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Small nucleolar RNA-guided post-transcriptional modification of cellular RNAs

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Introduction

For a long time it was believed that the nucleotide sequence of every RNA would represent a simple copy of its coding DNA. This axiom of molecular biology, however, has suffered several jolts during the last 25 years. The discovery of intervening or intronic sequences (splicing) and the finding that individual nucleotides can be inserted into and deleted from RNAs (RNA editing) demonstrated that genetic information can be changed at the RNA level. Post-transcriptional modification of individual ribonucleotides, namely deamination of adenosines and cytidines, can also change the readout of mRNAs (reviewed by Maas and Rich, 2000). Stable cellular RNAs, such as tRNAs, rRNAs and snRNAs, have long been known to contain a large number of post-transcriptionally synthesized irregular ribonucleotides (Limbach *et al*., 1994). In tRNAs, the modified nucleotides can facilitate the formation of correct anticodon–codon interaction and thereby increase the efficiency and fidelity of translation (reviewed by Agris, 1996). In rRNAs and spliceosomal snRNAs, methylation of the ribose moiety at the 2′-hydroxyl group and conversion of uridines into pseudouridine are the most prevalent nucleotide modifications. Since 2′-*O*-methylated nucleotides and pseudouridines are restricted to the functionally essential regions of rRNAs and snRNAs (Reddy *et al*., 1988; Maden, 1990), they are expected to contribute to the faithful function of the ribosome and the spliceosome. Consistent with this view, lack of ribosomal pseudouridines can reduce the growth rate or confer a selective disadvantage when it is competed against wild-type ribosomes (Raychaudhuri *et al*., 1999; Wrzesinski *et al*., 2000). More tellingly, nucleotide modifications in the 5′-terminal region of the U2 spliceosomal snRNA are absolutely essential for its function in pre-mRNA splicing (Yu *et al*., 1998).

Ribosomal 2′-*O*-methylated nucleotides and pseudouridines are synthesized by small nucleolar RNPs

The human 18S, 5.8S and 28S rRNAs together carry ~110 2′-*O*-methyl groups and almost 100 pseudouridines (Maden, 1990). Synthesis of this large number of modified nucleotides in rRNAs is directed by guide snoRNAs. The 2′-*O*-methylation and pseudouridylation guide snoRNAs possess distinct sequence and structural elements (Figure 1). The methylation guide snoRNAs carry the conserved box C (RUGAUGA, where R stands for any purine) and D (CUGA) motifs near their 5′ and 3′ ends, respectively (Figure 1A). The C and D boxes are frequently folded together by a short (4–5 bp) terminal helix. Additional, often imperfect copies of the C and D boxes, called C′ and D′ boxes, are located internally (Tycowski *et al*., 1996a; Kiss-László *et al*., 1998). The distance between the D′ and C′ boxes is restricted to 3–9 nucleotides, and frequently an internal stem brings these elements closer to each other. The 2′-*O*-methylation guide snoRNAs possess one or sometimes two 10–21 nucleotide antisense elements, which can form perfect double helices with rRNA sequences (Bachellerie *et al*., 1995). The ribosomal nucleotide positioned 5 bp upstream of the D or D′ box of the snoRNA is selected for 2′-*O*-methylation (Cavaillé *et al*., 1996; Kiss-László *et al*., 1996; Tycowski *et al*., 1996b).

The pseudouridylation guide snoRNAs consist of two hairpins and two short single-stranded regions, which contain the conserved H (ANANNA, where N stands for any nucleotide) and ACA boxes (Balakin *et al*., 1996; Ganot *et al*., 1997b) (Figure 1B). An internal loop in the 5′- and/or 3′-terminal hairpin of the snoRNA forms a complex pseudoknot structure with rRNA sequences. The substrate ribosomal uridine is positioned at the base of the upper stem closing the recognition loop of the snoRNA (Ganot *et al*., 1997a). The distance between the substrate uridine and the H or ACA box of the snoRNA (~14–16 nucleotides) is an important structural determinant for selection of the correct pseudouridylation site (Ni *et al*., 1997; Bortolin *et al*., 1999).

