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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 Gar1p-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 Gar1p. *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, Gar1p, 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).

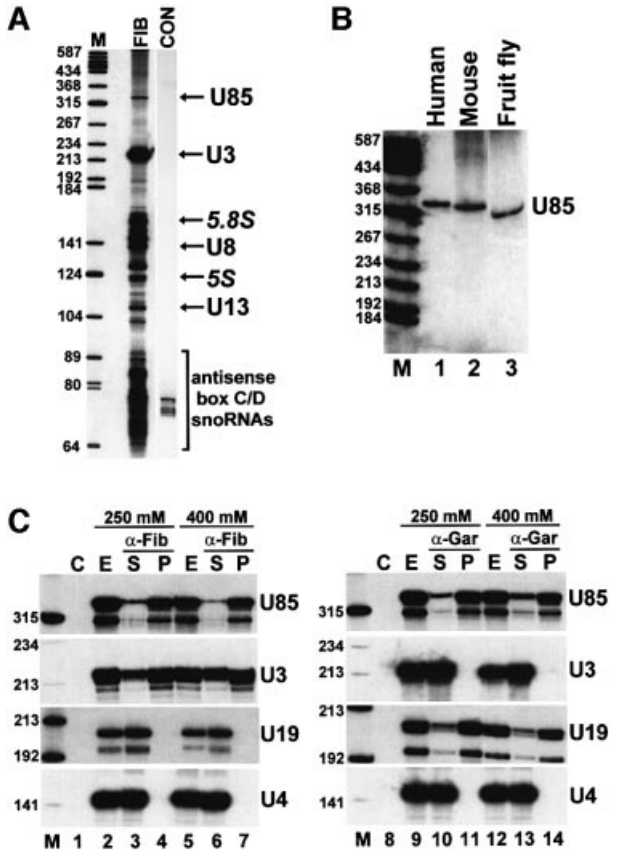

Fig. 1. Characterization of U85 snoRNA. (**A**) Human fibrillarin-associated snoRNAs. RNAs immunopreciptated with monoclonal anti-fibrillarin antibody (72B9) were 3′ end-labelled and fractionated on a 6% sequencing gel (lane FIB). Lane CON, control precipitation with monoclonal anti-Sp1 antibody. Lane 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

