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A Common Core RNP Structure Shared between the Small Nucleoar Box C/D RNPs and the Spliceosomal U4 snRNP

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Summary

The box C/D snoRNAs function in directing 2′-O-methylation and/or as chaperones in the processing of ribosomal RNA. We show here that Snu13p (15.5kD in human), a component of the U4/U6.U5 tri-snRNP, is also associated with the box C/D snoRNAs. Indeed, genetic depletion of Snu13p in yeast leads to a major defect in RNA metabolism. The box C/D motif can be folded into a stem-internal loop-stem structure, almost identical to the 15.5kD binding site in the U4 snRNA. Consistent with this, the box C/D motif binds Snu13p/15.5kD in vitro. The similarities in structure and function observed between the U4 snRNP (chaperone for U6) and the box C/D snoRNPs raises the interesting possibility that these particles may have evolved from a common ancestral RNP.

Introduction

Ribosomal RNAs (rRNAs) are synthesized from precursor rRNAs (pre-rRNAs) via a series of complex processing steps in the nucleolus. During these events, many nucleotides of the rRNA undergo covalent, post-transcriptional modification. The nucleolus contains a multitude of small nucleolar RNAs (snoRNAs), several of which are essential for pre-rRNA cleavage events; however, the majority function as sequence-specific guides in the modification of rRNA. Based on conserved sequence motifs, the majority of snoRNAs belong to either the box C/D or the H/ACA snoRNA family (Filipowicz et al., 1999; Venema and Tollervey, 1999; Weinstein and Steitz, 1999).

The box C/D snoRNAs contain two evolutionarily conserved sequence motifs, termed box C (box C′ in U3) and box D, that are essential for snoRNP biogenesis and function. Box C/D snoRNAs often contain a second sequence element homologous, but not identical, to boxes C and D which has been termed the box C′/D′ motif. The box C/D snoRNAs direct the modification of rRNA by base pairing with the 2′-O-methylation site, such that box D or D′ is positioned five base pairs from the target nucleotide (reviewed in Bachellerie and Cavaille, 1997; Filipowicz et al., 1999; Venema and Tollervey, 1999; Weinstein and Steitz, 1999).

Some box C/D snoRNAs, including U3, U8, and U14, contain significant regions complementary to sequences within the rRNA and/or pre-rRNA that are not 2′-*O*-methylated. These snoRNAs have been proposed to function solely as chaperones in rRNA processing steps. Indeed, as the majority of box C/D snoRNAs can form over ten continuous base pairs with the rRNA, it has been proposed that the snoRNAs involved in 2′-O-methylation also assist in the folding of the rRNA (Smith and Steitz, 1997). Comparisons can be drawn between these snoRNAs and the spliceosomal U4 snRNA. Within the U4/U6.U5 tri-snRNP complex, the U4 and U6 snRNAs are base paired. This interaction is disrupted during pre-mRNA splicing prior to step I of catalysis, after which the U4 snRNA is no longer needed. Thus, the U4 snRNA has been proposed to function as an RNA chaperone that delivers the U6 snRNA to the active center of the spliceosome (reviewed in Staley and Guthrie, 1998).

The box C/D snoRNAs are present in the nucleolus as RNP particles (Filipowicz et al., 1999; Venema and Tollervey, 1999; Weinstein and Steitz, 1999). Three proteins common to all box C/D snoRNPs have been thus far characterized, namely fibrillarin (Nop1p in yeast), Nop56p, and Nop58p/Nop5p (reviewed in Venema and Tollervey, 1999). Preliminary data suggest that boxes C and D function as a single protein binding motif; however, there is no direct evidence for this, and the structure of this RNA element has yet to be elucidated (Watkins et al., 1998b). To see whether additional, as yet unidentified, core proteins may mediate binding to the box C/D motif, we decided to isolate the yeast U3 snoRNP. To our surprise, this complex contained Snu13p, a protein component of the U4/U6.U5 tri-snRNP (Gottschalk et al., 1999; Stevens and Abelson, 1999). The human ortholog of Snu13p, 15.5kD, directly binds the 5′ stem-loop of the U4 snRNA (Nottrott et al., 1999). Here we show that Snu13p/15.5kD is associated with the box C/D snoRNAs. We also demonstrate that this protein directly binds the conserved box C/D motif and the U3 specific box B/C motif. Comparison of the three Snu13p/15.5kD binding sites showed that they all form

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an almost identical stem-internal loop-stem structure, suggesting a common mode of recognition by the protein. In addition, these data show that the U4 snRNP and the box C/D snoRNPs form homologous RNP structures, suggesting a common evolutionary origin.

Results

Snu13p, a Spliceosomal Tri-snRNP Protein, Copurifies with the Yeast U3 snoRNP

In order to purify the U3 snoRNP from the other RNPs, a specific affinity tag, the human U1A protein binding site, was inserted into stem-loop 4 of the yeast U3 snoRNA (see Experimental Procedures). The modified U3 snoRNA was then coexpressed together with a gene encoding an HA-tagged fragment of the human U1A protein in a yeast strain where the wild-type U3 gene is under the control of the Gal promoter. These cells grow on glucose-containing media, conditions under which the modified snoRNA is the only source of U3 in the cell. This demonstrates that the tag does not disrupt U3 snoRNP function. The m_3G-capped snoRNPs/snRNPs were first purified using an anti-cap antibody column (Fabrizio et al., 1994) and subsequently passed over an anti-HA-antibody column. The bound material was then eluted using an excess of the HA epitope peptide.