The 2′-*O*-methylation and pseudouridylation guide snoRNAs function in the form of small ribonucleoprotein particles (snoRNPs). Each snoRNP consists of a specific snoRNA and a set of associated proteins common to all box C/D or H/ACA snoRNPs. The conserved box C/D and H/ACA motifs are believed to function as protein binding signals. The box C/D 2′-*O*-methylation guide snoRNPs contain at least four evolutionarily conserved, essential proteins: fibrillarins Nop56p, Nop58p/Nop5p and Snu13p (Schimmang *et al*., 1989; Tyc and Steitz, 1989; Lafontaine and Tollervey, 1999, 2000; Lyman *et al*., 1999; Newman *et al*., 2000; Watkins *et al*., 2000). Fibrillarin shares a conserved domain with known *S*-adenosylmethionine-dependent methyltransferases (Niewmierzycka and Clarke, 1999; Wang *et al*., 2000). A point mutation in the putative methyltransferase domain of the yeast fibrillarin, Nop1p, inhibits the overall ribose methylation

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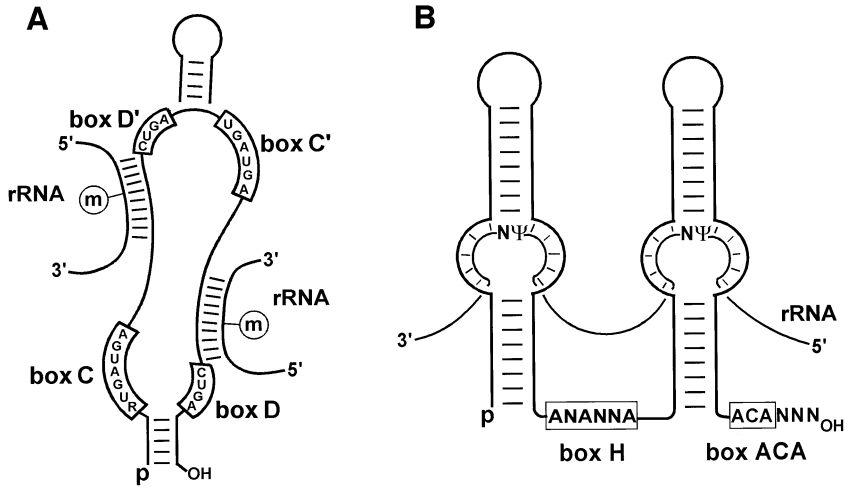

Fig. 1. Structure and function of (**A**) 2′-*O*-methylation and (**B**) pseudouridylation guide snoRNAs. The consensus sequences of boxes C, C′, D, D′, H and ACA are indicated (R is a purine and N stands for any nucleotide). Models for molecular selection of 2′-*O*-methylated nucleotides and pseudouridine were adopted from Kiss-László *et al*. (1998) and Ganot *et al*. (1997a), respectively.

of rRNAs (Tollervey *et al*., 1993), suggesting that fibrillarin is the methyltransferase in box C/D snoRNPs.

Thus far, four snoRNP proteins, Cbf5p, Gar1p, Nhp2p and Nop10p, have been identified in box H/ACA pseudouridylation guide snoRNPs (Balakin *et al*., 1996; Ganot *et al*., 1997b; Henras *et al*., 1998; Watkins *et al*., 1998). Cbf5p (Nap57p in mammals), which is highly homologous to the *Escherichia coli* tRNA:Ψ55 pseudouridine synthase, provides the pseudouridine synthase activity for the snoRNA-directed pseudouridylation reaction (Lafontaine *et al*., 1998; Zebarjadian *et al*., 1999). Gar1p, Nhp2p and Nop10p are also essential for the pseudouridylation reaction, but their function remains unknown (Bousquet-Antonelli *et al*., 1997; Henras *et al*., 1998).