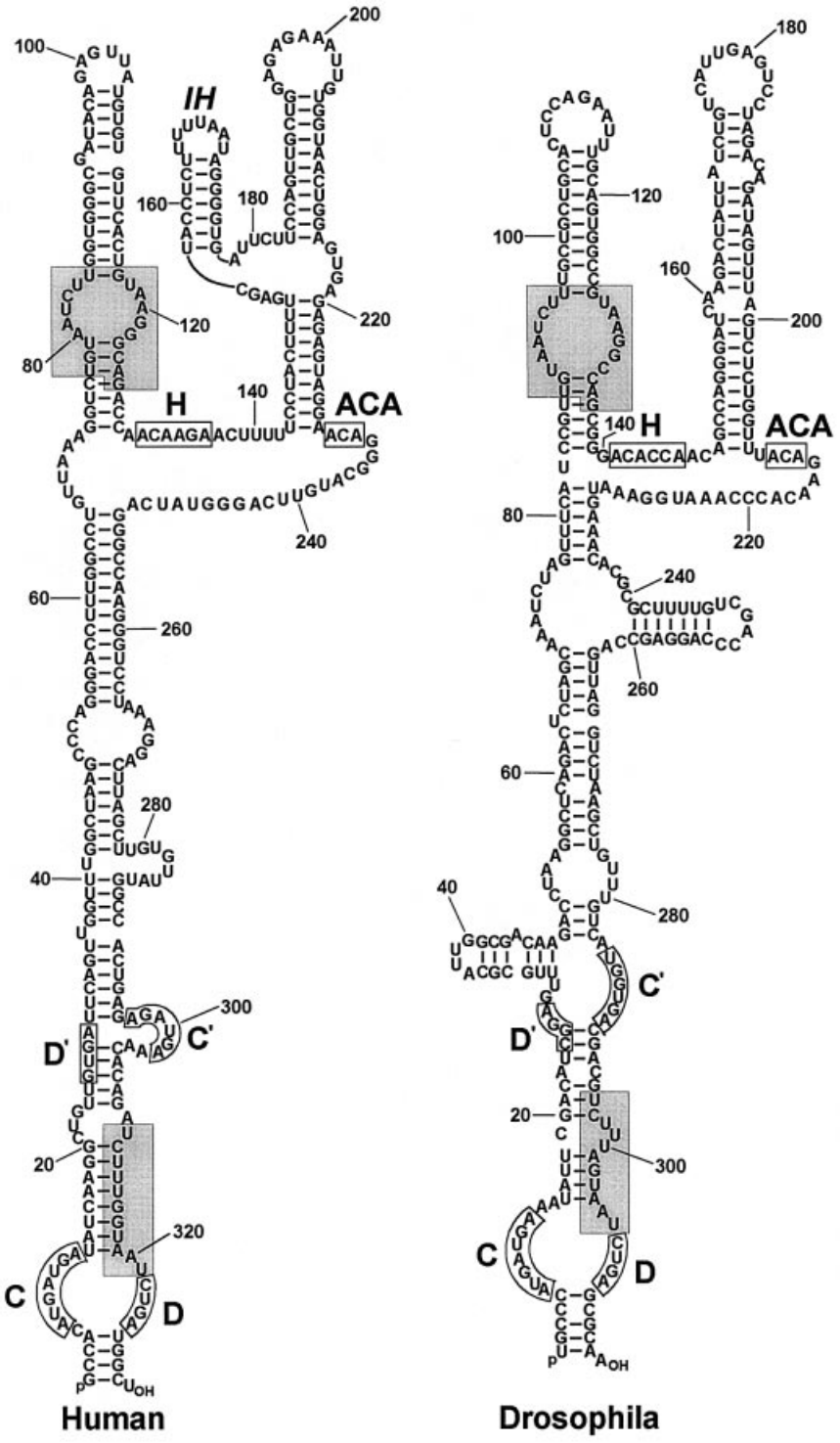

Fig. 2. Proposed secondary structures of the human and fruit fly U85 snoRNAs. The box C, C′, H, ACA, D and D′ motifs are boxed. Other sequences conserved between the two snoRNAs are shaded.

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

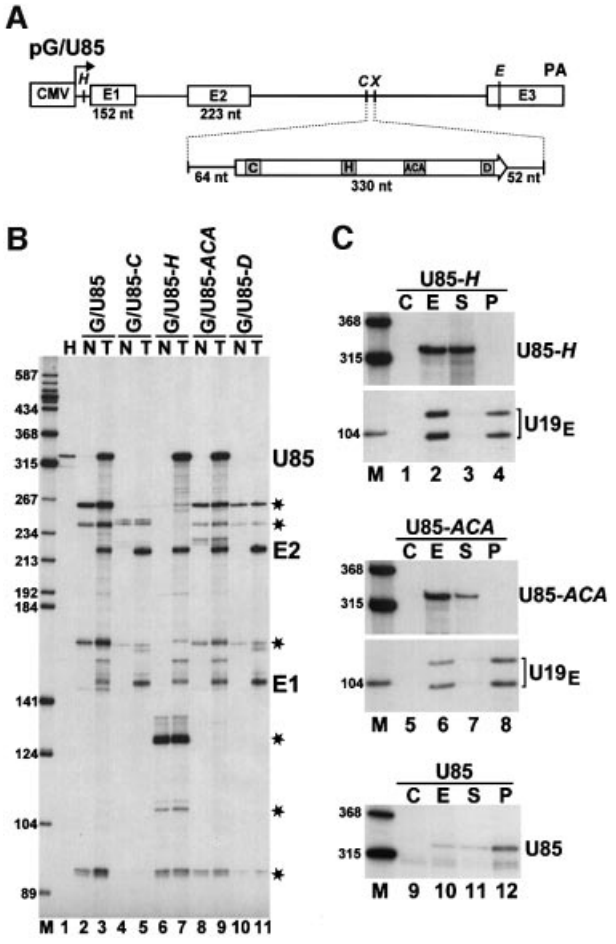

Fig. 3. Processing of the human U85 intronic snoRNA. (**A**) Structure of the expression constructs used for transfection of COS7 cells. The exons (E1, E2 and E3) and the polyadenylation site (PA) of the human β-globin gene and the promoter region of the cytomegalovirus (CMV) are indicated. Relevant 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*

