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Sequence and structural elements of methylation guide snoRNAs essential for site-specific ribose methylation of pre-rRNA

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Site-specific 2′-*O*-ribose methylation of eukaryotic rRNAs is guided by small nucleolar RNAs (snoRNAs). The methylation guide snoRNAs carry long perfect complementaries to rRNAs. These antisense elements are located either in the 5′ half or in the 3′ end region of the snoRNA, and are followed by the conserved D′ or D box motifs, respectively. An uninterrupted helix formed between the rRNA and the antisense element of the snoRNA, in conjunction with the adjacent D′ or D box, constitute the recognition signal for the putative methyltransferase. Here, we have identified an additional essential box element common to methylation guide snoRNAs, termed the C′ box. We show that the C′ box functions in concert with the D′ box and plays a crucial role in the methyltransfer reaction directed by the upstream antisense element and the D′ box. We also show that an internal fragment of U24 methylation guide snoRNA, encompassing the upstream antisense element and the D′ and C′ box motifs, can support the site-specific methylation of rRNA. This strongly suggests that the C box of methylation guide snoRNAs plays an essential role in the methyltransfer reaction guided by the 3′-terminal antisense element and the D box of the snoRNA.

Keywords: guide snoRNAs/modified nucleotides/nucleolus/pre-rRNA maturation/2′-*O*-ribose methylation

Introduction

In eukaryotes, synthesis and processing of rRNAs take place in the nucleolus (Hadjiolov, 1985). The primary rRNA transcripts (pre-rRNAs), in addition to the 18S, 5.8S and 25/28S rRNAs, contain long spacer sequences that are removed by a complex series of nucleolytic cleavages (Eichler and Craig, 1994; Venema and Tollervey, 1995; Sollner-Webb *et al.*, 1996). Before the nucleolytic processing, the rRNA-coding regions of the pre-rRNA undergo extensive post-transcriptional modifications. A large number of carefully selected nucleotides are methylated at the 2′-*O*-hydroxyl position and many uridine residues are isomerized into pseudouridines (Maden, 1990; Eichler and Craig, 1994). In vertebrates, mature rRNAs carry >100 2′-*O*-methyl groups and ~95 pseudouridine residues. Yeast rRNAs contain about half as many modified nucleotides (Maden, 1990; Ofengand *et al.*, 1995; Ofengand and Bakin, 1997). Although the function of the modified nucleotides is unknown, the facts that they cluster on the universal core regions of rRNAs and their positions show remarkable evolutionary conservation suggest that the 2′-*O*-methyls and pseudouridines contribute to folding or recognition events essential for ribosome function (Maden, 1990; Lane *et al.*, 1995; Ofengand *et al.*, 1995).

The nucleolus contains a large number of small nucleolar RNAs (snoRNAs) (Maxwell and Fournier, 1995; Sollner-Webb *et al.*, 1996; Tollervey and Kiss, 1997). Most of the snoRNAs can be divided into two families, based on the presence of common sequence motifs. The first group of snoRNAs is defined by two conserved sequence elements, the C and D boxes (Tyc and Steitz, 1989; Maxwell and Fournier, 1995). Members of the second group of snoRNAs are characterized by the conserved box H and ACA motifs (Balakin *et al.*, 1996; Ganot *et al.*, 1997b). Although some box C/D (U3, U8, U14, U22) and box H/ACA (snR30, U17, E2, E3) snoRNAs have been linked to the nucleolytic processing of pre-rRNA (Maxwell and Fournier, 1995; Enright *et al.*, 1996; Mishra and Eliceiri, 1997), most of the snoRNAs function as guide RNAs in the post-transcriptional modification of pre-rRNA. The box C/D snoRNAs direct the site-specific 2′-*O*-methylation of pre-rRNA (Cavaillé *et al.*, 1996; Kiss-László *et al.*, 1996; Tycowski *et al.*, 1996b; reviewed by Maden, 1996; Peculis and Mount, 1996; Tollervey, 1996; Bachellerie and Cavaillé, 1997; Tollervey and Kiss, 1997) and the box H/ACA snoRNAs guide the site-specific synthesis of ribosomal pseudouridines (Ganot *et al.*, 1997a; Ni *et al.*, 1997; reviewed by Maden, 1997; Peculis, 1997; Smith and Steitz, 1997).

Both methylation and pseudouridylation guide RNAs select the target nucleotides by forming direct base-pairing interactions with rRNA sequences. The methylation guide RNAs contain one or occasionally two stretches of 10–21 nucleotide long sequences that show perfect complementarities to mature rRNAs (Bachellerie *et al.*, 1995; Bachellerie and Cavaillé, 1997; Tollervey and Kiss, 1997). These so-called antisense elements are located either in proximity to the 3′ end or in the 5′ half of the snoRNA. Complementary sequences found in the 3′-terminal region of the snoRNA are always followed by the highly conserved D box (consensus: CUGA), and antisense elements in the 5′ half of the snoRNA are followed by a D box-like motif, designated the D′ box (Tycowski *et al.*, 1996a). The antisense element of the snoRNA forms an uninterrupted helix with the complementary rRNA sequence and positions the D or D′ box 5 bp from the methylated nucleotide (Kiss-László *et al.*, 1996; Nicoloso *et al.*, 1996). Apparently, the 2′-*O*-methylase enzyme that binds directly or indirectly to the D or D′ box of the snoRNA relies on this structural information to select the substrate nucleotide. Supporting this idea, novel methyl

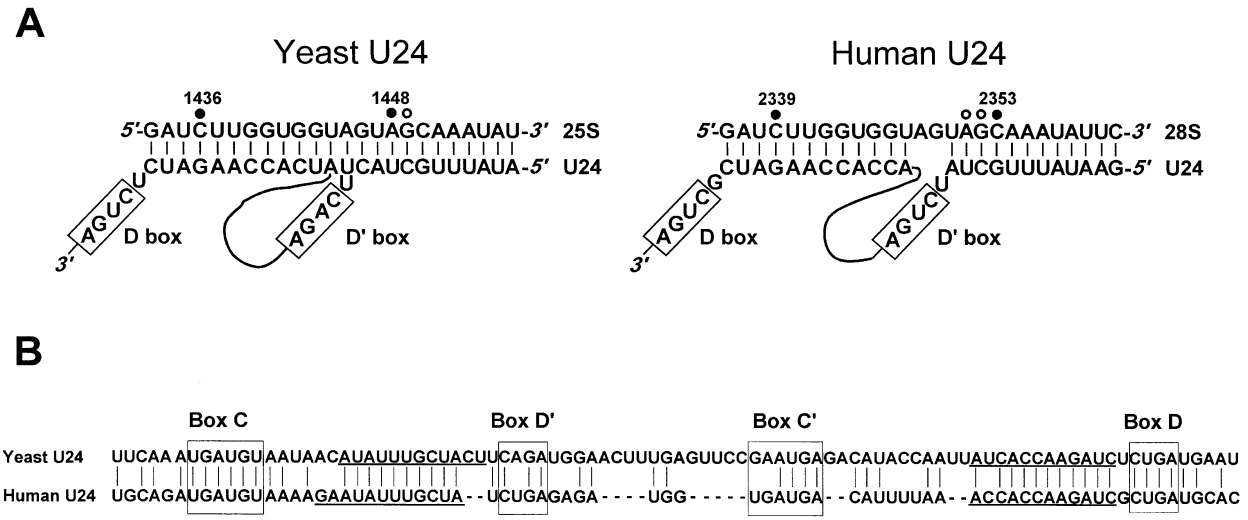

Fig. 1. Comparison of human and yeast U24 snoRNAs. (**A**) Selection of 2′-*O*-ribose-methylated nucleotides by yeast and human U24 snoRNAs in yeast 25S and human 28S rRNAs. The upper strands represent rRNA sequences in a 5′ to 3′ orientation. Ribose-methylated nucleotides with their canonical distance from the D or D′ box of the snoRNA are indicated by closed circles. Other 2′-*O*-methyl groups are indicated by open circles. The sequence of yeast 25S rRNA was taken from the database (DDBJ/EMBL/GenBank No. K01048). The positions of ribose-methylated nucleotides have been published by Veldman *et al.* (1981), and the sequences of human and yeast U24 snoRNAs have been taken from Qu *et al.* (1995). (**B**) Alignment of yeast (yU24) and human (hU24) U24 snoRNAs. Sequences complementary to 25S or 28S rRNAs are underlined. The evolutionarily conserved sequence motifs are boxed. Dashes stand for spacings introduced into the sequence of human U24 to improve the alignment.

groups have been introduced into yeast and mouse rRNAs either by shifting the position of the D box in the snoRNA–rRNA interaction or by manipulating the sequence of the antisense element of the snoRNA (Cavaillé *et al.*, 1996; Kiss-László *et al.*, 1996; J.Ni, A.G.Balakin and M.J. Fournier, personal communication).

