS7 cells transfected (Tr) or non-transfected (Nt) with the pGL/U2-*U5* and pGL/U2-*U5m* constructs were analysed by primer extension by using a terminally labelled oligonucleotide primer specific for the U2–*U5* and U2–*U5m* snRNAs. Closed and open arrows indicate the actual and expected positions of pseudouridines, respectively. Lanes A, G, C and U are dideoxy sequencing reactions. (**B**) Mapping of 2′-*O*-methyl groups. Cellular RNAs isolated from transfected (Tr) or non-transfected (Nt) cells were analysed by primer extension in the presence of 1.0 or 0.004 mM dNTPs as indicated above the lanes.

more tellingly, a mutant version of the U85 snoRNA (U85m) that carries five altered nucleotides in the putative pseudouridine recognition motif at positions 81–85 (see Figures 4A and 7A) failed to restore pseudouridylation of the 13-nt fragment (Figure 4B, lane 5). In conclusion, the U85 snoRNA can support *in vitro* pseudouridylation of the U5 snRNA, and this function of the U85 snoRNA depends on sequences that are predicted to position the U46 residue for pseudouridylation.

Chimeric U2–U5 snRNA is correctly modified in simian COS7 cells

Next, we tested whether the U85 snoRNA is capable of directing pseudouridylation and 2′-*O*-methylation of the U5 snRNA in living cells. To this end, a short fragment of the human U5 snRNA encompassing the C45 and U46 residues was inserted into the coding region of the U2 snRNA gene that is also transcribed by RNA pol II (Figure 5A). As a control, a mutant version of the U5 tag (*U5m*) carrying five altered nucleotides that are predicted to prevent base-pairings with the putative pseudouridylation and 2′-*O*-methylation guide sequences of the U85 snoRNA was also inserted into the U2 gene. Upon transfection into COS7 cells, the U2–*U5* and U2–*U5m* chimeric snRNAs were correctly expressed and accumulated in the nucleoplasm like the endogenous simian U2 snRNA (Figure 5B, lanes 4 and 10). As expected, the endogenous U3 snoRNA was found in the nucleolar fraction of COS7 cells (lanes 5 and 11).

The pol II-transcribed snRNAs are capped with a 5′-terminal TMG and are associated with Sm proteins. When extracts of COS7 cells expressing the U2–*U5* and U2–*U5m* RNAs were reacted with anti-TMG and anti-Sm antibodies, the U2–*U5* and U2–*U5m* RNAs were immunoprecipitated together with the endogenous U2 snRNA ($U2_E$) (Figure 5C, lanes 4, 6, 10 and 12). In comparison, neither the anti-TMG nor the anti-Sm antibody recognized the U69 H/ACA snoRNP. Since TMG formation and packaging with Sm proteins occur in the cytoplasm (Mattaj, 1986), we concluded that the U2–*U5* and U2–*U5m* snRNAs, prior to nucleoplasmic accumula-