snoRNPs function in modification of various classes of cellular RNAs

Besides synthesis of >200 modified nucleotides in rRNAs, snoRNPs also function in 2′-*O*-methylation and pseudouridylation of spliceosomal snRNAs. The five major mammalian spliceosomal snRNAs, U1, U2, U4, U5 and U6, carry 30 2′-*O*-methyl groups and 24 pseudouridines (Reddy and Busch, 1988). Involvement of snoRNPs in modification of spliceosomal snRNAs was first demonstrated by identification of two box C/D snoRNAs, mgU6-47 and mgU6-77, which function in 2′-*O*-methylation of the RNA polymerase (pol) III-transcribed U6 snRNA (Tycowski *et al*., 1998). Later, it was shown that all *trans*-acting factors directing the synthesis of the eight 2′-*O*-methylated nucleotides and three pseudouridines in the U6 snRNA are present and are functionally active in the nucleolus (Ganot *et al*., 1999). This observation, coupled with the finding that each U6 modification factor recognizes short sequences around the target nucleotide, led to the proposal that 2′-*O*-methylation and pseudouridylation of the U6 snRNA are mediated exclusively by snoRNPs.

An unusual ‘hybrid’ snoRNA, called U85, that contains both box C/D and H/ACA domains has been identified in human and *Drosophila* cells (Jády and Kiss, 2001). The U85 snoRNA is associated with both box C/D- and H/ACA-specific snoRNP proteins. *In vitro* and *in vivo* RNA modification experiments demonstrated that the U85 snoRNA functions in 2′-*O*-methylation and pseudouridylation of the RNA pol II-transcribed U5 snRNA. Current observations suggest that U85 is not the only snoRNA that directs modification of a pol II-specific spliceosomal snRNA. Several putative 2′-*O*-methylation and pseudouridylation guide snoRNAs with significant sequence complementarities to the U1, U2, U4 and U5 snRNAs have been identified (Hüttenhofer *et al*., 2001; B.E.Jády and T.Kiss, unpublished data). The new snoRNAs possess the potential to select known 2′-*O*-methylated nucleotides and pseudouridines in U1, U2, U4 and U5 snRNAs, further supporting the conclusion that snoRNAs function in post-transcriptional modification of both pol II- and pol III-transcribed spliceosomal snRNAs. To what extent snoRNPs participate in 2′-*O*-methylation and pseudouridylation of pol II-specific snRNAs remains unclear. However, it seems that synthesis of at least some pseudouridines is achieved by protein enzymes (Massenet *et al*., 1999; Jády and Kiss, 2001).

Current observations also suggest that the snoRNA-based guide mechanism is not limited to the modification of ribosomal and spliceosomal RNAs. Homologs of eukaryotic box C/D snoRNAs present in archaebacteria, besides directing ribose methylation of rRNAs, also function in 2′-*O*-methylation of tRNAs (Omer *et al*., 2000; B.Clouet-D’Orval and J.-P.Bachellerie, personal communication; C.Daniels, personal communication). In human and rodents, several novel putative 2′-*O*-methylation and pseudouridylation snoRNAs lacking significant complementarities to rRNAs, snRNAs or other known stable cellular RNAs have been identified (Cavaillé *et al*., 2000; Jády and Kiss, 2000; Hüttenhofer *et al*., 2001; B.E.Jády and T.Kiss, unpublished data). Most probably, these ‘orphan’ guide snoRNAs function in

