

Nucleolar localization signals of Box H/ACA small nucleolar RNAs

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

Keywords: coiled bodies/nucleolus/RNA transport/snoRNA/*Xenopus* oocyte

Introduction

In eukaryotes, the nucleolus is the site of assembly of ribosomal proteins and ribosomal RNA (rRNA) into ribosomal subunits. While ribosomal proteins are synthesized in the cytoplasm and transported to nucleoli, rRNAs are synthesized, modified and processed from precursors within the nucleolus (Scheer and Weisenberger, 1994; Shaw and Jordan, 1995). rRNA is heavily modified: >200 of the ~7000 nucleotides of mature human rRNA are

modified by isomerization of uridine to pseudouridine or methylation of the 2'-OH group of the ribose (Maden, 1990; Ofengand *et al.*, 1995; Ofengand and Bakin, 1997; Ofengand and Fournier, 1998). The purpose of 2'-O-methylation and pseudouridylation of rRNA is not known. While individual modifications have not been found to be essential for cellular viability (Maxwell and Fournier, 1995; Balakin *et al.*, 1996; Qu *et al.*, 1999), the positions of modified rRNA nucleotides are concentrated in the functional core of rRNA and are conserved, implying an important, perhaps collective function (Bakin *et al.*, 1994; Lane *et al.*, 1995). The modified precursor rRNA transcript is processed to produce three mature species (5.8S, 18S and 28S rRNA in vertebrates) that are incorporated into the large and small ribosomal subunits (Hadjiolov, 1985; Eichler and Craig, 1994; Venema and Tollervey, 1995; Sollner-Webb *et al.*, 1996).

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

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

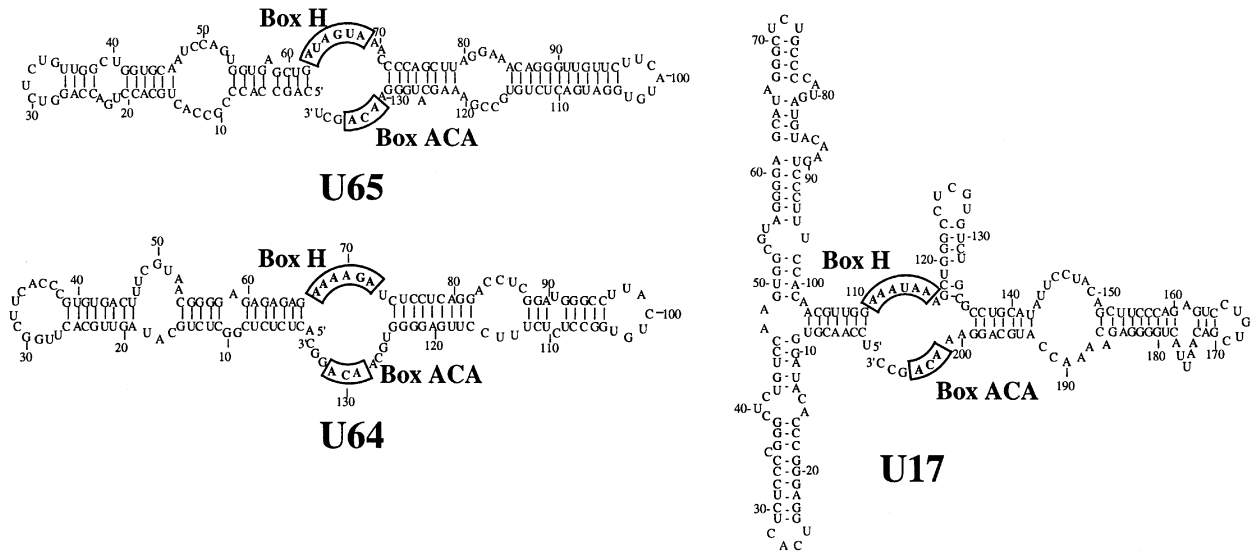


Fig. 1. Sequence and secondary structures of U65, U64 and U17 Box H/ACA snoRNAs (Ceconi *et al.*, 1994; Ganot *et al.*, 1997b; Bortolin *et al.*, 1999). The conserved Box H and Box ACA sequences are indicated.