The tagged U3 snoRNA was present in the eluted fraction (Figure 1A; confirmed by Northern blot, data not shown) along with some contaminating U2 snRNA (in a 3:1 ratio). The major protein bands in this eluate were identified by mass spectrometry peptide sequencing (Figure 1B) and the known U3 snoRNP proteins, Nop1p (fibrillarin), Nop56p, Nop58p, and Rrp9p (yeast homolog of the U3–55 kDa protein) (reviewed in Venema and Tollervey, 1999) were detected. This emphasizes that this novel method can be used to isolate RNP complexes and may prove especially useful for RNP complexes in which only the RNA component is known. The additional U3-specific proteins (Mpp10p, Lcp5p, Imp3p, Imp4p, and Sof1p) (reviewed in Venema and Tollervey, 1999) may have been lost during purification or present in substoichiometric amounts in the U3 snoRNP (Figure 1B). Surprisingly, the 13kD protein at the bottom of the gel was identified as Snu13p, a component of the yeast spliceosomal U4/U6.U5 tri-snRNP (Gottschalk et al., 1999; Stevens and Abelson, 1999). This protein is the likely ortholog of the human U4 snRNA binding 15.5kD protein (71% identity, 86% similarity), which was also shown to be present in the human spliceosomal U4/U6.U5 tri-snRNP (Nottrott et al., 1999).

Snu13p and 15.5kD Are Associated with the Box C/D snoRNAs in Yeast and HeLa Cells, Respectively

To investigate whether Snu13p is U3-specific or associated with other box C/D snoRNAs, we determined which RNAs are associated with this protein by immunoprecipitation. To this end, we prepared a yeast strain expressing HA-tagged Snu13p. For comparison, yeast strains expressing either protein A-tagged Nop1p (fibrillarin) or protein A-tagged Gar1p were also used. Immunoprecipitations were performed on whole cell extracts using either anti-HA antibodies or IgG agarose (Figure 2A).

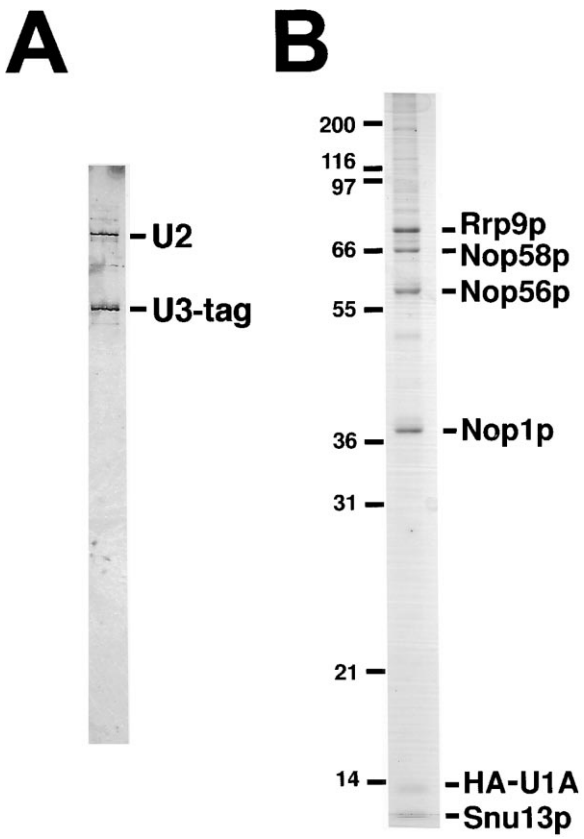

Figure 1. Purification and Characterization of the U3 snoRNP

Tagged U3 snoRNP (containing the U1A binding site) was purified as described in Experimental Procedures.

(A) RNA from the purified fraction was separated on an 8% polyacrylamide/7 M urea gel and visualized by silver staining. The position of the U2 and tagged U3 RNA is indicated on the right.

(B) The proteins present in this fraction were resolved on a 13% SDS polyacrylamide gel and visualized by staining with Coomassie. The identity of each major protein band, indicated on the right, was ascertained by mass spectroscopy peptide analysis. The position of the molecular weight markers (in kDa) is indicated on the left.

Strikingly, a very similar pattern of RNAs was coprecipitated with either tagged Snu13p or tagged Nop1p and no RNA was detected from the wild-type, nontagged strain. The RNAs coprecipitating with Snu13p and Nop1p not only include U3, but also the box C/D snoRNAs snR63, snR190, snR4, snR45, U14, and snR13 (Figure 2A). As Nop1p has been demonstrated to be a component of all box C/D snoRNPs, this implies that Snu13p is also associated with most if not all members of this family of complexes. In contrast, a completely different set of RNAs, which includes snR30, snR37, snR42, snR10, snR11, and snR31, was coprecipitated with H/ACA snoRNP protein Gar1p demonstrating that Snu13p is not a component of the H/ACA snoRNPs. In addition, Northern blot analysis confirmed that approximately 60% of the box C/D snoRNAs (such as U3, U14, U18, and snR190) present in the yeast extract, but not the H/ACA snoRNAs (such as snR30, snR10, and snR42), coprecipitate with HA-tagged Snu13p (data not shown). Two major RNA bands efficiently coprecipitated with Nop1p but not Snu13p (Figure 2A; marked *); how-

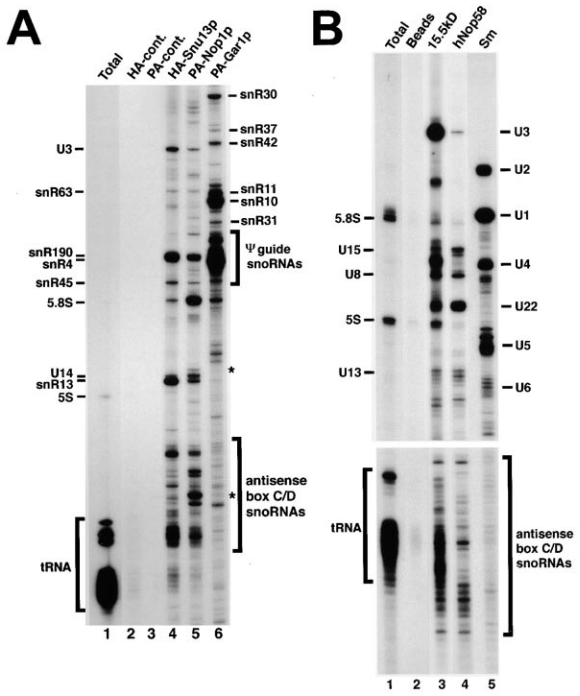

Figure 2. Snu13p and 15.5kD Are Associated with Yeast and HeLa Box C/D snoRNAs, Respectively