In vertebrates, methylation guide RNAs are processed from introns of pre-mRNAs (Tollervey and Kiss, 1997). The D box, together with the highly conserved C box (consensus UGAUGA), is essential for both the correct processing (Caffarelli *et al.*, 1996; Watkins *et al.*, 1996; Xia *et al.*, 1997) and the metabolic stability (Huang *et al.*, 1992; Peculis and Steitz, 1994; Terns *et al.*, 1995) of the snoRNA. Although the C and D boxes are positioned in the 5′- and 3′-terminal regions of the snoRNA, respectively, they frequently are brought together by a short 5′-, 3′-terminal helix (Tycowski *et al.*, 1993). The C and D boxes probably function as protein-binding signals, and the proteins bound to these elements are believed to protect the snoRNA from exonucleases responsible for the processing of intronic snoRNAs (Kiss and Filipowicz, 1995; Caffarelli *et al.*, 1996; Cavaillé and Bachellerie, 1996; Watkins *et al.*, 1996). Indeed, the C and D boxes are required (probably indirectly) for binding of an essential nucleolar protein, fibrillarin, that is common to this class of snoRNAs (Schimmang *et al.*, 1989; Baserga *et al.*, 1991; Tollervey *et al.*, 1993; Peculis and Steitz, 1994).

While structural elements essential for the processing of methylation guide snoRNAs have been studied extensively, we have little knowledge about the elements required for the site-specific methyltransfer reaction. In this study, we have identified a novel essential sequence element of methylation guide snoRNAs, termed the C′ box and, by using a chimeric box C/D–H/ACA snoRNA, we have determined the minimal core structure of methylation guide RNAs that is still capable of directing site-specific methylation of pre-rRNA.

Results

Human U24 snoRNA directs site-specific 2′-O-methylation of yeast rRNA

Human and yeast U24 snoRNAs possess two antisense elements that are complementary to two closely spaced regions of human 28S and yeast 25S rRNAs (Figure 1A). The antisense elements located in the 3′-terminal regions of human and yeast U24 snoRNAs seem to be functionally equivalent, since they possess the potential to select analogous C residues in the yeast 25S (C1436) and human 28S (C2339) rRNAs. The upstream antisense element of yeast U24 is required for methylation at A1448 and, unexpectedly, also at G1449 that is located one nucleotide downstream from the consensus position of 2′-*O*-methylated nucleotides (Kiss-László *et al.*, 1996). The upstream antisense element of human U24 is predicted to direct the synthesis of Cm2353 that is specific to human 28S rRNA.

To learn more about the molecular mechanism by which methylation guide snoRNAs direct rRNA methylation, we have assessed the ability of human U24 snoRNA to function in yeast cells. A cDNA of human U24 snoRNA was inserted into the intron of the yeast actin gene which had been placed under the control of the promoter and terminator of the yeast alcohol dehydrogenase gene (Figure 2A). The resulting construct was transformed into a yeast strain (ΔU24) that lacks a functional U24 locus (Kiss-László *et al.*, 1996), and accumulation of human U24 snoRNA in the resulting hU24 strain was examined by Northern blot analysis (Figure 2B). As compared with the control yeast U24 snoRNA (lane 1) and the authentic human HeLa U24 snoRNA (lane 4), the human U24 snoRNA was correctly and efficiently processed from the actin (pre-mRNA) in the transformed hU24 yeast strain (lane 3).

To test whether human U24 snoRNA is capable of directing methylation of yeast 25S rRNA, we compared the methylation patterns of the appropriate regions of 25S

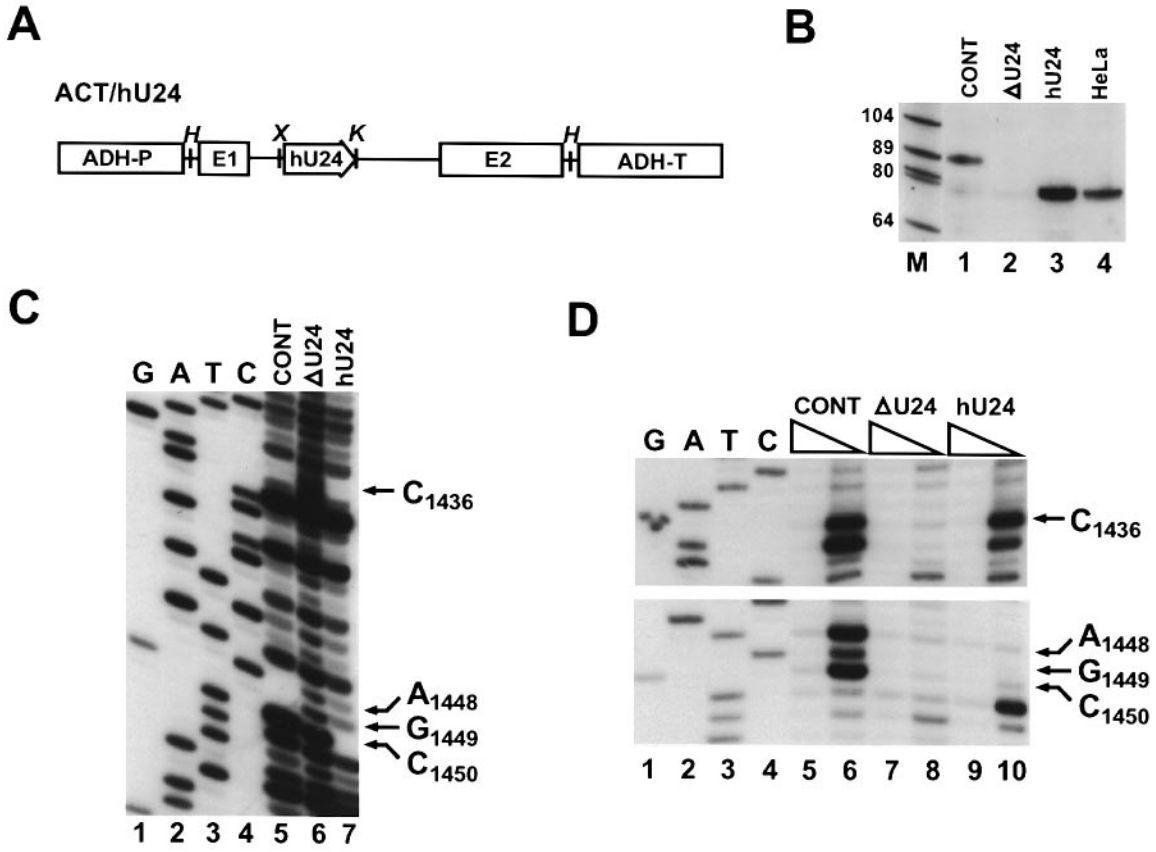

Fig. 2. Expression of human U24 snoRNA in yeast. (**A**) Schematic structure of the construct used to express human U24 snoRNA in yeast ΔU24 strain. A cDNA of human U24 (hU24) was inserted into the intron of the yeast actin gene controlled by the promoter (ADH-P) and terminator (ADH-T) of the yeast alcohol dehydrogenase gene (Kiss-László *et al.*, 1996). Relevant restriction sites are indicated (H, *Hind*III; K, *Kpn*I; X, *Xho*I). (**B**) Northern blot analyses. Cellular RNAs extracted from control CMY133 yeast cells (CONT), from cells carrying a disrupted U24 gene (ΔU24), from cells lacking a functional U24 gene but transformed with the ACT/hU24 construct (hU24) and from human HeLa cells (HeLa) were separated by denaturing polyacrylamide gel electrophoresis, and probed with a mixture of labelled oligonucleotides specific to human and yeast U24 snoRNAs. (**C**) Mapping of alkali-resistant 2′-*O*-methylated nucleotides by primer extension analysis. Cellular RNAs isolated from the control strain (lane 5), from the ΔU24 strain (lane 6) and from the hU24 strain (lane 7) were subjected to partial alkaline hydrolysis. A ^{32}P-labelled oligonucleotide complementary to yeast 25S rRNA from position 1463 to 1483 (oligonucleotide 29) was annealed to each RNA sample and extended with AMV reverse transcriptase. Lanes G, A, T and C represent dideoxy sequencing reactions performed on a recombinant plasmid carrying a fragment of the yeast 25S rRNA gene (Kiss-László *et al.*, 1996). (**D**) Primer extension mapping of 2′-*O*-methylated nucleotides in the presence of low concentration of dNTPs. RNA samples obtained from the control (lanes 5 and 6), the ΔU24 (lanes 7 and 8) or the hU24 (lanes 9 and 10) strain were analysed by primer extension of the ^{32}P-labelled oligonucleotide 29 in the presence of 1 (lanes 5, 7 and 9) or 0.004 mM dNTPs (lanes 6, 8 and 10). Lanes G, A, T and C represent sequencing ladders.