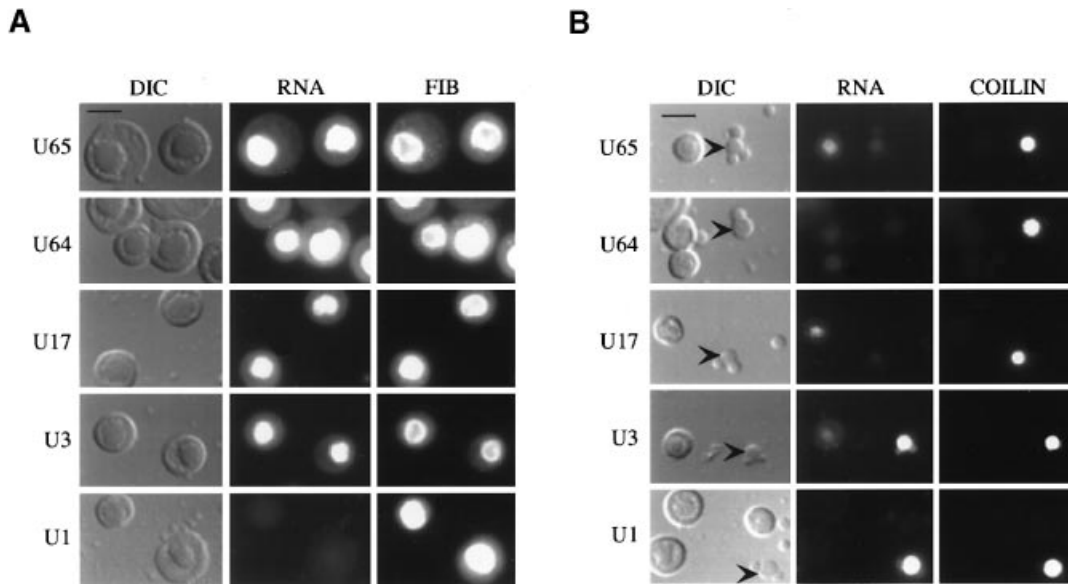


Fig. 2. Microinjected fluorescently labeled RNAs localized in *Xenopus* oocyte nuclear spreads. (A) Box H/ACA snoRNAs co-localize with endogenous fibrillarin in nucleoli. Fluorescently labeled U65, U64 and U17, as well as U3 and U1, were transcribed *in vitro* (see Materials and methods) and injected separately into *Xenopus* oocyte nuclei. m⁷G-capped U3 snoRNA and U1 snRNA served as positive and negative controls, respectively, for nucleolar localization. The nuclear spreads were prepared 1 h after injection. Endogenous fibrillarin was detected by indirect immunofluorescence using anti-fibrillarin antibody, 17C12 (Hultman *et al.*, 1994) and Texas Red-labeled secondary antibodies (FIB). The RNAs were observed by fluorescence microscopy. Differential interference contrast (DIC) and fluorescence images (RNA and FIB) are shown for each sample. The DIC panels show 2–5 nucleoli. Bar, 10 μ m. (B) Box H/ACA snoRNAs are not observed in coiled bodies. Nuclear spreads were made 15 min after the injection of fluorescently labeled RNAs. Coiled bodies were stained by indirect immunofluorescence using antibody H1 against p80 coilin (Tuma *et al.*, 1993) and Texas Red-labeled secondary antibodies (COILIN). A single coiled body is present in each panel (indicated by an arrow). The control U3 snoRNA and U1 snRNA localize to coiled bodies as reported previously (Narayanan *et al.*, 1999). DIC and fluorescence images (RNA and COILIN) are shown for each sample.

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

The sequences within Box H/ACA snoRNAs important for their accumulation and function in pseudouridylation have been investigated. These studies have been performed in both yeast and vertebrate systems, where the RNAs are

generated by distinct mechanisms. Vertebrate Box H/ACA snoRNAs (including U17, U64 and U65) are generated by processing from introns, whereas most Box H/ACA RNAs are transcribed independently in yeast (Maxwell and Fournier, 1995; Tollervey and Kiss, 1997). Accumulation of all Box H/ACA snoRNAs examined requires Box H and Box ACA, and the 3'-proximal stem has been demonstrated to be essential for stability in yeast (Balakin *et al.*, 1996; Ganot *et al.*, 1997b; Bortolin *et al.*, 1999).