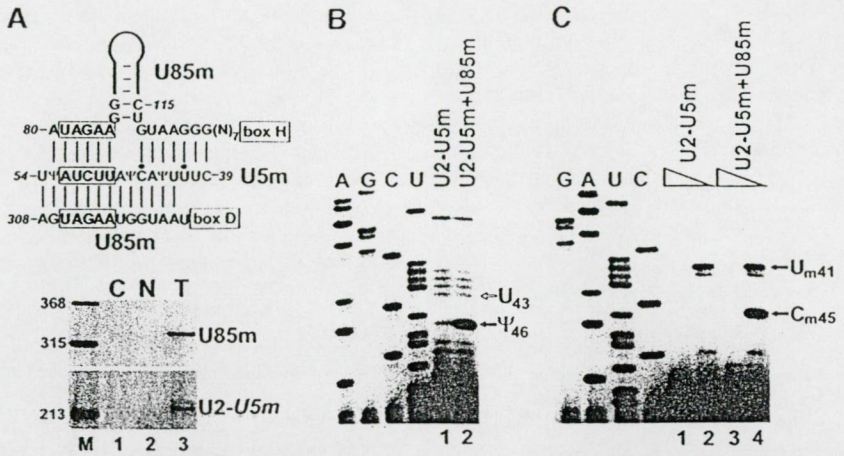

Fig. 7. Restoration of pseudouridylation and 2′-*O*-methylation of the U2–*U5m* snRNA. (**A**) Expression and predicted interaction of the U85m pseudouridylation and 2′-*O*-methylation guide snoRNA and the U2–*U5m* snRNA. RNAs from COS7 cells transfected (T) and non-transfected (N) with the pG/U85m/U2-*U5m* expression construct were mapped by RNase protection. Lane M, size markers. (**B**) Mapping of pseudouridines. CMC-alkali-treated cellular RNAs extracted from COS7 cells expressing the U2–*U5m* snRNA alone (lane 1) or together with the U85m snoRNA (lane 2) were analysed by primer extension. (**C**) Mapping of 2′-*O*-methylated nucleotides. Distribution of ribose-methylated nucleotides in the U2–*U5m* snRNA expressed in the absence (lanes 1 and 2) or presence of the U85m snoRNA (lane 3 and 4) was determined by primer extension analysis.

tion, went through the normal maturation pathway of pol II-specific snRNPs.

To locate pseudouridines in the U2–*U5* and U2–*U5m* chimeric snRNAs, the CMC [N^3-1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate] modification–primer extension method was used (Bakin and Ofengand, 1993). CMC specifically reacts with pseudouridine and arrests the reverse transcriptase 1 nt before the pseudouridylation site. Mapping of the U2–*U5* snRNA revealed that the U46 residue was correctly pseudouridylated in the U5 tag (Figure 6A, lane 1). However, alteration of the 3′ flanking sequences of the U46 residue in the U2–*U5m* RNA inhibited its pseudouridylation (lane 3, see also Figure 7B, lane 1). The same reason may account for the deficiency of formation of the Ψ43 residue that lacks its authentic 5′ flanking sequences both in the U2–*U5* and U2–*U5m* RNA (Figure 6A, lanes 1 and 3). Unfortunately, the U2-specific pseudouridines in the 5′ region of the U2–*U5* and U2–*U5m* snRNAs could not be unambiguously discerned, although synthesis of the Ψ54, Ψ58 and Ψ91 residues was apparent in both RNAs (data not shown).

Positions of 2′-*O*-methyl groups were mapped by the dNTP concentration-dependent primer extension procedure (Maden *et al.*, 1995). In low dNTP concentration, reverse transcriptase pauses 1 nt before and/or at the 2′-*O*-methylated nucleotide. Mapping of the U2–*U5* snRNA resulted in stop signals before the C45 and at the U41 residue, indicating that the U5 tag sequence of the U2–*U5* snRNA undergoes correct 2′-*O*-methylation (Figure 6B, lane 2). In comparison, no stops were observed in the presence of 1 mM dNTPs (lane 1) or in control mappings performed on RNAs from non-transfected cells (lanes 3 and 4). Mapping of the U2–*U5m* snRNA revealed that alteration of the U5 tag sequence abolished 2′-*O*-methylation of the C45 residue, but did not influence the synthesis of Um41 (lane 6, see also Figure 7C, lane 2). This latter observation could be explained by the fact that a putative guide snoRNA directing the 2′-*O*-methylation of U41 is predicted to possess an at least 9-nt-long complementarity to the U2–*U5m* snRNA (data not shown). Moreover, most, if not all, of the ribose- and base-methylated nucleotides reported for mammalian U2 snRNAs were also present in the U2–*U5* and U2–*U5m* snRNAs (data not shown). These results demonstrate that the short flanking sequences provide the necessary and sufficient information for the site-specific synthesis of the Cm45 and Ψ46 residues in the U5 snRNA and, therefore, strongly support the idea that selection of these nucleotides is a guide RNA-mediated process.