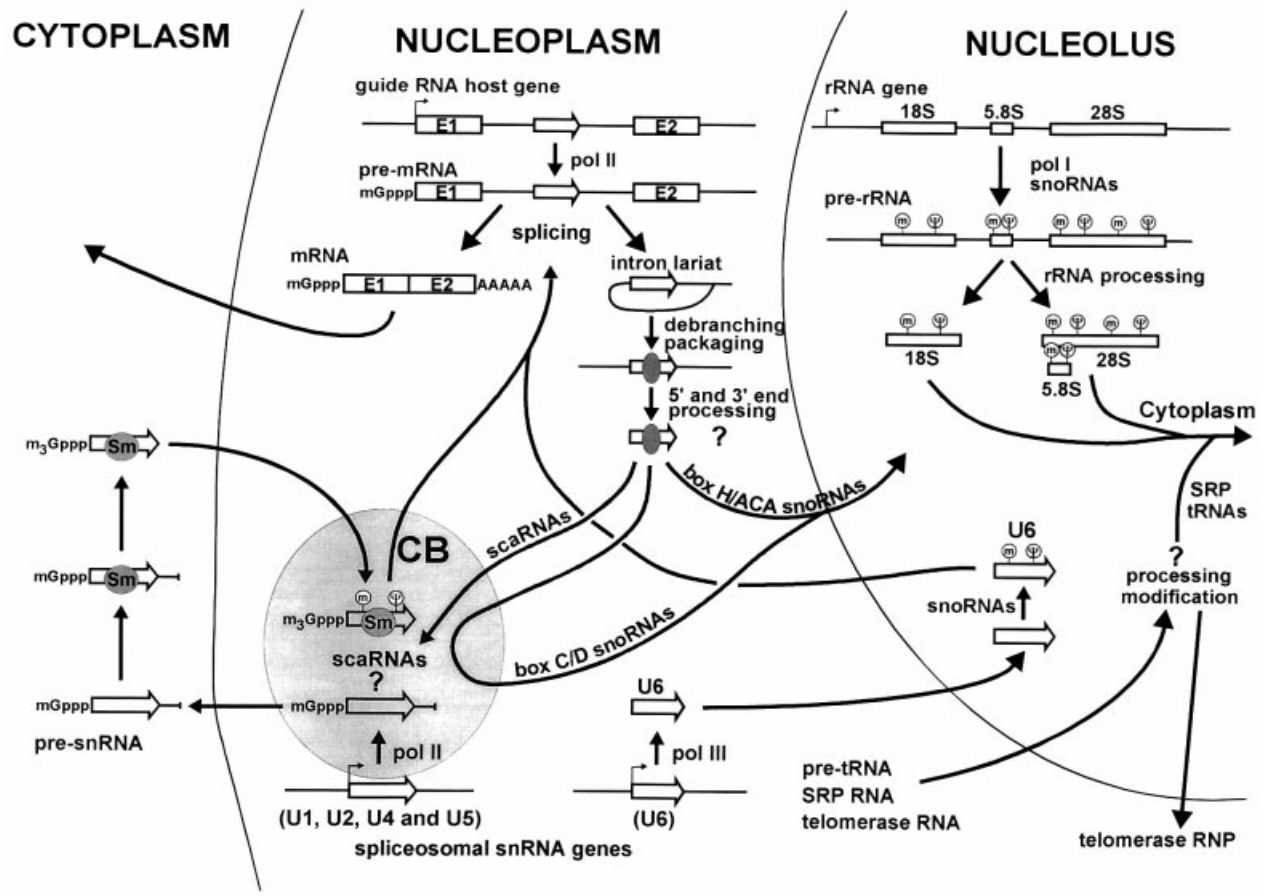

Fig. 2. Biogenesis and function of 2′-*O*-methylation and pseudouridylation guide snoRNAs. In mammalian cells, all guide RNAs are synthesized within introns of pre-mRNAs in the nucleoplasm. Most intronic snoRNAs are processed from the removed and debranched host intron by exonucleolytic activities. It remains unclear whether 5′ and 3′ end processing of snoRNAs occurs already in the nucleoplasm, or later in the nucleolus. Guide snoRNAs accumulating in the nucleolus direct 2′-*O*-methylation and pseudouridylation of the 18S, 5.8S and 28S rRNAs, the U6 snRNA and perhaps other cellular RNAs, including tRNAs, the signal recognition particle (SRP) and telomerase RNAs. It seems that box C/D, but not box H/ACA snoRNAs, transiently appear in the Cajal body before accumulating in the nucleolus. Some guide RNAs (scaRNAs) directing modification of the pol II-transcribed spliceosomal snRNAs accumulate in the Cajal body (CB). For other details, see the text.