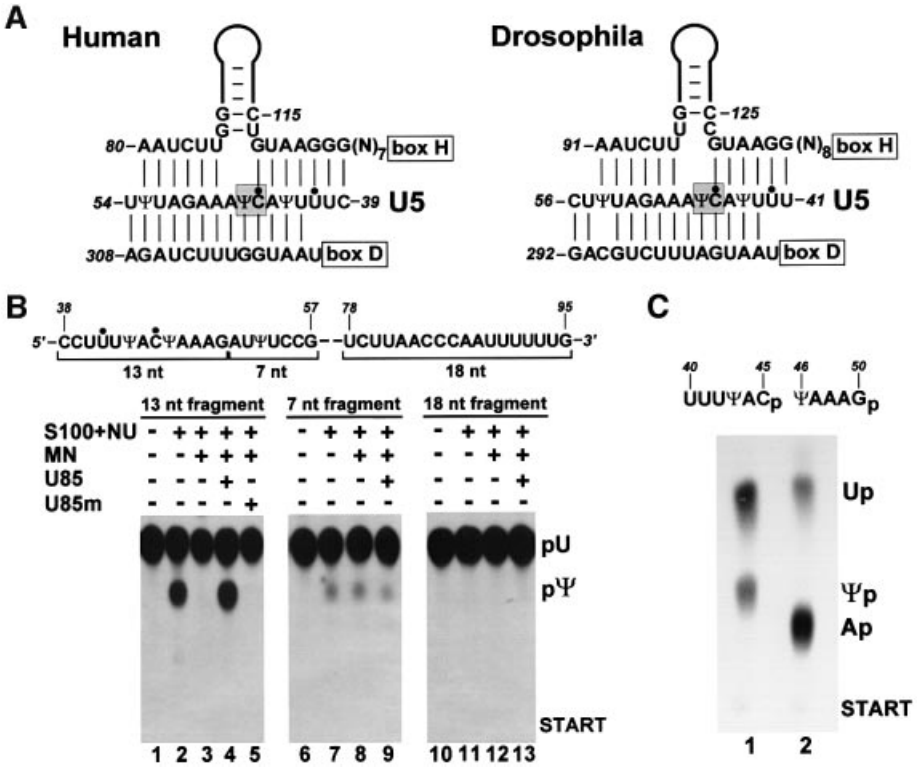

Fig. 4. The U85 snoRNA can direct modification of the U5 snRNA. (**A**) Potential hydrogen bonding between U85 and U5 RNAs in human and *Drosophila* cells. The 5′ hairpins in the H/ACA domains of the U85 snoRNAs are schematically represented. Pseudouridines (Ψ) and 2′-*O*-methyl groups (closed circles) 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