Compensatory base changes in the U85 snoRNA can restore modification of the U2–U5m snRNA

We investigated whether mutations in the U5-specific tag of the U2–*U5m* snRNA inhibiting 2′-*O*-methylation of the C45 and pseudouridylation of the U46 residue could be suppressed by compensatory base changes in the U85 snoRNA. Five nucleotides in the pseudouridylation (81-AUCUU-85) and 2′-*O*-methylation (310-AUCUU-314) guide sequences of the human U85 snoRNA were replaced with the UAGAA sequence motif. Figure 7A shows the postulated base pairing between the resulting U85m snoRNA and U2–*U5m* snRNA. The U85m gene was inserted into the pG expression construct (Figure 3A) that carried the U2–*U5m* gene (see Materials and methods). Upon transfection of the resulting pG/U85m/U2–*U5m* expression construct into COS7 cells, both U85m and U2–*U5m* RNAs accumulated (Figure 7A, lane 3).

Primer extension mapping of pseudouridines in the U2–*U5m* snoRNA resulted in a strong reverse transcriptase stop 1 nt before the U46 residue (Figure 7B, lane 2), demonstrating that expression of the U85m snoRNA re-established the site-specific synthesis of Ψ46. Likewise, 2′-*O*-methylation mappings revealed that methylation of the U2–*U5m* snRNA at the C45 residue was restored in the

presence of the U85m snoRNA (Figure 7C, lane 4). Control mappings performed on RNAs extracted from COS7 cells expressing only the U2–*U5m* snRNA failed to detect the Ψ46 (Figure 7B, lane 1) and Cm45 residues (Figure 7C, lane 2). These results demonstrate that compensatory base changes in the U85 snoRNA can restore the site-specific pseudouridylation and 2′-*O*-methylation of mutant U5 sequences expressed within the pol II-transcribed U2 snRNA.

Discussion

In the nucleolus, two structurally and functionally well defined families of snoRNAs function in the post-transcriptional modification of rRNAs. The box C/D snoRNAs direct 2′-*O*-methylation and the box H/ACA snoRNAs guide pseudouridylation of the 18, 5.8 and 28S rRNAs. We have identified a novel, evolutionarily conserved snoRNA that is composed of both box C/D and box H/ACA snoRNA domains. Instead of directing rRNA modification, several lines of evidence support the idea that the U85 snoRNA functions in 2′-*O*-methylation and pseudouridylation of the U5 spliceosomal snRNA. Sequences of the U85 snoRNA predicted to select the target nucleotides in the U5 snRNA for 2′-*O*-methylation and pseudouridylation are conserved during evolution. The U85 snoRNA is capable of directing the site-specific pseudouridylation of the U5 snRNA *in vitro*. Recognition of the U5 target nucleotides depends on short sequence motifs that are predicted to base-pair with the U85 snoRNA. Finally, the site-specific pseudouridylation and 2′-*O*-methylation of an altered U5 sequence can be restored by compensatory base changes introduced into the U85 snoRNA. The U85 snoRNA is the first example of a guide RNA that directs both pseudouridylation and 2′-*O*-methylation reactions and that functions in post-transcriptional modification of an RNA pol II-transcribed snRNA.

The human U85 snoRNA guides 2′-*O*-methylation of C45 and pseudouridylation of the U46 residues in the evolutionarily invariant terminal loop 1 of the U5 spliceosomal snRNA. During pre-mRNA splicing, nucleotides in the terminal loop 1 of the U5 snRNA interact with exon sequences immediately adjacent to the 5′ and 3′ splice sites (Sontheimer and Steitz, 1993). In the invariant loop 1 of the U5 snRNA, the modified Cm45 and Ψ46 residues, together with two additional 2′-*O*-methylated nucleotides (Gm37 and Um41) and a pseudouridine (Ψ43), show a striking evolutionary conservation. They are present in vertebrate, insect, plant, green alga, lime mold and protozoan U5 snRNAs (for references see Szkukalek *et al.*, 1995). These modifications are likely to have an important function in pre-mRNA splicing. They could strengthen the pre-mRNA–U5 snRNA interaction, or alternatively could stabilize the structure of U5 snRNA and/or facilitate its interaction with protein splicing factors. Interestingly, in the *S.cerevisiae* and *S.pombe* U5 snRNAs a C residue is present at the position corresponding to Ψ46 in mammalian RNAs. This, of course, could explain the lack of a U85-like RNA in yeast cells.