(A) Snu13p is associated with yeast box C/D snoRNAs. Whole cell yeast extracts containing either HA-tagged Snu13p (lane 4), protein A-tagged Nop1p (lane 5), protein A-tagged Gar1p (lane 6), or the wild-type, nontagged proteins were prepared and the tagged proteins were precipitated with either anti-HA antibodies or IgG agarose. Coimmunoprecipitated RNAs were recovered, labeled with [^{32}P]pCp, and then separated on an 8% polyacrylamide/7 M urea gel. Lane designations: Total: total RNA from the wild-type, nontagged strain (lane 1); HA-cont.: anti-HA precipitation of wild-type extract (lane 2); PA-cont.: IgG precipitation of wild-type extract (lane 3). The positions of the snoRNAs, ribosomal RNAs, and tRNAs are indicated as described previously (Watkins et al., 1998a). "Antisense box C/D snoRNAs" is used to denote the small box C/D 2′-*O*-methylase guide RNAs that include U18 and U24. The term Ψ guide snoRNAs is used to denote the complex pattern of H/ACA snoRNAs between 215 and 180 nt in length and include snR43, snR44, and snR189.

(B) The human 15.5kD protein is associated with box C/D snoRNAs. HeLa cell nuclear extract was immunoprecipitated using anti-15.5kD antibodies (lane 3), anti-hNop58p antibodies (lane 4), anti-Sm (Y12) antibodies (lane 5), or Protein A Sepharose beads alone (lane 2). Precipitated RNAs or total input RNA (lane 1) were recovered and analyzed as described above. The positions of the snoRNAs, snRNAs, ribosomal RNAs, and tRNAs are indicated in accordance with Lyman et al. (1999). "Antisense box C/D snoRNAs" is used to denote the small box C/D 2′-*O*-methylase guide RNAs that include U18 and U24.

ever, a longer exposure revealed that these RNAs are also coprecipitated with the tagged Snu13p (data not shown). These quantitative differences may reflect a difference in either the expression levels of individual snoRNAs in the two yeast strains, or the accessibility of various epitope tags in different complexes. In summary, our data demonstrate that Snu13p is a novel common core component of box C/D snoRNPs in addition to the previously characterized proteins Nop56p, Nop58p, and Nop1p (fibrillarin).

In view of the evolutionary conservation of Snu13p between yeast and mammals, we next investigated whether the human ortholog of Snu13p, the 15.5kD protein, is also an integral component of the human box C/D snoRNPs using 15.5kD specific antibodies for immunoprecipitation studies (Nottrott et al., 1999). As a comparison, HeLa extracts were also precipitated with anti-hNop58 (core box C/D protein) and anti-Sm (spliceosomal snRNP protein) antibodies. Coprecipitated RNAs were subsequently labeled with [^{32}P]pCp and analyzed on an 8% polyacrylamide/7 M urea gel (Figure 2B). Strikingly, the RNAs that were coprecipitated with hNop58-specific antibodies were also coprecipitated with antibodies specific for the 15.5kD protein (U3, U8, U13, U15, U22, and several RNAs between 60 and 90 nt in length; Figure 2B, lanes 3 and 4). Interestingly, several smaller RNAs were coprecipitated with 15.5kD but not hNop58 antibodies. These RNAs have not yet been identified and will be the subject of further investigations. In comparison, little or no RNA was detected when just protein A Sepharose was used (lane 2). Primer extension analysis of the precipitated RNAs confirmed that the box C/D snoRNAs (e.g., U3, U8, U13, and U15) but not the H/ACA snoRNAs (such as U17 and U64) are specifically coprecipitated with the 15.5kD protein (data not shown). This demonstrates that the 15.5kD protein is specific for the box C/D snoRNPs in both HeLa cells and yeast. Consistent with previous data, the U4/U6.U5 tri-snRNP was not precipitated by the anti-15.5kD antibodies (compare lanes 3 and 5), demonstrating that the 15.5kD protein is not accessible for antibody binding in the intact tri-snRNP (Nottrott et al., 1999). The difference between the anti-hNop58 and the anti-15.5kD antibodies in the precipitation efficiency of several individual snoRNAs (e.g., U3) is likely due to the selective accessibility of certain epitopes in specific snoRNP complexes.

Genetic Depletion of Snu13p Results in a Major Defect in RNA Metabolism

As common core snoRNP proteins are often essential for the stability/biogenesis of snoRNAs and ribosome biogenesis, we investigated whether genetic depletion of Snu13p would affect the steady-state levels of box C/D snoRNAs, H/ACA snoRNAs, ribosomal RNAs, and spliceosomal snRNAs. The growth of a strain containing a conditional allele of *SNU13* (*GAL1* promoter) is galactose-dependent, and the cells do not grow in glucose-containing media (Figure 3A). The growth rate of this strain was normal for the first 8 hr in glucose. Thereafter, there was a decrease, whereas the wild-type strain continued to grow normally in glucose media (Figure 3A). Western blot analysis using anti-15.5kD antibodies, that also recognize the yeast ortholog, revealed that this growth defect follows the depletion of Snu13p from the cell and after 8 hr this protein is no longer detectable (Figure 3B).