2′-*O*-methylation and pseudouridylation of thus far unidentified target RNAs. What types of cellular RNAs might be the substrates of the new guide snoRNAs? During the last decade, several non-coding RNAs with important catalytic and regulatory functions have been identified (Eddy, 1999; Filipowicz, 2000). We can anticipate that numerous additional non-coding RNAs have remained still undiscovered in eukaryotic cells. These RNAs, of course, might carry 2′-*O*-methylated nucleotides and pseudouridines that are synthesized by snoRNPs.

According to a current, even more fascinating concept, snoRNAs might also function in modification of mRNAs. Seven novel box C/D snoRNAs and a box H/ACA snoRNA with predominant expression in brain tissues have been identified in human and mouse (Cavaillé *et al*., 2000; de Los Santos *et al*., 2000; J.Cavaillé, P.Vitali, Z.Basyuk, J.-P.Bachellerie, J.Brosius and A.Hüttenhofer, personal communication). The function of the new brain-specific snoRNAs remains elusive. One of the novel box C/D snoRNAs carries an 18 nucleotide phylogenetically conserved target recognition element that is perfectly complementary to the serotonin receptor 5-HT_{2C} mRNA. Intriguingly, the putative 2′-*O*-methylation target nucleotide in the serotonin receptor mRNA is known to undergo an adenosine-to-inosine editing reaction, leading to the intriguing possibility that snoRNA-guided 2′-*O*-methylation might have a regulatory function in the expression of this important brain-specific protein (Cavaillé *et al*., 2000).

Cellular locations for snoRNA-directed RNA modification reactions

In vertebrates, all 2′-*O*-methylation and pseudouridylation guide snoRNAs are processed from pre-mRNA introns (reviewed in Tollervey and Kiss, 1997; Weinstein and Steitz, 1999) (Figure 2). The conserved box C/D and H/ACA motifs, through binding snoRNP proteins, direct the correct processing and nucleolar transportation of snoRNAs (Lange *et al*., 1998, 1999; Samarsky *et al*., 1998; Narayanan *et al*., 1999a,b). Since mature 2′-*O*-methylation and pseudouridylation snoRNPs are localized in the nucleolus, the most immediate issue that comes to mind is how they can function in post-transcriptional modification of various classes of cellular RNAs that accumulate in the cytoplasm or nucleoplasm. Recent attempts at

understanding this apparent enigma shed new light on the intracellular trafficking of RNAs.

Certainly, the snoRNA-directed 2′-*O*-methylation and pseudouridylation of rRNAs take place within the nucleolus where the 18S, 5.8S and 28S rRNAs are synthesized as long precursor rRNAs (pre-rRNAs) (Figure 2). Modification of the 18S, 5.8S and 28S rRNAs occurs during or immediately after transcription, but before nucleolytic processing of the pre-rRNA (Maden, 1990). This implies that each pre-rRNA transcript transiently interacts with ~200 modification guide snoRNPs.

The earlier finding that the U6-specific modification factors reside within the nucleolus raised the possibility that the U6 snRNA might cycle through the nucleolus to undergo snoRNP-mediated 2′-*O*-methylation and pseudouridylation (Tycowski *et al*., 1998; Ganot *et al*., 1999). Indeed, upon injection into the *Xenopus* oocyte nucleus, fluorescent U6 snRNA transiently localizes to the nucleolus before its accumulation in the nucleoplasm (Lange and Gerbi, 2000), supporting the idea that post-transcriptional modification of the U6 snRNA takes place in the nucleolus. Besides U6, several small stable RNAs, including RNase P RNA, signal recognition particle (SRP) RNA and telomerase RNA, have been detected in the nucleolus (reviewed by Pederson, 1998). In yeast cells, the RNase P-catalyzed endonucleolytic processing of the 5′ terminus of a major portion of tRNAs occurs within the nucleolus (Bertrand *et al*., 1998). Last, but not least, some mRNAs have also been reported to localize transiently to the nucleolus (Bond and Wold, 1993). These observations raised the fascinating possibility that various cellular RNAs can transit through the nucleolus to undergo nucleolar processing including snoRNA-directed nucleotide modifications.