The phylogenetic invariance of the structural organization of U85 snoRNA suggests that 'co-expression' of a pseudouridylation and a 2′-*O*-methylation guide snoRNA responsible for modification of two consecutive nucleotides in the U5 snRNA is advantageous for the cell. The *Drosophila* and most probably the human U85 snoRNA belong to the family of intron-encoded snoRNAs. The human U85 snoRNA is faithfully processed from the human β-globin pre-mRNA in COS7 cells, and its accumulation relies exclusively on the 5′,3′-terminal box C/D domain. In the absence of a functional C or D box, the H/ACA domain of the U85 snoRNA cannot accumulate independently. This might be a way to 'trap' the box H/ACA domain within the U85 snoRNA and to secure co-expression of two snoRNAs of different classes as 'Siamese twins' within a single snoRNP particle. Although the H and ACA boxes are dispensable for RNA accumulation, they play an essential role in the assembly of a functionally active box H/ACA snoRNP.

Concerning the molecular mechanism by which the U85 snoRNP mediates 2′-*O*-methylation and pseudouridylation of the U5 snRNA, many details remain to be answered. Apparently, the two modification reactions can be achieved only consecutively, since the 2′-*O*-methylation and pseudouridylation recognition motifs of the U85 snoRNA cannot base pair with the same target sequence at the same time. At the moment we cannot answer whether the same or two independent U85 snoRNP particles direct 2′-*O*-methylation and pseudouridylation of the C45 and U46 residues in the U5 snRNA. Clearly, packaging of the 2′-*O*-methylation and pseudouridylation activities into a common particle favours the idea that a single U85 particle can catalyse both reactions. If so, is there any obligate order of the two reactions? Can the 2′-*O*-methylation and pseudouridylation domains of the U85 snoRNP function in concert?

The evolutionarily conserved box elements have been demonstrated to act as nucleolar localization signals both for box C/D (Lange *et al.*, 1998; Samarsky *et al.*, 1998; Narayanan *et al.*, 1999a) and H/ACA (Lange *et al.*, 1999; Narayanan *et al.*, 1999b) snoRNAs. Since the U85 snoRNA possesses the box elements of both classes of snoRNAs, it is predicted to accumulate in the nucleolus. Interestingly, we have recently identified three other human snoRNAs that are predicted to act as guides for 2′-*O*-methylation and pseudouridylation of the U1, U4 and U2 snRNAs, respectively. The new snoRNAs, however, conform to the canonical structures of box C/D and H/ACA snoRNAs. Although the functional characterization of the new snoRNAs is still in progress, we can envisage that snoRNA-mediated 2′-*O*-methylation and pseudouridylation of RNA pol II-transcribed spliceosomal snRNAs is more common than has been anticipated (Ganot *et al.*, 1999; Massenet *et al.*, 1999).

A notion that snoRNAs can direct post-transcriptional modification of U5 and perhaps U1, U2 and U4 snRNAs raises the question of where in the cell modification of these RNAs occurs. Previously, three box C/D snoRNAs have been identified that function as guides in 2′-*O*-methylation of the RNA pol III-transcribed U6 snRNA (Tycowski *et al.*, 1998; Ganot *et al.*, 1999). Moreover, all factors required for synthesis of the eight 2′-*O*-methylated nucleotides and three pseudouridines of the U6 snRNA have been found within the nucleolus (Ganot *et al.*, 1999). Upon injection into the nucleoplasm of *Xenopus* oocytes, the U6 snRNA has been found to

accumulate transiently in the nucleolus (Lange and Gerbi, 2000). Therefore, the available data are most consistent with the idea that modification of the U6 snRNA occurs in the nucleolus (Ganot *et al.*, 1999; Lange and Gerbi, 2000).