The analysis of the RNA extracted at each time point revealed a dramatic reduction in most of the tested cellular RNAs between 8 and 12 hr growth in glucose media (Figure 3C). These RNAs not only include the box C/D snoRNAs, but also the H/ACA snoRNAs, the spliceosomal RNAs U2 and U4 (also U1, U5, and U6; data not shown), and the ribosomal RNAs. We also observed the loss of mRNAs encoding the ribosomal pro-

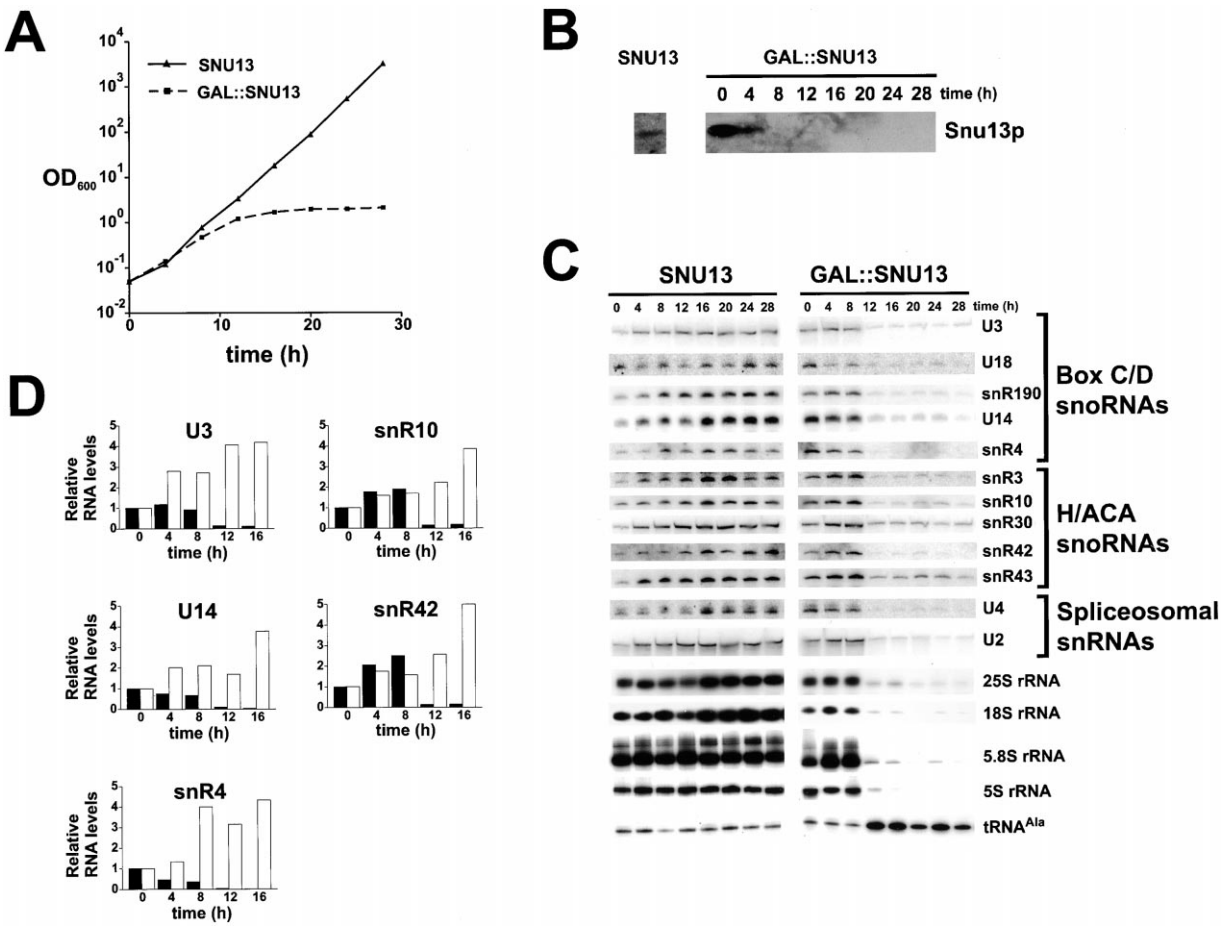

Figure 3. Genetic Depletion of Snu13p Results in a Major Defect in RNA Metabolism

(A) Growth of YGALSNU13 (*GAL::SNU13*) and YPF499 (*SNU13*) following transfer to glucose medium. The OD_{600} of each culture was monitored every 4 hr and plotted as a function of time.

(B) Western blot analysis of Snu13p levels in either the wild-type (*SNU13*) or galactose inducible strain YGALSNU13 (*GAL::SNU13*).

(C) Requirement of Snu13p for RNA metabolism. Total RNA was extracted from an equal number of yeast cells at various time points following the switch to glucose medium. Northern blot analysis was used to determine the relative levels of snoRNAs and snRNAs. The relative levels of $tRNA^{Ala}$ and the ribosomal RNAs were determined by primer extension analysis. The specific probe used is indicated to the right of each individual panel. The yeast strains used along with the respective incubation time are indicated at the top of the figure.

(D) Requirement of Snu13p to maintain box C/D snoRNA levels. The relative amount of several snoRNAs present in both the wild-type (*SNU13;* white bar) and galactose inducible strain (*GAL::SNU13*; black bar) as determined by phosphorimager analysis of the Northern blot, is represented graphically. In each case, the levels are normalized relative to the amount of RNA present at the zero hour time point. The horizontal axis, in each case, denotes time in hours after the shift to glucose containing medium.

teins S17A and S14A (genes *RP51A* and *CRY1*, respectively); however, we did not observe the accumulation of unspliced pre-mRNA (data not shown). The level of $tRNA^{Ala}$ is not depleted during this growth period and in contrast to the other RNAs, it appears to be significantly increased after 12 hr growth in glucose media in comparison to the wild-type strain. It is, however, important to note that the point at which we observe the severe reduction in the bulk of cellular RNA corresponds to the time at which cell growth is inhibited.