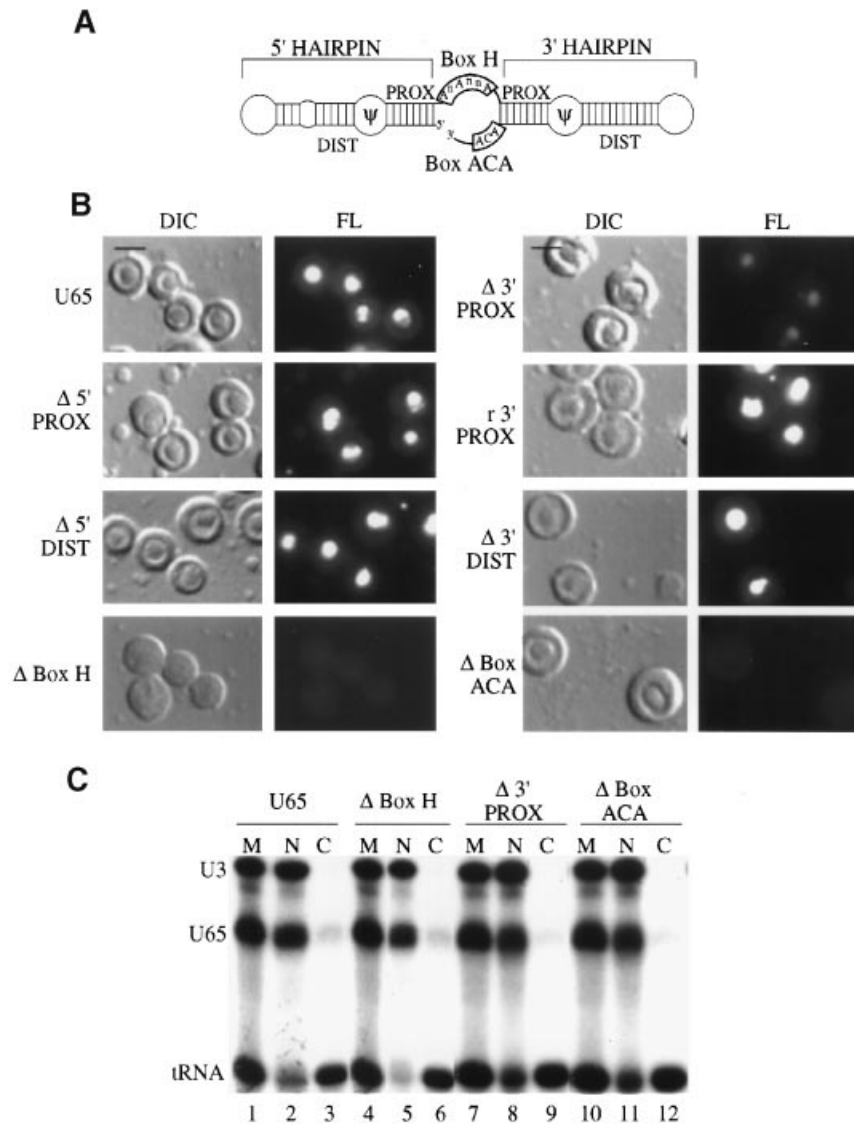


Fig. 3. Identification of *cis*-acting sequences essential for the nucleolar localization of U65 Box H/ACA snoRNA. (A) Schematic representation of the secondary structure of U65 snoRNA denoting the positions of Box H, Box ACA, and also the 5' and 3' hairpins. In addition, the stem region of each hairpin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

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

flank the pseudouridylation loops in the 5' and 3' hairpins (Bortolin *et al.*, 1999).