rRNAs obtained from the control CMY133 cells, from the ΔU24 and hU24 strains. Cellular RNAs isolated from each strain were subjected to mild alkali treatment and analysed by primer extension using a terminally ^{32}P-labelled oligonucleotide primer complementary to yeast 25S rRNA (Kiss-László *et al.*, 1996). Since 2′-*O*-methyl groups confer alkali resistance to the phosphodiester bonds (Smith and Dunn, 1959), ribose-methylated nucleotides appear as gaps in the ladder of primer extension products (Figure 2C). Alternatively, ribose-methylated nucleotides were visualized by a primer extension reaction performed in the presence of a low concentration of dNTPs (Figure 2D). In the presence of 0.004 mM dNTPs, reverse transcriptase stops one nucleotide before or, frequently, at the 2′-*O*-methylated nucleotides (Maden *et al.*, 1995). Therefore, in these mapping experiments, the position of the indicative stop signal and the actual position of the methylated nucleotide do not necessarily coincide.

Consistent with previous results (Kiss-László *et al.*, 1996), in 25S rRNA obtained from the control strain, three specific gaps (Figure 2C, lane 5) or stops (Figure 2D, lane 6) were detected for Cm1436, Am1448 and Gm1449 residues. These ribose-methylated nucleotides were clearly absent from 25S rRNA originating from the ΔU24 strain (Figure 2C, lane 6, and D, lane 8). However, expression of human U24 snoRNA restored the wild-type pattern of methylation of 25S rRNA at C1436 (Figure 2C, lane 7, and D, lane 10) and, as predicted by the rRNA methylation model, introduced a novel ribose-methylated nucleotide at C1450 (Figure 2C, lane 7, and D, lane 10) that is present in wild-type human 28S rRNA but is normally absent from yeast 25S rRNA (Maden, 1990). Note that the methylation state of the C1450 residue is indicated by a major stop at A1451. These results demonstrate that the human U24 snoRNA, although considerably shorter than its yeast counterpart (Figure 1B), possesses all of the sequence and structural elements that are essential for accumulation and function in yeast cells.

An internal C box-like element common to methylation guide snoRNAs

In the hope of identifying common functional domains, human and yeast U24 snoRNAs were aligned to maximum homology (Figure 1B). The 5′- and 3′-terminal regions of the two RNAs, that encompass the two antisense elements and the conserved C, D and D′ boxes, show >80% identity. Sequences in the middle regions of the two RNAs, mainly due to short spacer sequences inserted into yeast U24, show no significant conservation (38%). This central region of human U24 snoRNA carries a perfect copy of the C box sequence (UGAUGA) (Qu *et al.*, 1995). Interestingly, a similar sequence motif (GAAUGA) is also present in yeast U24 snoRNA, although it possesses two mismatches as compared with the sequence of the authentic C box.

Nevertheless, this observation encouraged us to re-investigate the sequences of human and yeast methylation guide snoRNAs reported thus far. To our surprise, we found that the seemingly non-conserved central regions of methylation guide snoRNAs always carry a sequence motif that is highly reminiscent of the C box (Figure 3A). This C box-like motif, termed the C′ box, carries one or frequently two altered nucleotides as compared with the canonical UGAUGA sequence of the C box of fibrillarin-associated snoRNAs. Only human U31 snoRNA represents an exception to this rule; the putative C′ box motif found in this snoRNA carries three mismatches.

Equally surprisingly, inspection of human and yeast antisense snoRNAs also revealed that all methylation guide snoRNAs possess a readily recognizable D′ box sequence motif (Figure 3A), indicating that this box element is not restricted to those snoRNAs that carry upstream complementarities to rRNAs (Kiss-László *et al.*, 1996; Nicoloso *et al.*, 1996; Tycowski *et al.*, 1996a). Similarly to the C′ box element, the putative D′ boxes also possess one or two altered nucleotides as compared with the consensus CUGA sequence of the authentic D box. A statistical analysis of the box D′ and C′ sequences shows that neither the D′ nor the C′ box possesses a perfectly conserved nucleotide (Figure 3B). In the D′ box sequences, the G3 and A4 nucleotides show the strongest conservation; >94% of the analysed D′ box sequences carry these nucleotides. In the C′ box sequence, the G5 and A6 residues show the strongest conservation; they are preserved in 86.3 and 80.4% of the C′ boxes, respectively. In the sequence, the D′ box always precedes the C′ box element and, normally, they are separated by 3–9 nucleotides. Interestingly, when the distance between these two box elements is longer than nine nucleotides (human U16, U18, U21, U35, U40 and U60, yeast snR40, snR41, U14, U18 and U24), potential intramolecular helix structures bring the D′ and C′ boxes closer to each other. This suggests that the D′ and C′ boxes work together, and the length of the spacer sequence separating these two elements is probably important for the optimal function of the snoRNA.

The C′ box of human and yeast U24 snoRNA is essential for the D′ box-dependent methylation of 25S rRNA

Since several lines of evidence indicate that sequences located between the C and D boxes of intron-encoded methylation guide snoRNAs have little or no effect on the processing and nucleolar accumulation of the snoRNA (Caffarelli *et al.*, 1996; Cavaillé and Bachellerie, 1996; Watkins *et al.*, 1996), it seems logical to hypothesize that the C′ and D′ box elements located in the central regions of the snoRNAs function in the site-specific methyltransfer reaction itself. To test this assumption, the C′ box region of yeast U24 was fully (yU24Δ1) or partially (yU24Δ2 and yU24Δ3) deleted and the sequence of the C′ motif of human U24 was altered (hU24m1) (Figure 4A). As a control, the D′ box of yeast U24 was masked by inverting its nucleotide sequence (yU24mD′). The resulting mutant snoRNAs were inserted into the intron region of the ADH/actin expression construct (see Figure 2A) and transformed into the ΔU24 yeast strain. As expected, Northern analyses (Figure 4B) confirmed that neither deletion of the C′ box element of yeast U24 (lanes 4–6) nor alteration of the C′ box of human (lane 9) and the D′ box of yeast (lane 7) U24 snoRNA affected the expression of U24 snoRNA. Since the electrophoretic mobilities of the mutant U24 snoRNAs were always tightly correlated with the predicted lengths of the snoRNAs, we concluded that the 5′ and 3′ termini of U24 snoRNA were processed correctly in the absence of an intact D′ or C′ box.

From each strain, RNA was extracted and the state of ribose methylation of 25S rRNA at C1436 and A1448-C1450 was tested by primer extension analyses performed in low concentration of dNTPs (Figure 4C). Similarly to the wild-type yeast (lane 2) and human (lane 14) U24 snoRNAs, each mutant U24 snoRNA restored the methylation of 25S rRNA at C1436 (lanes 6, 8, 10, 12 and 16). This demonstrates that neither the D′ box nor the C′ box plays an essential role in the ribose methylation reaction directed by the 3′-terminal antisense element and the D box of yeast and human U24 snoRNA. To ensure that 25S rRNA was intact in each sample and that the same amount of RNA was used for each reaction, we performed control mappings of 25S RNA at Am2279 and Am2280. These two consecutive modified nucleotides stop reverse transcriptase at Am2280 (Figure 4C; see also Kiss-László *et al.*, 1996).