In contrast to the pol III-transcribed U6 snRNA, maturation of the pol II-specific U1, U2, U4 and U5 snRNPs includes a cytoplasmic phase (Figure 2). The newly synthesized precursor snRNAs (pre-snRNAs) are exported to the cytoplasm, where the seven common Sm proteins bind to the pre-snRNAs before processing of their 3′ ends and hypermethylation of their primary monomethylguanosine cap to trimethylguanosine. After re-importation from cytoplasm, the newly assembled snRNPs transiently appear in Cajal (coiled) bodies before accumulating in the nucleoplasm (Carvalho *et al*., 1999; Sleeman and Lamond, 1999). Earlier, it was found that the actively transcribed human U1 and U2 genes frequently co-localize with Cajal bodies (Frey and Mateira, 1995; Smith *et al*., 1995; Frey *et al*., 1999). Although it seems that transcription does not occur within the Cajal body, the newly synthesized pre-U2 snRNA was also detected in this nucleoplasmic organelle (Smith and Lawrence, 2000). The Cajal body has long been considered as a possible site for assembly and/or modification of spliceosomal snRNPs (reviewed in Bohmann *et al*., 1995; Matera and Frey, 1998). We have recently investigated the subcellular distribution of the human U85 box C/D/H/ACA snoRNA that functions in both 2′-*O*-methylation and pseudouridylation of the U5 snRNA (Jády and Kiss, 2001). To our surprise, *in situ* hybridization experiments demonstrated that the U85 snoRNAs co-localize with Cajal bodies in human HeLa cells (X.Darzacq, B.E.Jády, C.Verheggen, E.Bertrand and T.Kiss, in preparation). Likewise, three other currently identified snoRNAs that are predicted to function in pseudouridylation of the U2 snRNA and 2′-*O*-methylation of the U1, U4 and U5 snRNAs were also found to accumulate within Cajal bodies. These observations raised the possibility that the novel small Cajal body-specific RNAs (scaRNAs) may direct modification of the U1, U2, U4 and U5 snRNAs within Cajal bodies either after the synthesis of nascent pre-snRNAs or after re-importation of the newly assembled snRNPs from the cytoplasm. A recent study, however, indicates that nucleolar factors may also contribute to the internal modification of the U2 snRNA (Yu *et al*., 2001). Therefore, several exciting questions remain to be answered in the future. Do all 2′-*O*-methylation and pseudouridylation guide RNAs that function in modification of pol II-specific snRNAs reside within Cajal bodies? Can bona fide nucleolus-localized RNAs also function in snRNA modification? What are the *cis*- and *trans*-acting factors that direct scaRNAs into the Cajal body?

Concluding remarks

Eubacterial rRNAs contain only a handful of 2′-*O*-methylated nucleotides and pseudouridines, which are synthesized by specific protein enzymes. To synthesize the numerous 2′-*O*-methylated nucleotides and pseudouridines in eukaryotic rRNAs, eukaryotes probably adopted the snoRNA-guided modification mechanism before the split between archaebacteria and eukaryotes (Gaspin *et al*., 2000; Omer *et al*., 2000; Watanabe and Gray, 2000). The primordial 2′-*O*-methylation and pseudouridylation guide snoRNAs likely derived from *cis*-acting rRNA or perhaps tRNA sequences which acquired the ability to function as *trans*-acting cofactors. The complex world of present-day guide snoRNAs probably evolved through duplications and random mutations of the snoRNA genes (Lafontaine and Tollervey, 1998). The guide snoRNA-based RNA modification mechanism provides several advantages for eukaryotic cells. Selection of modification sites by guide snoRNAs is independent of local structures of the substrate RNA, and guide RNAs can evolve much more rapidly than protein enzymes that are highly dependent on pre-existing modification sites. During evolution, therefore, the flexible snoRNA-based modification systems can continuously test novel 2′-*O*-methyl groups and pseudouridines in eukaryotic rRNAs, snRNAs, tRNAs and probably other cellular RNAs.

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