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

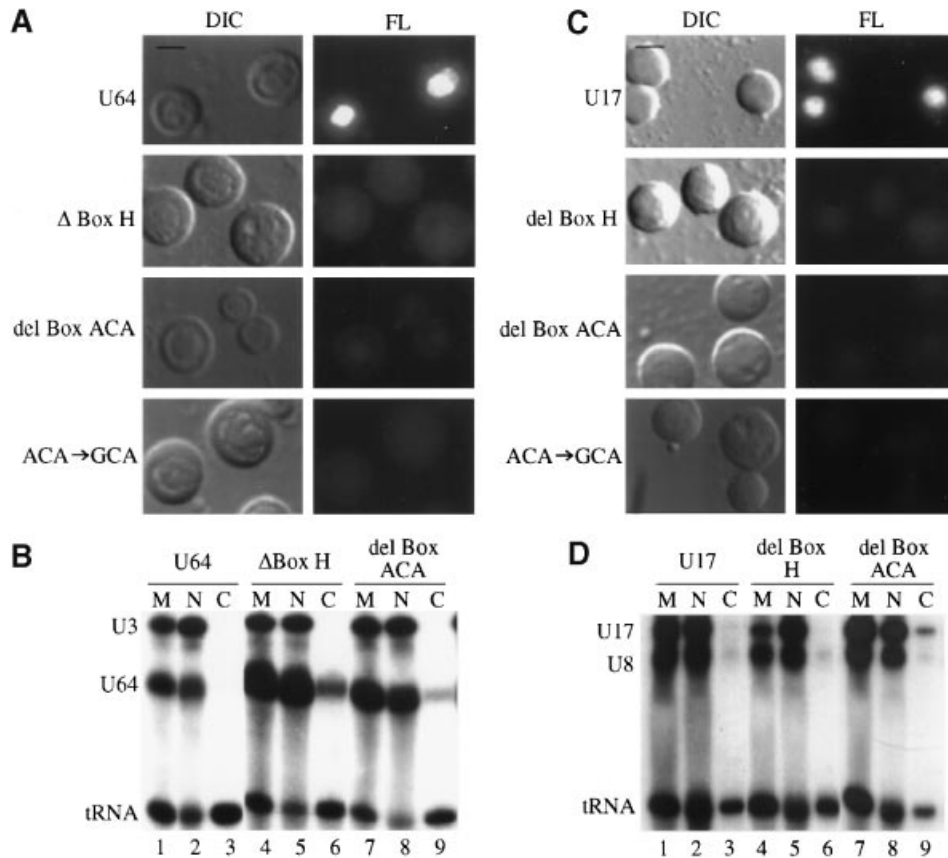


Fig. 4. Box H and Box ACA are required for nucleolar localization of U64 and U17. Variants of U64 and U17 Box H/ACA snoRNAs were injected into *Xenopus* oocyte nuclei. Nuclear spreads were prepared [(A) and (C)] and RNAs were analyzed by gel electrophoresis [(B) and (D)] 1 h after injection. Block substitution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

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

Results

Specific targeting of Box H/ACA snoRNAs to the nucleolus

Previously, we determined the sequences and structures critical for localization of Box C/D family snoRNAs to the nucleolus by injecting fluorescently labeled wild-type and variant RNAs into the nucleus of *Xenopus* oocytes and assessing the RNA localization in nuclear spreads (Narayanan *et al.*, 1999). Similarly, we have analyzed the nucleolar localization of three Box H/ACA family snoRNAs in this work. Fluorescently labeled human U65, U64 and U17 snoRNAs (Figure 1) are present in nucleoli 1 h following injection into *Xenopus* oocytes (Figure 2A). The RNAs co-localize with the nucleolar protein fibrillarin to the fibrillar region of the nucleolus (Figure 2A). The localization of the Box H/ACA RNAs is similar to that

observed with U3 Box C/D snoRNA (Figure 2A). U1 small nuclear RNA was not observed in nucleoli at any time point examined (Figure 2A; 15 min, 4, 8 and 24 h, A.Narayanan, R.Terns and M.Terns, unpublished data). In addition, while we found that Box C/D snoRNAs transiently localize to coiled bodies prior to nucleoli (Narayanan *et al.*, 1999), U65, U64 and U17 do not localize to coiled bodies at an early time point (15 min after injection; Figure 2B), or at any time point examined (including 1, 4 and 8 h after injection; A.Narayanan, R.Terns and M.Terns, unpublished data). Box H/ACA snoRNAs appear to be retained in the nucleus (Figure 3C) like Box C/D snoRNAs (Terns and Dahlberg, 1994; Terns *et al.*, 1995).