In contrast to the U6 snRNA, the pol II-transcribed U1 and U2 snRNAs show no major accumulation in the nucleolus after injection into the nucleoplasm of *Xenopus* oocytes (Lange *et al.*, 1999; Narayanan *et al.*, 1999a,b). However, Lange and Gerbi (2000) reported a small amount of U2 snRNA in the nucleolus that was above background. Moreover, test U2 snRNAs recovered from the nuclei of *Xenopus* oocytes were found to be fully modified, indicating that modification of the U2 and presumably all pol II-specific snRNAs occurs in the nucleoplasm (Yu *et al.*, 1998). A small fraction of U1, U2, U4/U6 and U5 snRNPs has long been known to colocalize with Cajal bodies in the nucleoplasm (Carmo-Fonseca *et al.*, 1992; Matera and Ward, 1993; Spector, 1993; Sleeman and Lamond, 1999). Cajal bodies are also known to contain box C/D snoRNAs (Samarsky *et al.*, 1998; Narayanan *et al.*, 1999b) as well as the fibrillarin and Nap57p snoRNP proteins, which are thought to provide the catalytic activities for the snoRNP-mediated RNA modification reactions (Raska *et al.*, 1991; Meier and Blobel, 1994). These observations make Cajal bodies a possible nuclear locale where modification of pol II-transcribed snRNAs might occur (Yu *et al.*, 1998). Intriguingly, a remarkable structural and functional relationship exists between Cajal bodies and nucleoli. As well as sharing common antigens, Cajal bodies are frequently found within nucleolar structures (Lyon *et al.*, 1997), leading to the fascinating concept that snRNPs might be delivered into the nucleolus by Cajal bodies to undergo snoRNA-mediated modifications (Yu *et al.*, 1998).

In summary, identification of a novel type of snoRNA that is composed of a box C/D and H/ACA domain and that functions in 2′-*O*-methylation and pseudouridylation of the RNA pol II-transcribed U5 spliceosomal snRNA raises exciting questions. Is the U85 snoRNA the first member of a new family of snoRNAs? If so, how many box C/D+H/ACA 'Siamese' snoRNAs exist in the cell? How many composite snRNAs featuring structural domains of distinct classes of small RNAs exist in the cell? In this context, it is noteworthy that the human and yeast telomerase RNAs have recently been demonstrated to contain box H/ACA and Sm domains, respectively (Mitchell *et al.*, 1999; Seto *et al.*, 1999). These questions clearly indicate that much still remains to be learned about the complex and fascinating world of small nuclear RNAs.

Materials and methods

General procedures

Unless otherwise stated, all techniques used for manipulating RNA, DNA and oligonucleotides were as described by Sambrook *et al.* (1989). The following oligonucleotides were used in this study: 1. GCCATCAGATTACCAAAGATC; 2. AATAGGAT(C)$_{18}$; 3. ATAATCGATGGAAGGTGTTTGTTATC; 4. ATACTCGAGTTTCACTCACTTCTTTC; 5. CAACAGCCTTGATAGGGGGGTGTGGCTGACAAAATGT; 6. CTGTATCGCCCACCTTCTATACAGACCTTTA; 7. CTTGCAGCCATCAGATTACCATTCTACTGTGTTTCATCT; 8. ACAGATCTTTGGTAATCCCCTGGCTGCAAGTGCTGGGTG; 9. TTTCTCGAGTA-CGAACAAGGAAGTGG; 10. TTTGAGCTCCTATTCCATCTCCC; 11. TTTACTAGTTGCTCCGTCCACTCCACG; 12. TTTGGTACCTCAGGGAAGCAGTTAAGCT; 13. TTTACTAAAGATTTGACA; 14. CTAGTGTCAAATCTTTAGTAAAAGCT; 15. TTTACTATTCTATTGACA; 16. CTAGTGTCAATAGAATAGTAAAAGCT; 17. CTGTATCGCCCACCTTCTATACAGACCTTTA; 18. CTTGCAGCCATCAGATTACCATTCTACTGTGTTTCATCT; 19. GGACGGAGCAACTAGTGT; 20. ATAGTCGACATAGTCGGACCTCGAGTACGAACAAGGAAG; 21. ATATCTAGAGGATCCCCGGGTACCTCAGGGAAGCAGTTA; 22. ATAGAATTCTGTAATACGACTCACTATAGGATACTCTGGTTTCTCTTCA; 23. ATATCTAGATGCATGGCCTTGCCAAGGCAAGG.