Predictably enough (Cavaillé *et al.*, 1996; Kiss-László *et al.*, 1996), alteration of the D′ box sequence of yeast U24 snoRNA (yU24mD′) completely abolished the methylation of 25S rRNA at A1448 and G1449 (lane 12). More surprisingly, the mutant yU24Δ1, yU24Δ2 and yU24Δ3 yeast U24 snoRNAs that possessed an intact D′ box motif, but carried either fully or partially deleted C′ box regions, failed to mediate the site-specific methylation of 25S rRNA at A1448 and G1449 (lanes 6, 8 and 10). Likewise, when the wild-type sequence of the C′ box of human U24 snoRNA (UGAUGA) was replaced with the CCAUGG sequence, methylation of yeast 25S rRNA was completely obliterated at C1450 (lane 16). These results demonstrate that the C′ and D′ boxes of yeast and human U24 snoRNAs function largely independently of the C and D box elements. The C′ and D′ boxes are not essential for the processing or accumulation of the snoRNA, and neither of these elements contribute significantly to the rRNA methylation reaction directed by the 3′-terminal antisense element and the D box of U24 snoRNA. However, our results demonstrate that the C′ box motif, together with the D′ box, plays a crucial role in the rRNA

A

	box D'	box C'
Human		
U14-6	*41-CAAA*agcuuggc	UAAUGA
U15	*26-*CUGAcuugg	GGAUGU
U16	*25-CUUA*cucuguucucagcgacaguugccugcugucaguaagcugguacagaaggu	UGACGA
U18	*37-CUGA*accacaugguuuucuc	GGAUGU
U20	*21-*CUGAgaaauaau	UGAUGA
U21	*23-*CUGAgcagucaguaguugguccuuugguugcaua	UGAUGC
U24	*30-CUGA*gagaugg	UGAUGA
U25	*34-CUGA*gcuccg	UGAGGA
U26	*37-CUGA*uggauuag	UGGAGA
U27	*35-CUGA*acuuucaag	UGAUGU
U28	*16-*UUGAuaagc	UGAUGU
U29	*29-AUGA*ccgacag	UGAAAA
U30	*33-CUGA*ugacuugc	UGUUGA
U31	*31-CUGA*ucaau	GUGUGA
U32	*35-UUGA*gucucacgg	CCAUGA
U33	*41-*CCGAccaug	AGAUGA
U34	*33-UUGA*ucc	UCAUGA
U35	*33-CGGA*uugacugcgggaa	UGGCGA
U36a	*23-*CUGAauucaaccu	UGAAGU
U37	*35-CAGA*uguc	UGUUGA
U38	*35-CUGA*caguaag	UGAAGA
U39	*36-*CCGAgugcgcg	UGGGGA
U40	*22-*CUUAggcgugguugugccgucu	UGGUCA
U41	*17-*CUGUgac	UGUUGA
U42	*25-*UGGAaaagaaug	ACAUGA
U43	*30-CAGA*aacugug	UGCUGA
U44	*22-*CUGAcugaaca	UGAAGG
U45a	*28-CUGA*aucuauugc	UGAUGU
U47	*27-AUGA*aaua	UAAUGA
U48	*24-*CUGAgugugucgc	UGAUGC
U49	*34-CAGA*agcgauaac	UGACGA
U50	*26-CUGA*acuuc	UGUUGA
U51	*38-CUGA*guucg	UGAUGG
U52	*30-CCGA*ugcugg	UCAUGA
U53	*36-*CUGAguuucag	AGAUGA
U54	*26-*UUGAguaacgg	UGAUAA
U55	*35-*CCGAgugcgcgg	UGGGGA
U56	*29-CAGC*aguucacc	UAGUGA
U57	*18-*CUGAgccugaccu	UGUAGA
U58a	*30-UGGA*uuuaccg	UGAAAA
U59	*39-CUGA*guu	UGCUGA
U60	*38-AUGA*aaacugcacgugcaguc	UGAUUA
U61	*22-*UUGAucgucugaca	UGAUAA
Yeast		
snR38	*42-CUGA*auggg	UAAUAA
snR39	*37-*UCGAcuagucau	UGACGA
snR40	*32-CUGA*acauccaugccacaaguacucagguguc	CUAUGA
snR41	*45-CUGA*auaguggguuuaacuaca	UGUCGA
snR190	*36-CCGA*uucgacca	UGACGA
U14	*44-*CGGUaggaguacgcuuacgaacccaucguuaguacucu	CGGUGA
U18	*35-AUGA*ugggaaguaagacccccg	AUAUGA
U24	*31-CAGA*uggaacuuugaguucc	GAAUGA

B

	Box D' sequence				Box C' sequence					
Nucleotide	C_1	U_2	G_3	A_4	U_1	G_2	A_3	U_4	G_5	A_6
C	76.5%	11.8%	2.0%	2.0%	5.9%	7.8%	3.9%	11.8%	2.0%	3.9%
U	15.7%	68.6%	3.9%	3.9%	78.4%	5.9%	11.8%	68.6%	2.0%	11.8%
G	0.0%	7.8%	94.1%	0.0%	7.8%	76.5%	15.7%	5.9%	86.3%	3.9%
A	7.8%	11.8%	0.0%	94.1%	7.8%	9.8%	68.6%	13.7%	9.8%	80.4%

Fig. 3. Structure of box D′ and C′ motifs of human and yeast methylation guide snoRNAs. (**A**) Compilation of sequences of putative box D′ and C′ motifs of methylation guide snoRNAs. Boxes D′ and C′ are shown in upper case. The D′ box elements that are known to be preceded by complementary sequences to rRNAs are italicized. Spacer sequences are indicated by lower case letters. Potential intramolecular helices are underlined. Numbers indicate the positions of the first nucleotides of the D′ box elements. The sequences of human and yeast methylation guide snoRNAs have been obtained from the literature. For references, see Kiss-László *et al.* (1996) and Nicoloso *et al.* (1996). Please note that human U40 and U46, that have been characterized independently by Nicoloso *et al.* (1996) and Kiss-László *et al.* (1996), represent the same snoRNA. (**B**) Nucleotide conservation in the proposed D′ and C′ box motifs of human and yeast methylation guide RNAs. The most conserved nucleotides in the box D′ and box C′ sequences are indicated in italics.