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

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

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

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

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

Minimal elements sufficient for localization of RNA to the nucleolus

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

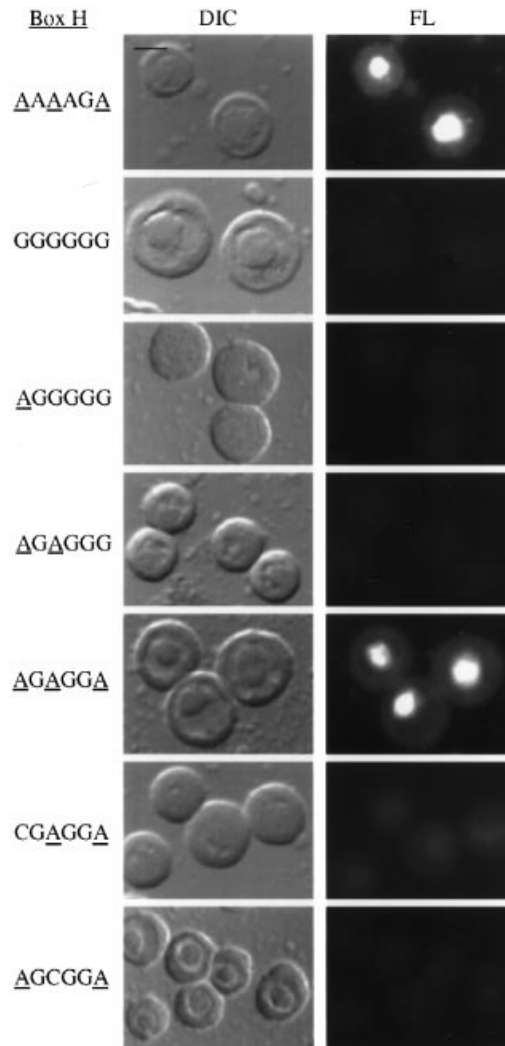


Fig. 5. All three conserved adenine nucleotides of the Box H region of U64 are essential for nucleolar localization. *In vitro* transcribed, fluorescently labeled RNAs were injected into oocytes and nuclear spreads were prepared 1 h after injection and analyzed by fluorescence microscopy. The Box H sequence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

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

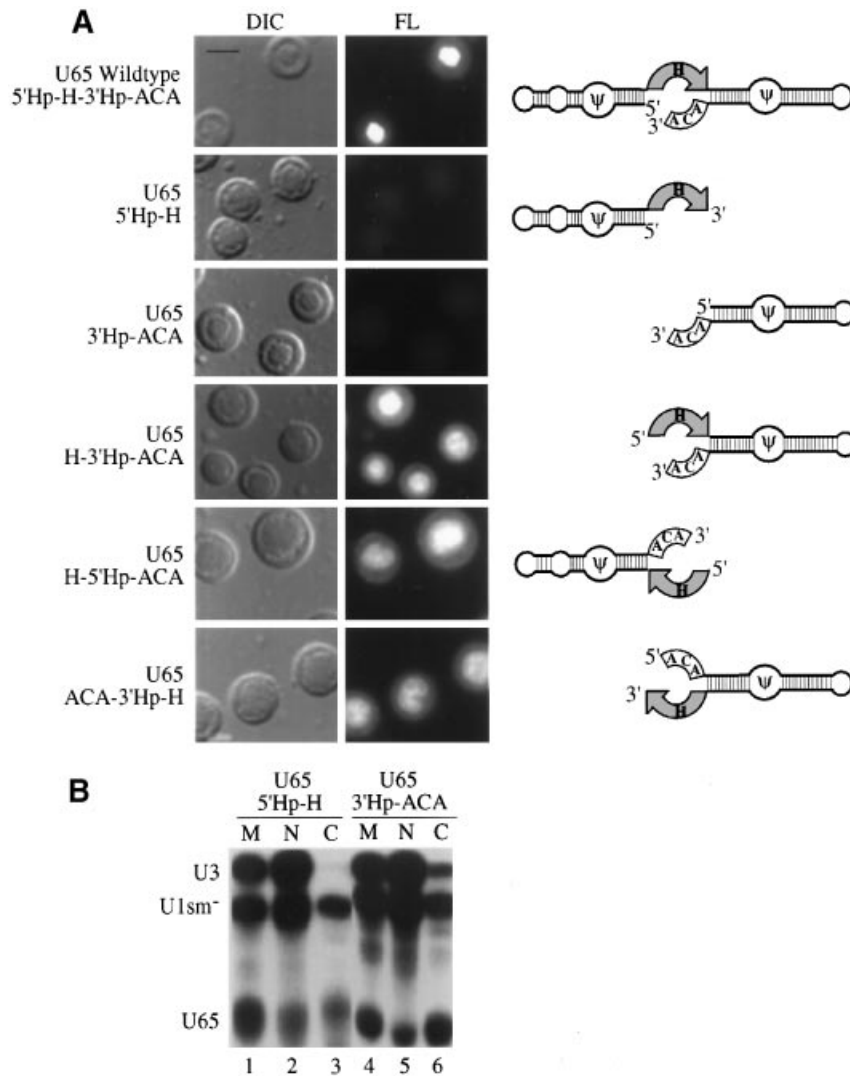


Fig. 6. RNAs consisting of Box H, Box ACA, and either the 5' or the 3' hairpin are sufficient for nucleolar localization. **(A)** Fragments of U65 that contain the 5' hairpin and Box H (5'Hp-H), or the 3' hairpin and Box ACA (3'Hp-ACA), were analyzed. In addition, a larger fragment of U65 consisting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