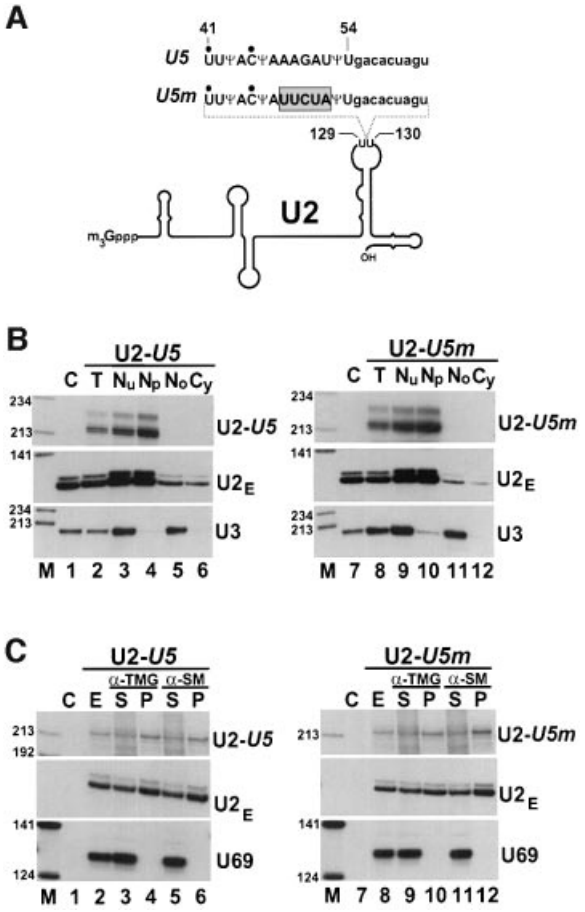

Fig. 5. Expression of chimeric U2 snRNAs in COS7 cells. (**A**) Schematic structure of the U2–*U5* and U2–*U5m* snRNAs. Sequences of the *U5* and *U5m* tags and positions of pseudouridines (Ψ) and 2′-*O*-methylated nucleotides (closed circles) are shown. The authentic U5 sequences are in upper case. 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.

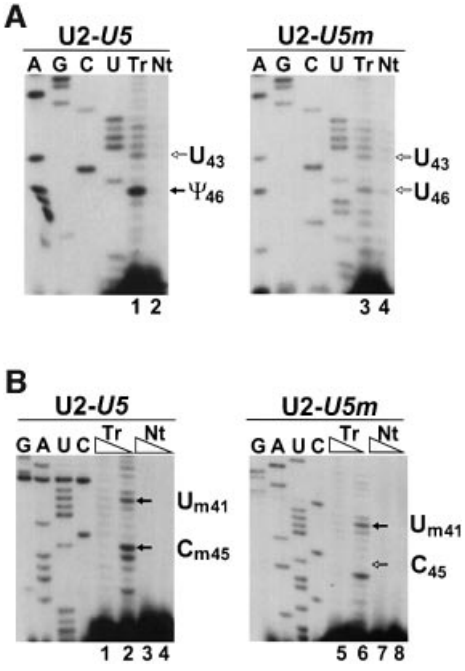

Fig. 6. Primer extension mapping of pseudouridines and 2′-*O*-methylated nucleotides in the U2–*U5* and U2–*U5m* snRNAs. (**A**) Mapping of pseudouridines. CMC-alkali-treated cellular RNAs extracted from COS7 cells transfected (Tr) or non-transfected (Nt) with the pGL/U2-*U5* and pGL/U2-*U5m* constructs 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-

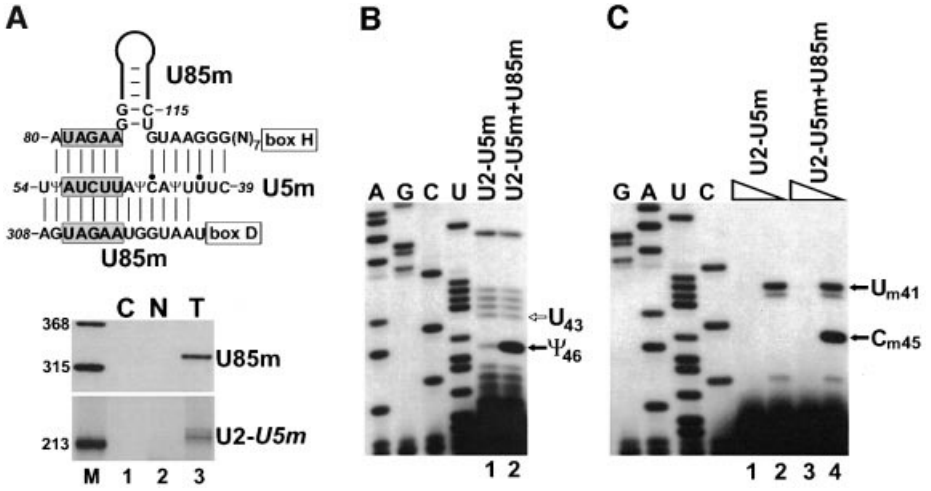

Fig. 7. Restoration of pseudouridylation and 2′-*O*-methylation of the U2–*U5m* snRNA. (**A**) Expression and predicted interaction of the U85m pseudouridylation and 2′-*O*-methylation guide snoRNA and the U2–*U5m* snRNA. RNAs from COS7 cells transfected (T) and non-transfected (N) with the pG/U85m/U2-*U5m* 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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