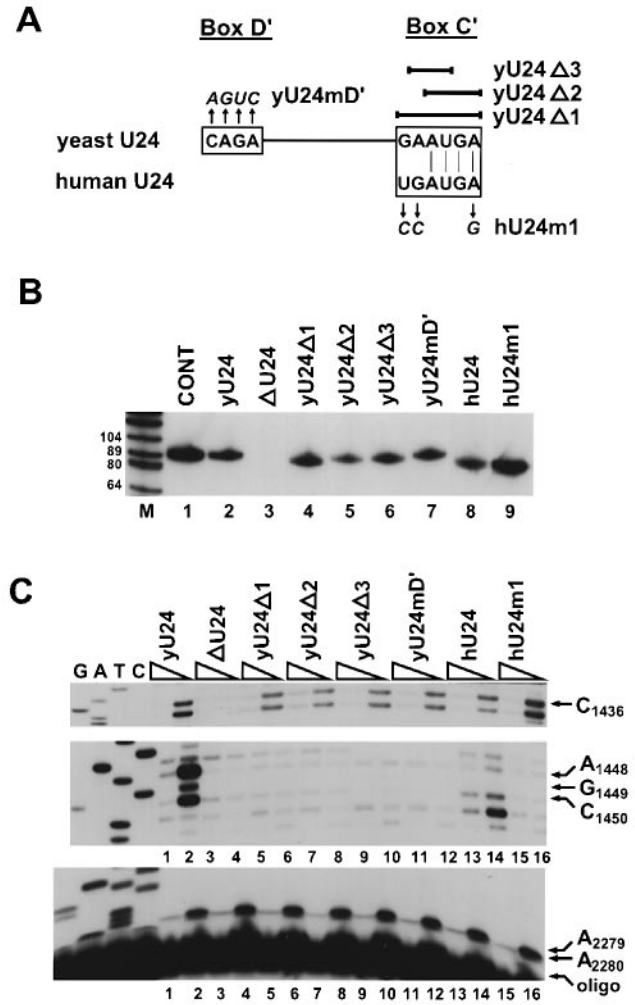

Fig. 4. Expression and activity of human and yeast U24 methylation guide snoRNAs with modified D′ and C′ box elements. (**A**) Construction of mutant human and yeast U24 snoRNAs. Deletions and nucleotide changes (in italics) are indicated above or below the authentic D′ and C′ sequences of yeast and human U24 snoRNAs. (**B**) Northern blot analyses of expression of human and yeast U24 snoRNAs with modified D′ and C′ elements. RNAs isolated from the control CMY133 cells (lane 1), from the ΔU24 strain (lane 3) or from ΔU24 cells transformed with the ADH/actin expression construct that carried either the wild-type yeast U24 (lane 2), the wild-type human U24 (lane 8), the yeast yU24Δ1, yU24Δ2, yU24Δ3, yU24mD′ (lanes 4–7, respectively) or the human hU24m1 (lane 9) snoRNAs were subjected to Northern blot analyses. For details, see legend to Figure 2B. (**C**) Primer extension mapping of ribose-methylated nucleotides in yeast 25S rRNA. RNAs obtained from the ΔU24 strain (lanes 3 and 4), from yeast cells expressing either the wild-type human U24 (lanes 13 and 14), the wild-type yeast U24 (lanes 1 and 2), the yeast yU24Δ1 (lanes 5 and 6), yU24Δ2 (lanes 7 and 8), yU24Δ3 (lanes 9 and 10), yU24mD′ (lanes 11 and 12), or the human hU24m1 (lanes 15 and 16) snoRNAs were analysed by primer extension in the presence of 1 or 0.004 mM dNTPs as indicated above the lanes. For other details, see legend to Figure 2D.

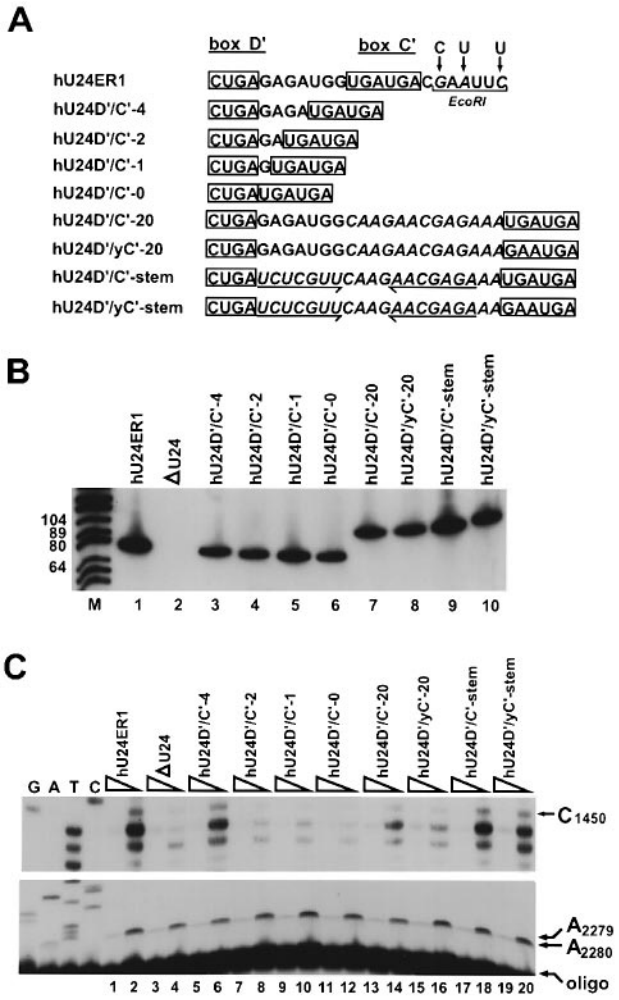

Fig. 5. Expression and activity of human U24 methylation guide snoRNA with modified D′ and C′ box spacer sequences. (**A**) Sequences of the modified regions of human U24 snoRNA. To facilitate cloning, a novel *Eco*RI site was introduced downstream of the C′ box of U24 snoRNA. The non-natural sequences are italicized. In the hU24D′/yC′-20 and hU24D′/yC′-stem RNAs, the C′ box of human U24 was replaced with the C′ box of yeast U24 snoRNA. The inverted arrows indicate putative intramolecular helices. (**B**) Northern blot analyses. RNAs isolated either from the ΔU24 strain (lane 2) or from its derivative strains (lanes 1 and 3–10) transformed with the ADH/actin expression construct carrying the mutant human U24 snoRNAs (as indicated above the lanes) were separated on a denaturing gel and probed with a radiolabelled oligonucleotide complementary to human U24 snoRNA. (**C**) Mapping of ribose methylations in yeast 25S rRNA. RNAs isolated from yeast strains indicated above the lanes were analysed by primer extension. For other details, see legend to Figure 2D.

methylation reaction directed by the upstream antisense elements of human and yeast U24 snoRNA.

Correct spacing of the D′ and C′ boxes of U24 snoRNA is required for efficient rRNA methylation

Normally, the D′ and C′ boxes of methylation guide snoRNAs are separated by 3–9 nucleotides (Figure 3A). In snoRNAs where the distance between the D′ and C′ boxes is longer than nine nucleotides, potential intramolecular helices can fold the two elements closer to each other. To test whether the distance between the D′ and C′ boxes is really essential for optimal snoRNA function, a series of D′–C′ spacing mutants of human U24 snoRNA were constructed and expressed in the ΔU24 yeast strain (Figure 5A and B).

As expected, deletion or insertion of sequences within the D′–C′ spacer region of U24 had no effect on the accumulation of snoRNA (Figure 5B) and did not influence the D box-dependent methylation of 25S rRNA at C1436 (data not shown). However, changing the distance between the D′ and C′ boxes of U24 had detrimental effects on

the D′ box-dependent methylation of 25S rRNA at C1450 (Figure 5C). The hU24D′/C′-4 snoRNA, in which the D′ and C′ boxes are separated by four nucleotides, still supported the methylation of 25S rRNA (lane 6) at the levels of the wild-type U24 snoRNA (lane 2). However, placing the C′ box two or one nucleotide downstream of the D′ box (hU24D′/C′-2 and hU24D′/C′-1) or fusing the two box elements (hU24D′/C′-0) almost completely abolished the methylation of 25S rRNA (lanes 8, 10 and 12, respectively).

Similarly, increasing the distance between the D′ and C′ boxes of U24 by insertion of a 13 nucleotide long additional spacer sequence (hU24D′/C′-20) significantly decreased the efficiency of rRNA methylation at C1450 (lane 14). Interestingly, replacement of the C′ box of hU24D′/C′-20 (UGAUGA) with the C′ box of yeast U24 (GAAUGA) further decreased the methylation activity of the resulting hU24D′/yC′-20 (lane 16). More tellingly, replacement of the spacer sequences following the D′ box of hU24D′/C′-20 and hU24D′/yC′-20 snoRNAs with sequences that are capable of forming a 7 bp uninterrupted helix with downstream spacer sequences (hU24D′/C′-stem and hU24D′/yC′-stem), thereby bringing closer the D′ and C′ boxes of U24 snoRNA, restored the wild-type levels of rRNA methylation at C1450 (lanes 18 and 20). Control mappings at Am2279 and Am2280 proved that the 25S rRNA was intact in each RNA sample. These results demonstrate that the sterical arrangement of the D′ and C′ boxes in the snoRNA structure can seriously influence the efficiency of the methyltransfer reaction directed by the upstream antisense element and the D′ box.