Closer examination of the relative levels of individual RNAs revealed that the box C/D snoRNAs are affected prior to the major loss of total RNA but remain unaffected in the wild-type strain (Figure 3C). In the wild-type strain, the relative levels of most of the RNAs tested increases with time, reaching a plateau between 16 and 20 hr (Figures 3C and 3D). In the *GAL::SNU13* strain, the levels of the box C/D snoRNAs remain constant or are reduced during the first 4 hr, and at 8 hr are significantly reduced compared to the wild-type strain (Figure 3D). This inhibition in the biosynthesis of box C/D snoRNAs correlates with the observed depletion of Snu13p and is consistent with this protein being essential for box C/D snoRNA biogenesis (Figure 3B). In contrast, the H/ACA snoRNAs and spliceosomal snRNAs that were tested during this study are unaffected by the loss of Snu13p during this initial incubation (Figures 3C and 3D). Therefore, the depletion of Snu13p results in a specifc reduction, or lack of accumulation, of the box C/D snoRNAs prior to the loss of the bulk of cellular RNA. In conclusion, Snu13p is not only required to maintain the levels of box C/D snoRNAs but also appears to be required to maintain the steady-state levels of the majority of cellular RNAs which results in an inhibition of cell growth.

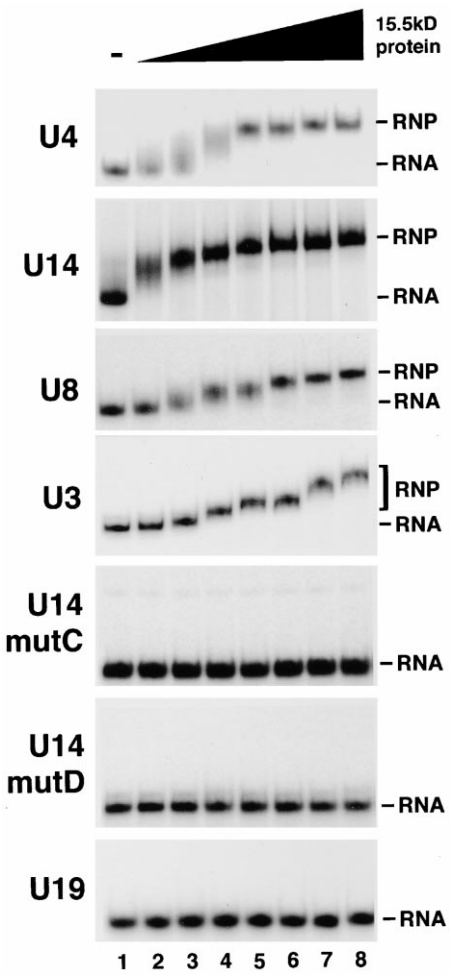

Figure 4. Recombinant Human 15.5kD Protein Directly Binds Box C/D but not H/ACA snoRNAs In Vitro

In vitro transcribed, ^{32}P-labeled snRNA/snoRNAs were incubated with recombinant 15.5kD protein and the resulting complexes resolved on a 6% native polyacrylamide gel. Lane 1: no protein. In lanes 2, 3, 4, 5, 6, 7, and 8 the RNAs were incubated with 15.5kD at a concentration of 8, 16, 32, 64, 128, 256, and 512 nM, respectively. The RNA used is indicated at the left of each panel. The position of 15.5kD-RNA complexes (RNP) and free RNA (RNA) is indicated on the right.

The Human 15.5kD Protein Interacts Directly with Box C/D snoRNAs

The human 15.5kD protein has been recently shown to directly interact with the 5′ stem-loop of the U4 snRNA (Nottrott et al., 1999). We therefore investigated whether the recombinant human 15.5kD protein would bind in vitro transcribed human box C/D snoRNAs in a gel electrophoresis retardation assay. As shown in Figure 4, the recombinant 15.5kD indeed forms a complex with the U3, U8, and U14 box C/D snoRNAs as well as the control U4 snRNA substrate (see also Nottrott et al., 1999) with apparent K_ds of 30, 40, 8, and 20 nM, respectively. Importantly, this protein does not bind to the H/ACA snoRNAs U19, U17, and U64 (Figure 4 and data not shown), demonstrating that the interaction with the box C/D snoRNAs is specific. Interestingly, as the concentration of recombinant 15.5kD in the binding reaction is increased in the presence of the U3 snoRNA, first the normal-shift and then a second "super-shift" was observed with an apparent K_d of 240 nM. This second shift was not observed with U4, U8, or U14 suggesting that the U3 snoRNA contains two 15.5kD protein binding sites.

As the box C/D motif is the only common sequence motif shared between the box C/D snoRNAs, it is the most likely binding site in the snoRNAs for the 15.5kD protein. Indeed, mutation of the highly conserved and essential GA dinucleotide present in either box C (U14mutC) or box D (U14mutD) resulted in an RNA that no longer interacts with the recombinant protein (Figure 4), thus indicating that the 15.5kD protein directly binds the box C/D motif. This then suggests that 15.5kD is the primary box C/D binding protein.

Boxes C and D Can Adopt an Asymmetric Internal-Loop Structure Very Similar to the U4 15.5kD Binding Site that Is Sufficient for Protein Binding

As the 15.5kD protein requires an intact box C/D motif for binding, we decided to investigate whether the box C/D motif could form a structure similar to the 15.5kD binding site in the U4 snRNA 5′ stem-loop. The 15.5kD binding site within the U4 snRNA has recently been shown to be comprised of two base-paired stem structures interrupted by an asymmetric (2 + 5) internal loop (Figure 5A; Nottrott et al., 1999). The GA at positions 6 and 7 and the UGA at nucleotide positions 3–5 of the internal loop of the U4 5′ stem-loop are evolutionarily extremely conserved (Figure 5A) and essential for 15.5kD binding in vitro (Nottrott et al., 1999). In addition, the A at position 1 in the internal loop is highly conserved, while a purine is always present at position 2 (Figure 5A). Finally, while the formation of both stems I and II is essential for protein binding there is no requirement for a particular sequence (Figure 5A; Nottrott et al., 1999).