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

We further tested the requirement for the proximal stem (PROX), pseudouridylation loop (LOOP) and distal stem region (DIST) of the hairpin in the localization of a minimal RNA to the nucleolus. Substitutions were introduced into the fragment of U65 consisting of Box H, the 3' hairpin and Box ACA (H-3'Hp-ACA) to disrupt a stem structure (Δ) and compensatory changes were made to restore the stem (r). Disruption of either the proximal (Δ PROX) or distal (Δ DIST) stem resulted in loss of nucleolar signal (Figure 7A) and RNA stability (Figure

7B). However, RNAs in which the stem structures were restored by substitution of the sequence of the opposite strand of the stem are targeted to the nucleolus (r PROX and r DIST; Figure 7A), indicating that the sequences of the stems are not important for targeting of the fragment. In addition, we introduced substitutions into one strand of the pseudouridylation loop which were expected to result in base-pairing with the opposite strand and loss of the loop structure (Δ LOOP). Although the stability of the Δ LOOP RNA was compromised, sufficient RNA remained in the nucleus (based upon experiments in which we titrated the amount of RNA required in the nucleus to produce a significant nucleolar signal; A.Narayanan, R.Terns and M.Terns, unpublished data), but did not localize to nucleoli (Figure 7A and B). When compensatory mutations were introduced into the sequence of the opposite strand to disrupt base-pairing and restore the

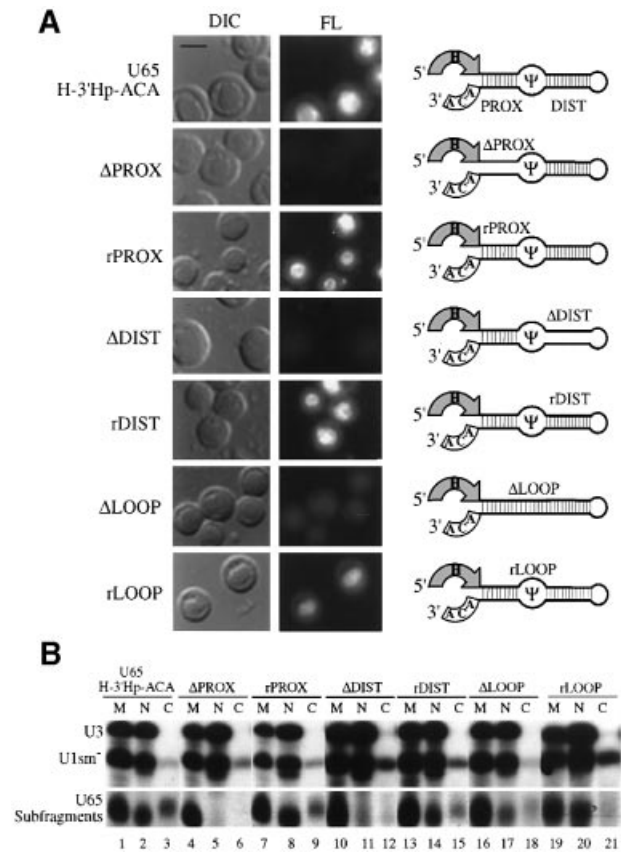


Fig. 7. Essential elements of a fragment of U65 sufficient for nucleolar localization. (A) Substitutions were made in the sequence of a fragment of U65 that localizes to the nucleolus (H-3^Hp-ACA) to disrupt (Δ) and restore (r) the primary substructures of the hairpin: the stem proximal to the Box H/ACA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

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

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

Telomerase RNA functions to maintain telomeres at the ends of chromosomes (Blackburn and Greider, 1995). Potential sequence and structural similarities of telomerase RNA to the Box H/ACA snoRNAs have recently been identified (Mitchell *et al.*, 1999). In addition, a small fraction of telomerase RNA has been reported in nucleoli in steady-state biochemical fractionation studies (Mitchell *et al.*, 1999). We injected fluorescently labeled human telomerase RNA into *Xenopus* oocytes and observed specific localization of the RNA to the fibrillar regions of