A minimal core structure of U24 snoRNA competent in site-specific rRNA methylation

The experiments presented thus far, although clearly demonstrating that the C′ and the D′ boxes of human and yeast U24 snoRNA play crucial roles in the methyltransfer reaction, do not exclude the possibility that additional snoRNA elements, for example the C and D boxes, also contribute to the rRNA methylation reaction directed by the upstream antisense element of U24. To assess this important question, a short fragment of human U24 snoRNA encompassing the upstream antisense element and the D′ and C′ boxes was inserted into the yeast *SNR5* snoRNA gene (Figure 6A). The snR5 snoRNA belongs to the family of box H/ACA snoRNAs. Being synthesized by the RNA polymerase II, the 5′ terminus of snR5 is blocked by the trimethylguanosine cap structure that is characteristic of all polymerase II-transcribed snRNAs (Hernandez, 1992). Therefore, sequences inserted into the 5′-terminal leader of snR5 that precedes the evolutionarily conserved hairpin–hinge–hairpin–tail secondary structure of box H/ACA snoRNAs have presumably no effect on the accumulation of the chimeric snR5/U24 snoRNA (Balakin *et al.*, 1996; Ganot *et al.*, 1997b). Since the snR5 snoRNA functions as guide RNA in the site-specific pseudouridylation of rRNA (Ganot *et al.*, 1997a), one could assume that the snR5 snoRNP particle would deliver the U24-specific tag into the right subnucleolar compartment where pseudouridylation and, most probably, ribose methylation of pre-rRNA also takes place (Maden, 1990; Eichler and Craig, 1994).

The expression construct carrying the chimeric *SNR5/U24* gene was transformed into the ΔU24 yeast strain. Northern hybridization (Figure 6B) showed that the snR5/U24 snoRNA accumulated efficiently in the transformed cells (lane 3). Mapping the ribose methylation sites in the 25S rRNA obtained from the snR5/U24 strain (Figure 6C) demonstrated that expression of the chimeric snR5/U24 snoRNA at least partially restored the methylation of the 25S rRNA at the C1450 residue. As compared with the wild-type 25S rRNA (lane 2), ~40% of the nucleotides at the C1450 position were methylated in the 25S rRNA obtained from the snR5/U24 strain (lane 6). As revealed by control mappings at A2279 and A2280, the RNA samples analysed in this experiment contained the same amounts of apparently intact 25S rRNAs. This experiment demonstrates that a short fragment of a methylation guide snoRNA containing the complementary element followed by the D′/D and C′/C boxes can provide sufficient information for the snoRNP proteins to perform the site-specific methylation of pre-rRNA.

Discussion

In eukaryotic rRNAs, many nucleotides are post-transcriptionally methylated at the 2′-*O*-hydroxyl position (Maden, 1990). Since the locations of these modified nucleotides lack any conserved sequence motifs or local secondary structures, the mechanism underlying the specific recognition of the numerous methylation sites had long been obscured (Maden, 1990). Only recently, it became evident that each rRNA methylation site is recognized by a specific methylation guide snoRNA that can form a 10–21 bp perfect double helix with the target rRNA sequence (Figure 7). This snoRNA–rRNA duplex, in conjunction with the adjacent D or D′ box of the snoRNA, provides the structural information necessary for the methyltransferase activity to methylate the rRNA nucleotide opposite the fifth base upstream from the D or D′ box (Cavaillé *et al.*, 1996; Kiss-László *et al.*, 1996).

The focus of this study was to determine those elements of methylation guide snoRNAs that, in addition to the antisense regions and the D and D′ boxes, are required for the site-specific methylation of pre-rRNA. An examination of human and yeast snoRNA sequences revealed that the internal region of all methylation guide snoRNAs contains a D′ box motif that is always followed by a C box-like motif, termed the C′ box (Figure 7). Usually, the D′ and the C′ boxes contain one or two base substitutions as compared with the consensus sequences of the authentic D (CUGA) and C (UGAUGA) boxes (Figure 3). Deletions and nucleotide substitutions introduced into the D′ and C′ boxes of U24 snoRNA demonstrated that these elements are absolutely required for the rRNA methylation reaction guided by the upstream antisense element of U24 (Figure 4). We also noticed that the D′ and C′ boxes that are positioned more than nine nucleotides apart from each other could be folded together by potential internal helices (Figures 3 and 7). Indeed, deletion and insertion analyses proved that an appropriate juxtapositioning of the D′ and C′ elements can greatly improve the efficiency of the methyltransfer reaction (Figure 5). Our results indicate that the D′ and C′ boxes, similarly to the authentic C and D boxes (Caffarelly *et al.*, 1996; Watkins *et al.*, 1996), probably establish a protein-binding site. Consistent with

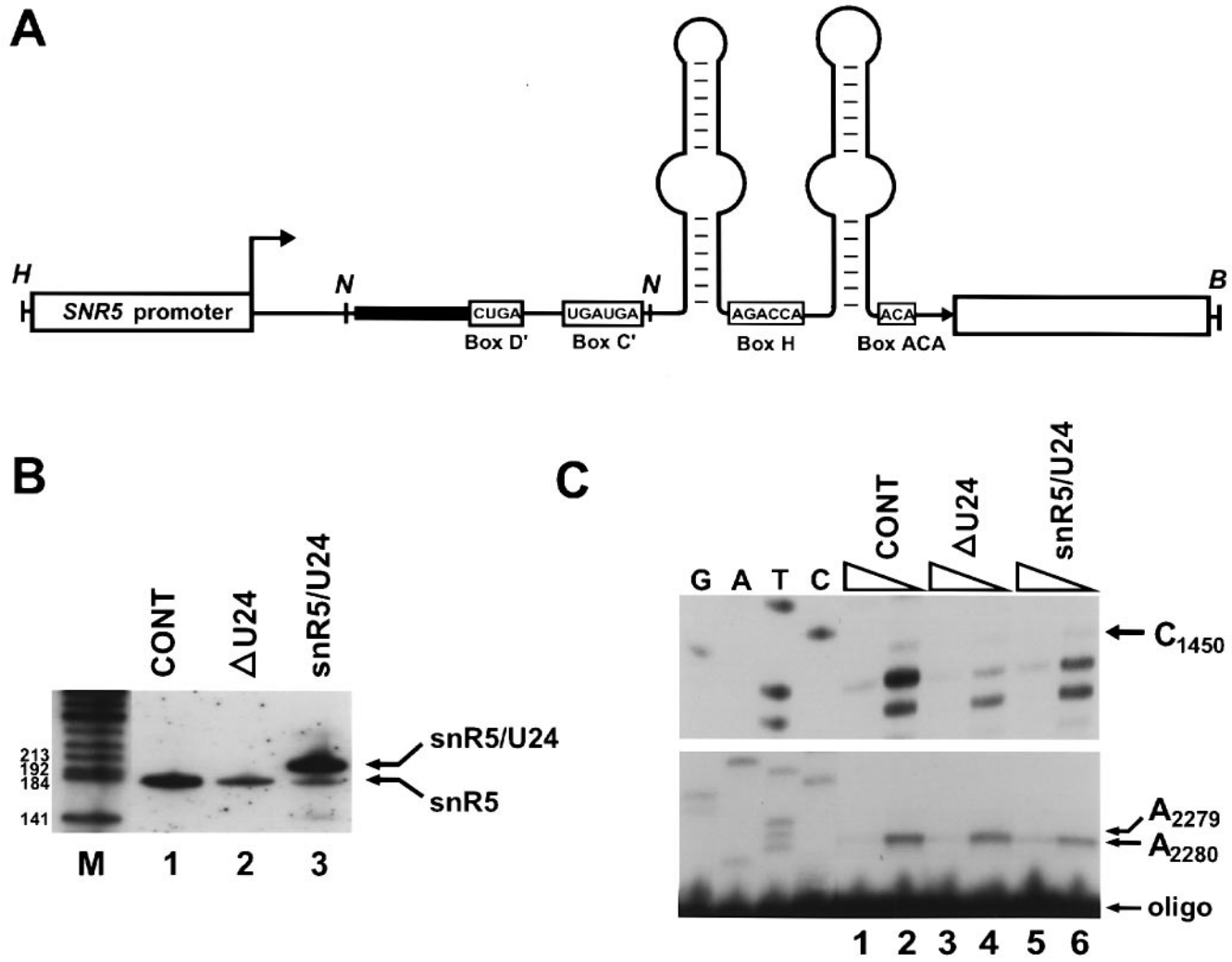

Fig. 6. Minimal snoRNA sequence requirements for methylation of pre-rRNAs. (**A**) Schematic structure of the *SNR5/U24* chimeric snoRNA gene. A fragment of the human U24 snoRNA encompassing the upstream antisense element (thick line) and the D′ and C′ boxes was inserted into the snR5-coding region of the yeast *SNR5* gene. The promoter and downstream flanking regions of the *SNR5* gene are indicated by open boxes. The schematic secondary structure and the evolutionarily conserved H and ACA box elements of snR5 RNA are shown. The relevant restriction sites are indicated (B, *Bam*HI; H, *Hin*dIII; N, *Nco*I). (**B**) Northern blot analyses of the expression of the snR5/U24 chimeric snoRNA. Cellular RNAs obtained from the CMY133 control strain (lane 1), from the ΔU24 strain (lane 2), and from the snR5/U24 strain (lane 3) were probed with an snR5-specific probe. Since the *SNR5/U25* gene cloned into the pFL36 shuttle vector (Bonneaud *et al.*, 1991) is expected to be present in multiple copies in the cell, in lanes 2 and 3 we analysed 2 μg rather than 10 μg of cellular RNAs. (**C**) Mapping of ribose-methylated nucleotides in yeast 25S rRNAs obtained from the control and the snR5/U24 strains. For other details, see legend to Figure 2D.