We discovered that if the 3′ end of box C is base paired with the 5′ end of box D, a stem-internal loop-stem structure can be formed that is virtually identical to that of the U4 snRNA 15.5kD binding site (Figures 5A and 5B). The most striking similarity seen between the U4 5′ stem-loop and the box C/D motif is in the internal loop. In both structures, the short side of the loop is a GA dinucleotide, whereas the long side of the loop has the sequence AAUGA in U4 and NRUGA in the box C/D motifs. The complete box D sequence (CUGA) and the xxGAxGx nucleotides of box C (RUGAUGA) are essential for box C/D motif function (Xia et al., 1997). The conservation and essential nature of these nucleotides is consistent with this proposed box C/D motif structure. The highly conserved and essential GA dinucleotides of boxes C and D are found at positions 4, 5, 6, and 7 in the asymmetric internal loop, positions essential for 15.5kD binding to the U4 5′ stem-loop. The additional essential nucleotides within boxes C and D are involved in the formation of stem II, thus creating the stem-internal loop-stem structure.

Stem II in the box C/D motif is conserved in sequence and consists of three base pairs (Figure 5B). The C-G base pair is highly conserved, and both nucleotides are essential for box C/D function. The U-U non-Watson-

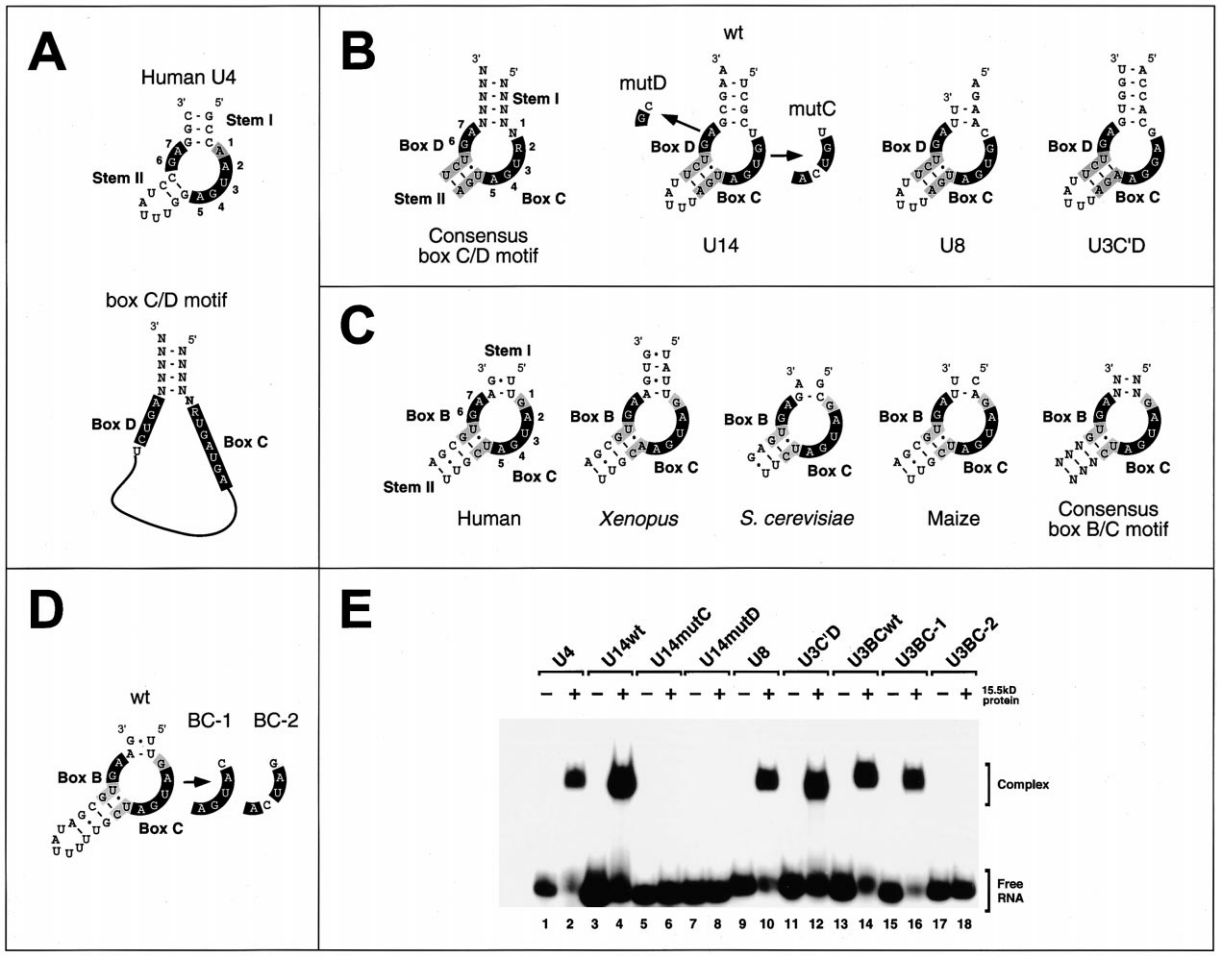

Figure 5. The Box C/D Motif and the Box B/C Motif Can both Form a Structure Very Similar to the U4 5′ Stem-Loop that Are Capable of Binding the 15.5kD Protein

(A) The sequence and structure of the U4 5′ stem-loop and the consensus box C/D motif. The U4 5′ stem-loop necessary for 15.5kD protein binding is represented schematically. The highly conserved nucleotides required for 15.5kD binding to the U4 snRNA are indicated in white on a black background. Gray boxes indicate the remaining conserved nucleotides. The nucleotides within the internal loop and the two stem structures are numbered. The box C/D motif is drawn as predicted from previous publications with the conserved motif nucleotides indicated in white on a black background (reviewed in Weinstein and Steitz, 1999).