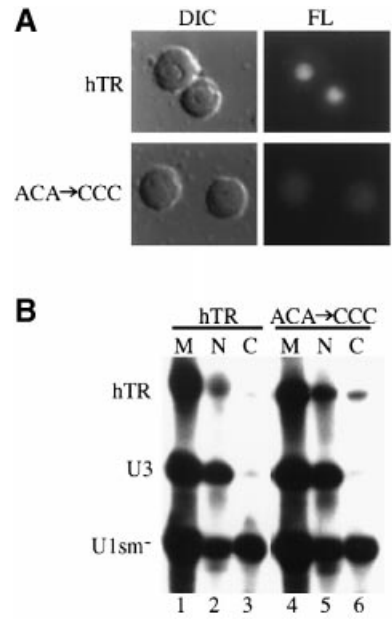


Fig. 8. Localization of telomerase RNA to the nucleolus depends on Box ACA. (A) Fluorescently labeled human telomerase RNA was microinjected into *Xenopus* oocyte nuclei. Wild-type (hTR) and Box ACA mutant (ACA→CCC) telomerase RNAs were injected. Nuclear spreads were prepared 1 h after injection and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

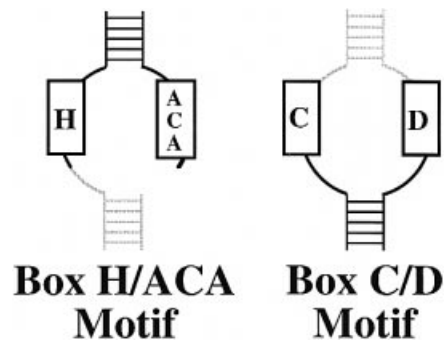


Fig. 9. Sequence/structural elements essential for the nucleolar localization of the two major snoRNA families. In the case of both the Box H/ACA and Box C/D snoRNAs, we have found that a motif comprised of the signature conserved sequence elements (Box C and Box D, or Box H and Box ACA) and an adjacent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

nucleoli (Figure 8A), indicating that telomerase RNA does associate with nucleoli. Furthermore, mutation of the putative Box ACA sequence located three nucleotides from the 3' terminus of the RNA blocked targeting of telomerase RNA to the nucleolus (ACA→CCC; Figure 8A) similar to U65, U64 and U17 Box H/ACA snoRNAs

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

Discussion

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

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

H, Box ACA and a single hairpin still localizes to the nucleolus, it seems unlikely that it could direct rRNA pseudouridylation, because snoRNAs carrying a destroyed distal or proximal 5' stem are already inactive in pseudouridylation guiding (Bortolin *et al.*, 1999).

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

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

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

Materials and methods

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

Oligodeoxynucleotides used in this study are listed in Table I.

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

Table I. Oligodeoxynucleotides used in this study^a

1.	5' <u>TAATACGACTCACTATAGGG</u> TCAGCCACCCGCCACTGC3'
2.	5' <u>TAATACGACTCACTATAGGG</u> TGTCGGTGGCGCCAC3'
3.	5'AGCTGTTCCCATGCTTTCG3'
4.	5'AGCGGGTCCCATGCTTTCG3'
5.	5'AGCTGTTGGGTACGAATCGG3'
6.	5' <u>TAATACGACTCACTATAGGG</u> ATAGTAAACCCAGCTTAGG3'
7.	5' <u>TAATACGACTCACTATAGGG</u> CCCAGCTTAGGAAACAGG3'
8.	5' <u>TAATACGACTCACTATAGGG</u> ATAGTAAACCCAGCCACCCGCCAC3'
9.	5' <u>TAATACGACTCACTATAGGG</u> ACAGCTCCCAGCTTAGGAAACAGG3'
10.	5' <u>TAATACGACTCACTATAGGG</u> ATAGTAAACCCGGTTCGAAAGGAAACAG3'
11.	5' <u>TAATACGACTCACTATAGGG</u> ATAGTAAACCCAGCTTTCCTTACAGGGTTG3'
12.	5'GTTTACTATCAGCTCACCAC3'
13.	5'AGCTGTTTCAGCTCACCCTGG3'
14.	5'GTTTACTATCCCATGCTTTCGGCACAG3'
15.	5'AGCTGTTCCCATGCTTAGGAAACAGAGTC3'
17.	5'GCCTGTTGCACCCCTCAAGG3'
18.	5'GCCTGCACCCCTCAAGG3'
19.	5'TAATACGACTCACTATAGGG3'
20.	5'ATAGAATTTCGTAATACGACTCACTAT3'
21.	5'ATATGCGCATGTGTGAGCCGAGTCCTGGGTG3'
22.	5'ATAAAGCTTGTAAATACGACTCACTAT3'
23.	5'ATATGCGCAGGGGTGAGCCGAGTCCTGGGTG3'

^aT7 promoter sequences are underlined.