this idea, a C′ box element, when it is located at a suboptimal distance from the D′ box element, but possesses the consensus C box sequence (UGAUGA), can support the rRNA methylation more efficiently than a C′ box carrying deviations from the consensus C box sequence (Figure 5). Finally, we have shown that an internal fragment of U24 snoRNA encompassing the upstream antisense element and the D′–C′ box regions is able to support the site-specific methylation of yeast 25S rRNA, indicating that this short fragment of U24 carries all the elements that are necessary to select the correct rRNA nucleotide and to recruit the protein factors required for rRNA methylation.

At the moment, the function of the putative D′ and C′ box motifs found in those methylation guide snoRNAs which lack upstream complementarties to rRNAs is entirely speculative. Apparently, snoRNAs lacking functional D′ and C′ boxes are still processed correctly, they accumulate efficiently and successfully support the rRNA methylation reaction guided by the 3′-terminal antisense element and the D box motif (Figure 4). It is possible that the internal D′ and C′ boxes, through their ability to bind snoRNP proteins, can improve either the stability, the nucleolar transportation or the methyltransferase activity of these snoRNPs. A more fascinating hypothesis would be that the D′ and C′ motifs of these snoRNAs may be involved in the site-specific ribose methylation of substrate RNAs other than rRNAs. Notably, a recently characterized human box C/D snoRNA that lacks a significant complementarity to rRNAs possesses an 11 nucleotide long region that is perfectly complementary to human U6 snRNA. Intriguingly, the position of the D′ box of this putative methylation guide RNA allows the selection of the ribose-methylated A53 nucleotide in human U6 snRNA (Z.Kiss-László and T.Kiss, unpublished data).

Since the C and D boxes of methylation guide snoRNAs

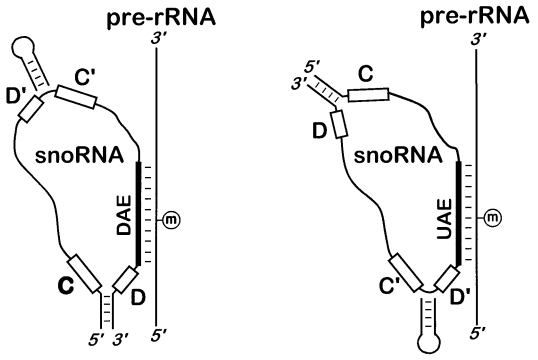

Fig. 7. A model proposed for the involvement of the C, D, C′ and D′ box elements of methylation guide snoRNAs in the site-specific ribose methylation of pre-rRNA. The downstream antisense element (DAE) or the upsteam antisense element (UAE) of the guide snoRNA forms a 10–21 bp double helix with the complementary rRNA sequences. These highly specific and strong snoRNA–pre-rRNA interactions are followed either by the terminal D and C or the internal D′ and C′ boxes. The D and C as well as the D′ and C′ boxes frequently are brought together by intramolecular helices. Putative protein factors bound to the DAE helix boxes D/C or the UAE helix boxes D′–C′ structural motifs accomplish the site-specific methyltransfer reaction. Few snoRNAs, for example U24, are able to form both DAE and UAE types of interactions.

are absolutely required for the processing and accumulation of the snoRNA (Caffarelli *et al.*, 1996; Watkins *et al.*, 1996; Xia *et al.*, 1996), involvement of these elements in the rRNA methylation reaction cannot be assayed by direct mutational analyses. However, the demonstration that the C′ box of U24 snoRNA plays a crucial role in the rRNA methylation reaction directed by the upstream antisense element and the D′ box strongly suggests that the authentic C box, in conjunction with the D box motif, functions in the methyltransfer reaction guided by the downstream antisense element. In a previous work (Cavaillé *et al.*, 1996), in order to study the function of the D box of U20 methylation guide snoRNA, a second copy of the D box was introduced downstream of the authentic D box and the 3′-terminal antisense element of U20 and, thereby, the original D box of U20 was converted into a D′ box-like motif. The resulting snoRNA, although devoid of a C′ box motif, supported the 'D′ box'-dependent methylation of rRNA. However, any single mutation introduced into the canonical CUGA sequence of the 'D′ box' drastically inhibited the methylation reaction. This was rather unexpected, since many naturally occurring D′ box sequences possess one or two altered nucleotides (Figure 3). A possible interpretation of these results is that the C box may also contribute to the methyltransfer reaction directed by the upstream antisense element and the D′ box. It is easily imaginable that as soon as the snoRNA–rRNA double helix has been formed, the C box may establish a protein-mediated interaction with the D′ box. Of course, the mutant D′ boxes carrying altered nucleotides cannot compete successfully with the authentic D box motif that possesses the canonical CUGA sequence.

The nucleotide boxes C/C′ and D/D′ function apparently through binding specific protein factors. The snoRNP proteins bound to the box C and D sequences are essential for the processing of the intron-encoded snoRNAs (Watkins *et al.*, 1996) and the nucleolar retention and hypermethylation of the polymerase II-synthesized box C/D snoRNAs (Terns *et al.*, 1995). It would be very interesting to determine whether the D–C and the D′–C′ boxes of methylation guide snoRNAs can recruit the same set of snoRNP protein(s). The C and D boxes have been implicated in binding of fibrillarin (Baserga *et al.*, 1991, 1992; Peculis and Steitz, 1994), but no evidence supporting the existence of a direct snoRNA–fibrillarin interaction has been presented. Recently, immunoprecipitation of internally deleted mouse U14 snoRNAs suggested that, in addition to the C and D boxes, the middle part of U14 snoRNA also contains some structural elements that are essential for fibrillarin binding (Watkins *et al.*, 1996). Now, in the light of our results, it becomes evident that the deletion mutants of U14 snoRNA that failed to bind fibrillarin lacked the putative D′ and C′ boxes of U14 snoRNA. Whether the the D′ and C′ boxes of methylation guide snoRNAs are involved in the binding of fibrillarin or other snoRNP proteins remains an interesting question.

In summary, we have identified a novel common structural element of methylation guide snoRNAs, the C′ box, that is required for the 2′-*O*-ribose methylation of pre-rRNA. The C′ box, together with the D′ box and the upstream antisense element, constitute a minimal core structure of the snoRNA that can support the selection and methylation of the target rRNA nucleotide. This argues that the authentic C box of methylation guide snoRNA functions in the rRNA methylation reaction guided by the 3′-terminal antisense element and the D box motif. In the near future, identification of the putative methyltransferase enzyme and the snoRNP proteins involved in the snoRNA-guided 2′-*O*-methylation of rRNAs will provide an exciting challenge.