(B) The box C/D motif can be folded into a structure similar to the U4 5′ stem-loop. The consensus U14, U8, and U3 box C/D motifs are drawn to demonstrate their structural similarity with the U4 5′ stem-loop. The nucleotides conserved between the U4 and box C/D motif are indicated in white on a black background. Gray boxes indicate the remaining conserved nucleotides of boxes C and D. The loop sequence used to join each of the box C/D motifs is derived from the U4 5′ stem-loop (see above).

(C) The proposed structure of the U3-specific box B/C motif. The box B/C motifs derived from human, *Xenopus*, *S. cerevisiae*, and maize U3 snRNAs are drawn schematically to demonstrate their potential structural similarity with the U4 5′ stem-loop. Also included is the consensus box B/C motif derived from the known U3 snoRNA sequences. The positions of nucleotide boxes B and C are indicated. The nucleotides conserved between the U4 and box B/C motif are indicated in white on a black background. Gray boxes indicate the remaining conserved nucleotides of boxes B and C.

(D) Sequence of the oligos used to investigate the interaction of the 15.5kD protein with the box B/C motif. The RNA sequences are drawn and labeled as described above. The loop nucleotides used to join the box B/C motif are derived from the U4 5′ stem-loop (see above).

(E) Gel mobility shift analysis of the interaction between the 15.5kD protein and the box C/D motif oligos. The RNA oligos with the sequences shown in (B) and (D) were incubated with recombinant 15.5kD protein and the resultant protein-RNA complexes resolved on a 6% native polyacrylamide gel. The presence or absence of the 15.5kD is indicated above each lane by a + or −, respectively. The position of the protein-RNA complex and the free RNA is indicated on the right. The RNA oligo used is indicated above each lane. The U4 5′ stem-loop oligo is included as a comparison (Nottrott et al., 1999).

Crick base pair closing stem II is also highly conserved (Figure 5B). While the U4 snRNA contains a G-C base pair at this position in stem II, the U4 5′ stem-loop still binds the 15.5kD protein when this is replaced by a U-U base pair even though this stem only contains one Watson-Crick base pair (Nottrott et al., 1999). We have also included an additional U at the 5′ end of the box D sequence (UCUGA), to form the final base pair with the last A in box C of stem II; this position is 80% conserved and a base pair is found in 80% of box C/D motifs, however, neither of the two positions are essential for box C/D function (Xia et al., 1997; data not shown).

Oligonucleotides containing the box C/D motif from several mammalian snoRNAs were synthesized to test whether this motif would be sufficient for 15.5kD binding in a gel mobility shift assay (Figure 5B). It was decided to use the loop from the U4 5′ stem-loop, which is not conserved and does not contact the protein (Nottrott et al., 1999), to connect the box C/D sequences. As seen in Figure 5E (lanes 1–4 and 9–12), the recombinant 15.5kD protein binds the box C/D motif oligonucleotide derived from U3, U14, and U8 as efficiently as the U4 5′ stem-loop oligonucleotide.

To demonstrate the specificity of binding to the U14 box C/D motif, oligonucleotides were synthesized with a point mutation in either box C or box D. The nucleotides chosen for mutation were the highly conserved and essential A (position 7) in box D and the 5′-most G (position 4) in box C (Figures 5A and 5B). The mutated U14 oligonucleotides were incapable of interacting with the 15.5kD protein, confirming the specificity of the box C/D interaction (Figure 5E; lanes 5–8). Importantly, mutation of the equivalent nucleotides in the internal loop of the U4 5′ stem-loop also inhibits 15.5kD binding, providing evidence that these motifs form the same stem-internal loop-stem structure (Nottrott et al., 1999). This provides evidence that boxes C and D form a single protein binding motif.

The 15.5kD Protein also Binds the U3-Specific Nucleotide Boxes B and C

From the gel shift data, it appears that the full-length U3 snoRNA has two 15.5kD binding sites (Figure 4B). The U3 snoRNA contains a second proposed protein binding domain that is centered around nucleotide boxes B and C and is thus a potential binding site for the 15.5kD protein (Méreau et al., 1997; Parker and Steitz, 1987; Samarsky and Fournier, 1998). Inspection of the evolutionary conservation and covariation of nucleotides in and around boxes B and C revealed that this motif forms a stem-internal loop-stem structure virtually identical to the box C/D motif and the U4 5′stem-loop (Figure 5C and data not shown). Furthermore, the internal loop nucleotides are almost identical to those present in the box C/D and U4 motifs, with the only variation being the conserved G at position 1 of the internal loop of the box B/C motif. Stem I is generally shorter in this U3-specific motif, and the conserved extension of the box B/C stem II is probably necessary to maintain the conformation of this motif prior to protein binding (Figure 5C).

Oligonucleotides corresponding to the human U3 box B/C motif were synthesized and analyzed for 15.5kD binding (Figure 5D). As with the box C/D motif, the five-nucleotide loop of the U4 RNA was used to join the two RNA strands of this motif. As shown in Figure 5E (lanes 13 and 14), the box B/C motif also binds the 15.5kD protein. Interestingly, the position of the box C sequence within this structural motif is somewhat different from the box C/D motif. In the box C/D motif, the first GA dinucleotide at positions 4 and 5 in the internal loop (RUGAUGA) is essential for protein binding. However, in the box B/C motif, box C is arranged such that the second GA dinucleotide (UGAUGA) is at positions 4 and 5 in the loop. To confirm the specificity of binding to this motif, oligonucleotides were synthesized in which either the G at position 1 or 4 of the internal loop of the U3 box B/C was mutated to C (Figure 5D). Mutation of the G at position 4 in the internal loop inhibited protein binding (Figure 5E; lanes 17 and 18), while the mutation of the G at position 1 in the internal loop had no effect (Figure 5E; lanes 15 and 16) confirming the novel position of box C within this structure. Consistent with this, it has previously been demonstrated that the first GA dinucleotide of box C is not essential for U3 function (Samarsky and Fournier, 1998). Based on these results, we propose that the box B/C motif adopts a structure almost identical to the U4 5′ stem-loop that is also a binding site for Snu13p/15.5kD in vivo.