Characterization of U85 snoRNA

RNAs extracted from pellets of immunoprecipitation reactions performed on a HeLa cell extract with anti-fibrillarin antibodies were labelled using [5′-^{32}P]pCp and T4 RNA ligase. The labelled RNAs were fractionated on a 6% polyacrylamide gel containing 8 M urea. The U85 snoRNA was further purified on a 12% native polyacrylamide gel and subjected to chemical sequencing. Human and *Drosophila* U85-specific cDNAs were synthesized by AMV (avian myeloblastosis virus) reverse transcriptase using an oligonucleotide primer (oligo 1) complementary to the human U85 snoRNA from position G309 to C329. A homopolymer G tail was added to the resulting cDNAs by terminal deoxynucleotidyltransferase. The cDNAs were PCR-amplified using oligos 1 and 2 as primers and cloned into the *Sma*I–*Bam*HI sites of the pBluescribe vector (Stratagene). Folding of U85 RNAs was performed by using the RNAdraw computer program (http://rnadraw.base8.se).

Construction of plasmids for transfection of COS7 cells

To generate the pG/U85 expression construct, a 446 bp human genomic fragment encompassing the U85 gene and its 64/52 bp flanking sequences was PCR amplified (oligos 3 and 8). The amplified fragment was digested with *Cla*I and *Xho*I and inserted into the same sites of the pG$_{CXM}$ expression vector (Ganot *et al.*, 1997b). Construction of pG/U85-*C*, pG/U85-*H*, pG/U85-*ACA* and pG/U85-*D* was performed by asymmetric PCR amplifications using the pG/U85 construct as a template. 'Megaprimers' were generated by PCR amplification reactions performed with oligos 3/5 (pG/U85-*C*), 3/6 (pG/U85-*H*), 4/7 (pG/U85-*ACA*) and 4/8 (pG/U85-*D*). Oligos 5, 6, 7 and 8 carried appropriate sequences to replace the box C, H, ACA or D sequences with short C stretches. The amplified fragments were used as megaprimers in the second amplification reaction, where oligos 4 (pG/U85-*C* and pG/U85-*H*) and 3 (pG/U85-*ACA* and pG/U85-*D*) were used as 5′ and 3′ end-specific primers, respectively. The amplified mutant U85 genes were inserted into the *Cla*I and *Xho*I sites of pG$_{CXM}$.

To obtain pGL/U2-*U5* and pGL/U2-*U5m*, two fragments of the human U2 gene (DDBL/EMBL/GenBank accession No. K03023), positions −326 to +129 and +130 to +307 were PCR amplified using oligos 9/10 and 11/12 as primers, respectively. The amplified 5′- and 3′-terminal fragments of the U2 gene were digested with *Xho*I–*Sac*I and *Spe*I–*Kpn*I, respectively. Annealed oligos 13/14 (pGL-U2-*U5*) and 15/16 (pGL-U2-*U5m*) were inserted between the *Sac*I and *Spe*I sites and the resulting tagged U2 genes were cloned into the *Xho*I and *Kpn*I sites of the pGL2-Promoter vector (Promega). The U85m gene was created by three consecutive PCR reactions in which the pG/U85 construct was used as a template. In the first reaction, a 5′-terminal fragment of the U85m gene was generated with oligos 3 and 17, which resulted in an altered pseudouridine recognition motif of U85. The amplified fragment was used as a megaprimer in the second amplification reaction, in which the 3′ primer (oligo 18) introduced an altered 2′-*O*-methylation recognition motif. This fragment was purified and used as a megaprimer with oligo 4 to amplify the full-length U85m gene that was inserted into the *Cla*I and *Xho*I sites of pG$_{CXM}$, resulting in pG/U85m. To obtain pG/U85m/U2-*U5m*, the U2-*U5m* gene was PCR amplified with oligos 20 and 21 using the pGL/U2-*U5m* construct as a template. The amplified fragment was digested with *Sal*I and *Xba*I and inserted into the same sites of the pG/U85m construct. Transfection of COS7 cells has been described (Ganot *et al.*, 1997b).