Materials and methods

Strains and general procedures

Growth and genetic manipulations of yeast *Saccharomyces cerevisiae* were done by standard techniques (Sherman *et al.*, 1983). Construction of the yeast Δ*U24* strain (*a, trp1.Δ, his3.Δ, ura3.52, lys2.801, ade2.101, URA3::U24*) has been described (Kiss-László *et al.*, 1996). Recombinant DNA work was performed according to standard laboratory protocols (Sambrook *et al.*, 1989). The identity of all constructions was verified by sequence analysis. The following oligodeoxynucleotides were used in this study: (1) TCGAGAATATTGGTACCG; (2) TCGACGGTACCAATATTC; (3) ATACTCGAGTGTGCAGATGATGTAAAAG; (4) ATAGGTACCTGGTGCATCAGCGATCTTGGT; (5) ATACTCGAGCCATTCAAATGATGTAA; (6) ATAGGTACCCCATTCATCAGAGATCTTGG; (7) GTGGTTTGAATTCTCATCA; (8) TTCGGAATTCAAAGTTCC; (9) TGATGAGAATTCAAACCAC; (10) GGAACTTTGAATTCCGAA; (11) TAAAATGCCATGGCCATC; (12) GATGGCCATGGCATTTTA; (13) AACTTTGAATTCCGACATACCAA; (14) CTTTGAATTCCGAGACATACCAA; (15) CTTTGAATTCCGGAGACATACCAA; (16) TCGGAATTCAAAGTTCCAGACTAAGTAGCA; (17) TTTGAATTCTCATCATCTCTCAGATAG; (18) TTTGAATTCTCATCATCTCAGATAGCA; (19) TTTGAATTCTCATCACTCAGATAGCAA; (20) TTTGAATTCTCATCATCAGATAGCAAA; (21) TTTGAATTCTCATCATTTCTCGTTCTTGCCATCTCTCAGAT; (22) TTTGAATTCTCATCATTTCTCGTTCTTGAACGAGATCAGATAGCAAAT; (23) TTTGAATTCTCATTCTTTCTCGTTC; (24) TTTCCATGGTCATCACCATCTCTC; (25) TTTCCATGGGAATATTTGCTA; (26) CTTTTACATCATCTGCA; (27) GTTATTACATCATTTGA; (28) ATAGACATATGGAGGCGTG; (29) GCTTCAGTCTTCAAAGTTCTC.

Construction of expression plasmids

Construction of the yeast expression vector, pFL45/ACT, has been described (Kiss-László *et al.*, 1996). Briefly, the yeast actin gene was placed under the control of the promoter and terminator of the yeast

alcohol dehydrogenase gene and inserted into the pFL45 yeast shuttle vector (Bonneaud *et al.*, 1991). To facilitate cloning, the *Sma*I, *Kpn*I, *Sac*I and *Eco*RI sites were removed from the polylinker region of pFL45/ACT by digestion with *Sma*I and *Eco*RI, followed by religation after Klenow treatment. A new *Kpn*I site was introduced into the intron region of the actin gene by insertion of a synthetic DNA fragment (oligonucleotides 1 and 2) into the *Xho*I site of pFL45/ACT.

To obtain pFL45/ACT/hU24 and pFL45/ACT/yU24, the snoRNA-coding regions of human and yeast U24 genes were PCR amplified using oligonucleotides specific for the 5′- and 3′-terminal regions of human (oligonucleotides 3 and 4, respectively) and yeast (oligonucleotides 5 and 6, respectively) U24 snoRNAs. The amplified fragments were introduced into the *Xho*I–*Kpn*I sites of the modified pFL45/ACT vector using the PCR-introduced *Xho*I and *Kpn*I sites.

To facilitate mutational analyses of human and yeast U24 snoRNAs, a new *Eco*RI site was introduced into the U24 snoRNA-coding sequence by PCR mutagenesis. The 5′ halves of human and yeast U24 snoRNAs were amplified using 5′ end-specific primers (oligonucleotides 3 and 5, respectively) and primers complementary to either human (from position 41 to 59; oligonucleotide 7) or yeast (from position 35 to 53; oligonucleotide 8) U24 snoRNAs. The 3′ halves of human and yeast U24 snoRNAs were amplified using 3′ end-specific primers (oligonucleotides 4 and 6, respectively) and primers encompassing either human (from position 41 to 59; oligonucleotide 9) or yeast (from position 35 to 53; oligonucleotide 10) U24 snoRNA sequences. The internal primers contained appropriate nucleotide changes to introduce a new *Eco*RI site. The amplified fragments were digested with *Eco*RI. The 5′ and 3′ halves of human or yeast snoRNAs were ligated together, digested with *Xho*I and *Kpn*I and inserted into the *Xho*I–*Kpn*I sites of the modified pFL45/ACT, resulting in pFL45/ACT/hU24ER1 and pFL45/ACT/yU24ER1, respectively. A similar approach was used to construct pFL45/ACT/hU24m1. The 5′ half of human U24 was amplified using oligonucleotides 3 (5′ primer) and 11 (3′ primer containing an new *Nco*I site), and the 3′ half was obtained by using oligonucleotides 4 (3′ primer) and 12 (5′ primer containing a new *Nco*I site). The amplified fragments were cut with *Nco*I, ligated together, and inserted into the *Xho*I–*Kpn*I site of pFL45/ACT.

To obtain pFL45/ACT/yU24Δ1, pFL45/ACT/yU24Δ2 and pFL/45/ACT/yU24Δ3, the *Eco*RI–*Kpn*I fragment of pFL45/ACT/yU24ER1 containing the 3′ half of the yeast U24 snoRNA gene was replaced with PCR-amplified fragments generated by using a combination of a common 3′ end-specific primer (oligonucleotide 6) and appropriate mutagenic 5′ primers encompassing the *Eco*RI site of yU24ER1 (oligonucleotides 13, 14 and 15, respectively). To create pFL45/ACT/yU24mD′, the *Xho*I–*Eco*RI fragment of pFL45/ACT/yU24ER1 containing the 5′ half of the yeast U24 snoRNA gene was replaced with a mutant sequence generated by PCR amplification with a 5′ end-specific primer (oligonucleotide 5) and an appropriate mutagenic 3′ primer (oligonucleotide 16) containing the *Eco*RI site of yU24ER1.

To create pFL45/ACT/hU24D′/C′-4, pFL45/ACT/hU24D/C′-2, pFL45/ACT/hU24D′/C′-1, pFL45/ACT/hU24D′/C′-0, pFL45/ACT/hU24D′/C′-20 and pFL45/ACT/hU24D′/C′-stem, the 5′ half of human U24 snoRNA was amplified using a common 5′ end-specific primer (oligonucleotide 3) and appropriate mutagenic primers (oligonucleotides 17, 18, 19, 20, 21 and 22, respectively). The amplified fragments were cut with *Xho*I and *Eco*RI and were used to replace the *Xho*I–*Eco*RI fragment of pFL45/ACT/hU24ER1. The same approach was used to generate pFL45/ACT/hU24D′/yC′-20 and pFL45/ACT/hU24D′/yC′-stem, except that pFL45/ACT/hU24D′/C′-20 and pFL45/ACT/hU24D′/C′-stem were used as templates and oligonucleotide 23 was used as a common 3′ primer. Finally, the *Xho*I–*Eco*RI fragments of the PCR template constructs were replaced with the amplified fragments.

Construction of pFL45/SNR5 was reported earlier (Ganot *et al.*, 1997b). A novel *Nco*I restriction site was introduced into the snoRNA-coding region of the snR5 gene at position 8 by PCR mutagenesis, resulting in pFL45/SNR5Nco. A portion of human U24 snoRNA encompassing the upstream antisense element, the box D′ and box C′ elements was PCR amplified using oligonucleotides 24 (5′ specific) and 25 (3′ specific). The amplified fragment was inserted into the *Nco*I site of pFL45/SNR5Nco using the PCR-introduced *Nco*I sites.

RNA analysis

Total yeast RNA was isolated by guanidinium thiocyanate/phenol–chloroform extraction (Tollervey and Mattaj, 1987). For Northern analyses, unless stated otherwise, 10 μg of yeast or 5 μg of human HeLa cellular RNAs were separated on an 8% sequencing gel, and electroblotted onto a Hybond-N nylon membrane (Amersham). The blots were probed with phosphorylated oligonucleotides complementary to either human U24 (oligo 26), yeast U24 (oligo 27) or yeast snR5 (oligo 28) snoRNAs. Mapping of 2′-*O*-ribose-methylated nucleotides C1436, A1448–C1450 in yeast 25S rRNA was performed by primer extension analyses either on partially hydrolysed RNA (Kiss-László *et al.*, 1996) or on intact RNA in the presence of a low concentration of dNTPs (Maden *et al.*, 1995). Control mappings of 25S rRNA at positions Am2279 and Am2280 were performed as described by Kiss-László *et al.* (1996).

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