Table II. snoRNAs used in this study

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

Plasmids encoding wild-type human U65 (pFL45/SNR/U65/U24) and mutated derivatives of U65 (previously called U65-5'Br, U65-5'Ur, U65-5'Ud, U65-5'Bd, U65-H, U65-3'Br, U65-3'Ur, U65-3'Ud, U65-3'Bd and U65-ACA) have been described previously (Bortolin *et al.*, 1999). Likewise, plasmids encoding wild-type human U64 (pU64) and mutant forms of human U64 (pU64H series and pU64ACA) were reported previously (Ganot *et al.*, 1997b). A plasmid encoding human U17a (pHU17; F.Dragon and W.Filipowicz, unpublished) was used to generate Hinge and ACA mutant constructs. In all cases, block substitution mutations (Δ) indicate that each nucleotide of a conserved Box element or one side of a stem structure was replaced with the complementary nucleotide. To restore (r) the stem structures, the sequences of the opposite side of the mutated stem were changed to their Watson-Crick complement. In a few cases, deletion mutants (del) were analyzed. A recombinant pUC19 plasmid carrying a full-length cDNA of the human telomerase RNA (hTR) (kindly provided by Professor W.Filipowicz,

Friedrich-Miescher Institut, Basel, Switzerland) was used as a template for PCR amplification of cDNAs of the wild-type (oligos 20 and 21) and ACA→CCC mutant (oligos 22 and 23) hTR RNAs. The wild-type and mutant hTR cDNAs were inserted into the *EcoRI-SmaI* or *HindIII-SmaI* sites of pUC19, respectively. After *FspI* digestion, the linear DNAs were used as templates for transcription with T7 RNA polymerase.

In vitro RNA synthesis

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

Injection of RNAs into *Xenopus* oocytes

Detailed protocols for microinjection and micromanipulation of *Xenopus* oocytes have been described previously (Terns and Goldfarb, 1998). Briefly, oocyte clusters were subjected to collagenase treatment (2 mg/ml collagenase in 1× MBSH minus calcium) for 90–120 min and washed well with 1× MBSH buffer. Model PL1-100 picoinjector microinjector (Medical Systems Corporation) and a glass needle with a 10 μm outer-diameter tip were used for microinjections. RNA samples for injection were prepared by drying using a Savant speed vacuum unit and resuspended in a filter-sterilized solution of blue dextran (20 mg/ml, 2 × 10⁶ mol. wt; Sigma) in microinjection buffer (10 mM NaH₂PO₄ pH 7.2, 70 mM KCl, 1 mM MgCl₂). Stage V/VI oocytes were injected with 10 nl of solution containing 1 fmol of each of the test RNAs (fluorescently and ³²P labeled) and ~1 fmol each of the control RNAs (³²P labeled). Injected oocytes were maintained at 18°C in 1× MBSH buffer. Nuclear injections were monitored using the blue dextran in the injected solution, which turns nuclei blue (Jarmolowski *et al.*, 1994). The injected oocytes were then subjected to two different assays: nucleolar localization (described below) and gel electrophoresis (to determine the stability and nucleocytoplasmic distribution of the RNA). Gel electrophoresis of the injected RNA was carried out as described previously (Narayanan *et al.*, 1999). Briefly, RNA was prepared from nuclear (N) and cytoplasmic (C) fractions of injected oocytes, and one

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

Nucleolar localization assay, indirect immunofluorescence and microscopy

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

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

Acknowledgements

We thank Witold Filipowicz for kindly providing unpublished constructs (hTR and U17), and Elisa Izzaualde (tRNA), Brenda Peculis (U8) and Paul Romaniuk (5S) for providing plasmids. Joseph Gall (p80 coilin mAb H1), K.Michael Pollard and Eng Tan (Fibrillarin 17C12) are thanked for providing antibodies used in this study. We are grateful to Claiborne V.C.Glover III for critical reading of this manuscript, Sharon Mattox for technical assistance, and Sharon Mattox, Dave Brown and Syed Ali for assistance in the preparation of figures. B.E.J. has been supported by the French Government and the Hungarian Research Foundation (OTKA, T 029042). This work was supported by a University of Georgia Faculty Research Grant to R.M.T., a grant from la Ligue Nationale Contre le Cancer to T.K., and grants from the National Institute of Health (GM54682) and National Science Foundation (RR61345F) to M.P.T.

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Received June 28, 1999; revised and accepted July 22, 1999