RNA analysis

RNAs were isolated by the guanidinium thiocyanate/phenol–chloroform extraction procedure (Goodall *et al.*, 1990). For northern analysis, ~5 μg of human and mouse and 10 μg of *Drosophila* cellular RNAs were fractionated on a 6% denaturing gel and electroblotted onto Hybond-N nylon membrane (Amersham Pharmacia Biotech). The filter was probed with an antisense RNA probe complementary to the human U85 RNA (see below). The filter was washed in 0.1× SSC containing 0.5% SDS at 50°C. RNase A/T1 protection was performed as described by Goodall *et al.* (1990). Antisense RNA probes used for mapping of human U4, U3, U19 and U69 RNAs have been described (Ganot *et al.*, 1997b). To obtain sequence-specific probes for the U85, U85m, U85-*C*, U85-*H*, U85-*ACA*

and U85-*D* RNAs, the *Hin*dIII–*Eco*RI fragments of the pG/U85, pG/U85m, pG/U85-*C*, pG/U85-*H*, pG/U85-*ACA* and pG/U85-*D* constructs were inserted into the same sites of the pBluescribe vector. The resulting recombinant plasmids were cut with *Hin*dIII and transcribed by T7 RNA polymerase. To generate antisense RNA probes for the U2–*U5* and U2–*U5m* snRNAs, the *Xho*I–*Kpn*I fragment of pGL/U2-*U5* and pGL/U2-*U5m* was cloned into the corresponding sites of the pBluescript KS-vector (Stratagene), and probes were synthesized from the *Xho*I-cut recombinant plasmids by T3 RNA polymerase. Pseudouridines and 2′-*O*-methylated nucleotides were mapped as described by Bakin and Ofengand (1993) and Maden *et al.* (1995), respectively. Kinase-labelled oligo 19 was used as a primer for mapping of modified nucleotides in the U2–*U5* and U2–*U5m* chimeric snRNAs.

Immunoprecipitation and in vitro pseudouridylation

Preparation of HeLa and COS7 cell extracts for immunoprecipitation reactions was performed essentially as described by Ganot *et al.* (1997b). Cells were sonicated in 40 mM Tris–HCl pH 7.5, 0.05% NP-40 containing 250 or 400 mM NaCl. The anti-hGAR1 antipeptide, the anti-fibrillarin monoclonal (72B9) and the anti-TMG polyclonal antibodies were kindly provided by Drs W.Filipowicz, J.A.Steitz and R.Lührmann, respectively. Monoclonal anti-Sp1 antibody was purchased from Santa Cruz Biotechnology. *In vitro* pseudouridylation of U5 snRNA was performed as described (Patton, 1991, 1994). For hydrolyses of nucleic acids, 100 µl of S100 or nuclear extract were treated with 15 µg of MN at 30°C for 15 min. MN was inhibited by adding EGTA to 3 mM final concentration. To generate a U5 substrate RNA, the coding region of the human U5 snRNA was PCR amplified by using oligos 24 and 25 as 5′ and 3′ end-specific primers, respectively. The amplified DNA fragment that carried the U5 gene and the T7 RNA polymerase promoter was cloned into the *Eco*RI and *Xba*I sites of pUC19. The resulting pT7-U5 recombinant plasmid was linearized with *Nsi*I and transcribed by T7 RNA polymerase.

Accession numbers

The DDBJ/EMBL/GenBank accession Nos for the human and *Drosophila* U85 snoRNAs are AF308283 and AF308282, respectively.

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