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-pairing 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 snoRNAs 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
Nucleolar localization of Box H/ACA snoRNAs

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

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

one of the two pseudouridylation loops of a Box H/ACA snoRNA and sequences flanking the modification site in rRNA (Ganot et al., 1997a). The pseudouridylation loops are located within the hairpins of Box H/ACA guide RNAs flanked by the proximal and distal stem regions. The sequences within Box H/ACA snoRNAs important for their accumulation and function in pseudouridylation have been investigated. These studies have been performed in both yeast and vertebrate systems, where the RNAs are generated by distinct mechanisms. Vertebrate Box H/ACA snoRNAs (including U17, U64 and U65) are generated by processing from introns, whereas most Box H/ACA RNAs are transcribed independently in yeast (Maxwell and Fournier, 1995; Tollervey and Kiss, 1997). Accumulation of all Box H/ACA snoRNAs examined requires Box H and Box ACA, and the 3’-proximal stem has been demonstrated to be essential for stability in yeast (Balakin et al., 1996; Ganot et al., 1997b; Bortolin et al., 1999).
On the other hand, the 5′ cap is important for the accumulation of independently transcribed Box H/ACA snoRNAs, but not for intron-derived RNAs, which do not contain 5′ caps (Bortolin et al., 1999). The 5′-proximal stem is essential for accumulation of intronic Box H/ACA snoRNAs, but not for capped RNAs (Bortolin et al., 1999). Box H and Box ACA are also essential for generating correctly processed 5′ and 3′ snoRNA termini (Balakin et al., 1996; Bortolin et al., 1999). Finally, the ability of the snoRNAs (including U64 and U65) to function in pseudouridylation depends on the integrity of Box H, Box ACA and each of the four stem regions that flank the pseudouridylation loops in the 5′ and 3′ hairpins (Bortolin et al., 1999).

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

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 pseudouridy-
alation, 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 (ΔH and ΔACA; Figure 3B). Importantly,
each of the variant RNAs that is not observed in nucleoli
is present in the nucleus at the time of analysis 1 h after
injection (Figure 3C). Thus, Box H, Box ACA and the
3′-proximal stem formed by sequences adjacent to Box H
and Box ACA are important for localization of U65
to nucleoli.

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

We tested whether the conserved sequence elements Box
H and Box ACA were also involved in the nucleolar
localization of two additional Box H/ACA snoRNAs. We
found that disruption of either Box element in U64 or in
U17 blocked localization of the RNAs to nucleoli (Figure
4A and C). Again, although the RNAs did not localize to
nucleoli, they were present in nuclei at 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 AnAmA) 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 (AAAAAGA) and each
U64 variant RNA is indicated to the left (Ganot et al., 1997b).
Conserved adenine residues are underlined. DIC indicates differential
interference contrast panels and FL indicates fluorescence panels. Bar,
10 μm.](Image 340x528)

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
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-3Hp-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 accurate 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 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. 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
Although the Box ACA mutant telomerase RNA was not observed in nucleoli 1 h after injection, it was present in the nucleus (Figure 5B). 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).
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Table I. Oligodeoxynucleotides used in this study

<table>
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<th>snoRNA</th>
<th>DNA templates</th>
<th>5' + 3' oligos</th>
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<td>U65 wild-type</td>
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<td>U65-5' Br</td>
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<td>6 + 5</td>
</tr>
<tr>
<td>Δ LOOP</td>
<td>pFL45/SNR/U65/U24</td>
<td>6 + 15</td>
</tr>
<tr>
<td>r LOOP</td>
<td>Δ LOOP</td>
<td>11 + 15</td>
</tr>
<tr>
<td>U64 wild-type</td>
<td>pUC46</td>
<td>19 + 17</td>
</tr>
<tr>
<td>U64 Δ Box H</td>
<td>pUC46H series</td>
<td>19 + 17</td>
</tr>
<tr>
<td>U64 del Box ACA</td>
<td>pUC46ACA</td>
<td>19 + 18</td>
</tr>
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</table>

Plasmids encoding wild-type human U65 (pFL45/SNR/U65/U24) and mutated derivatives of U65 (previously called U66) were used as templates 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–Smal or HindIII–Smal 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 (BoxH), the 5' and 3' subfragments (5'-H and 3'-ACA) of U64 (Pdi), and wild-type and mutant U17 constructs (Nael). All transcriptions of Box H/ACA snoRNAs were driven by a T7 promoter. Xenopus U18, U1 and U1sm−, U3 and rRNA/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 submitted 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 × 104 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

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–Smal or HindIII–Smal sites of pUC19, respectively. After FspI digestion, the linear DNAs were used as templates for transcription with T7 RNA polymerase.

Table II. snoRNAs used in this study

<table>
<thead>
<tr>
<th>snoRNA</th>
<th>DNA templates</th>
<th>5' + 3' oligos</th>
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<tr>
<td>U65 wild-type</td>
<td>pFL45/SNR/U65/U24</td>
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<tr>
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<td>U65-5' Ur</td>
<td>1 + 3</td>
</tr>
<tr>
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<td>U65-5' Ud</td>
<td>1 + 3</td>
</tr>
<tr>
<td>Δ 5' PROX</td>
<td>U65-5' Bd</td>
<td>1 + 3</td>
</tr>
<tr>
<td>r 3' PROX</td>
<td>U65-3' Br</td>
<td>1 + 3</td>
</tr>
<tr>
<td>r 3' DIST</td>
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</tr>
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</tr>
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</tr>
</tbody>
</table>
Nucleolar localization of Box H/ACA snoRNAs

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; Narayanam et al., 1999). Indirect immunofluorescence was performed on fixed nuclear spreads as described previously (Wu and Gall, 1997, Narayanam 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 Immunoresearch Laboratories, Inc.) at 1:150 dilution (in 1× PBS) were used for the detection of the primary antibodies. A Zeiss Axiover 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 Spectroscopic software.

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References


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