Discussion

In this manuscript we have shown that the U4/U6.U5 tri-snRNP protein 15.5kD/Snu13p is an integral component of the box C/D snoRNPs in both yeast and man. Consistent with this, we have also shown by immunofluorescence studies that the 15.5kD protein is found in the nucleolus of HeLa cells (data not shown). The box C/D motif can form a structure similar to the previously characterized U4–15.5kD binding site (Figures 5A and 5B) that directly binds the 15.5kD protein. The striking feature of this proposed structure is that the highly conserved and essential nucleotides of the box C/D motif are present in the internal loop and probably directly involved in 15.5kD binding. The conserved nature of the two distinct motifs suggests that Snu13p/15.5kD recognizes each in a similar manner. The fact that both boxes C and D are required to generate this structure is consistent with previous mutagenesis data which suggested that these two sequence elements function as a single RNA motif (Watkins et al., 1998b; Xia et al., 1997).

The box C/D RNP complex also contains Nop56p, Nop58p, and fibrillarin (Nop1p) (see Introduction). Interestingly, a box C/D snoRNP complex, assembled in mouse nuclear extract, has recently been purified and shown to contain three proteins of 65 (Nop56 and Nop58), 55, and 50 kDa (Watkins et al., 1998b). We have shown, by coimmunoprecipitation, that the U14 snoRNP assembled in a HeLa cell nuclear extract contains the 15.5kD protein (data not shown). Of the core box C/D proteins, only Nop58p and Snu13p are essential for the accumulation/stability of all tested box C/D snoRNAs in yeast, suggesting that they form a basic core complex with the snoRNA (this work; Venema and Tollervey, 1999).

During the 2′-*O*-methylation of rRNA, the snoRNA base pairs with the substrate such that the target ribose is positioned 5 base pairs downstream of box D (see Introduction). In this context, our novel structure of the box C/D motif containing Snu13p/15.5kD has several significant implications regarding snoRNP function. The 15.5kD/Snu13p bound to the box C/D motif may function as a binding site for the methyltransferase. This enzyme, which has been proposed to be fibrillarin (Nop1p) (Wang et al., 2000), may interact via protein-protein and/or protein-RNA interactions with this RNP complex to define the target nucleotide for modification. The structure of

the U4 5′ stem-loop RNA/15.5kD complex has recently been solved by X-ray crystallography (I. Vidovic, S. N., K. Hartmuth, R. L., and R. Ficner, unpublished data). Indeed, the U4 snRNA 5′ stem-loop adopts a stem-internal loop-stem in which the internal loop adopts a unique fold. In this structure, the protein primarily recognizes the nucleotides present in the conserved asymmetric internal loop, leaving both flanking stems accessible for potential contacts with other components of the U4/U6 snRNP. By analogy to this crystal structure, we would predict that Snu13p/15.5kD also binds primarily to the internal loop nucleotides of the box C/D motif. Previous mutagenesis data have demonstrated that the sequence of stem II in the U4 snRNA is not essential for the binding of 15.5kD (Nottrott et al., 1999). The fact that the nucleotides forming stem II in the box C/D motif are conserved (Figure 5B) raises the interesting possibility that this stem may play a role in the binding of one or more additional core proteins of the box C/D snoRNP. In this respect, it is interesting to note that recent data have suggested that recombinant *Xenopus* fibrillarin binds weakly to the box C/D motif of the U16 snoRNA (Fatica et al., 2000).

Previous mutagenesis data demonstrated the essential role of the U (U at position 3; Figure 5A) in the internal loop for 15.5kD binding to the U4 RNA 5′ stem-loop. In analyzing the box C/D sequences, we noticed that a small fraction of the box C/D sequences (less than 5%), and in particular the vertebrate U3 box C′ sequences, differ from the consensus and do not contain a U at position 3 in the internal loop (Xia et al., 1997; Figure 5B). Interestingly, the human U3 snoRNA box C′/D motif, which has a G at position 3 in the internal loop, also binds the 15.5kD protein. It is possible that either the loop adopts a slightly different conformation, or that the additional base pair in stem II stabilizes the RNA structure, allowing variations in the internal loop nucleotides of the box C/D motif.

The box C/D snoRNAs often contain a box C′/D′ element, which possesses a similar consensus as the box C/D motif and also directs 2′-*O*-methylation. However, the level of sequence conservation is lower, with the box C′ and D′ sequence elements often containing one or two deviations from the consensus (Kiss-Laszlo et al., 1998). It will be interesting to see whether the snoRNPs contain two sets of each of the core box C/D motif proteins.

We have also shown that the conserved U3-specific box B/C motif binds the 15.5kD protein in vitro and can fold into a similar stem-loop-stem structure, reminiscent of the U4 and box C/D structures. The box B/C motif has been shown to be a second protein binding site in the U3 snoRNP in vivo (Méreau et al., 1997; Parker and Steitz, 1987; Samarsky and Fournier, 1998) that is required for U3–55 kDa and fibrillarin binding (Baserga et al., 1991; Lübben et al., 1993). Recent results demonstrate that the box B/C motif alone can target the RNA to the coiled bodies but not the nucleolus (Narayanan et al., 1999). While this indicates that the protein composition of the box B/C complex is different from the box C/D complex, the exact components associated with this U3-specific motif have yet to be elucidated.

The U4/U6 snRNP is associated with Prp3p, Prp4p (90kD and 60kD in human), and Prp31p (for a recent compilation of U4/U6.U5 tri-snRNP and U4/U6 proteins see Gottschalk et al., 1999; Stevens and Abelson, 1999). These proteins are not found in the free U6 particle suggesting that these proteins are primarily integrated into the U4/U6 snRNP particle via direct or indirect interactions with the U4 snRNA. Prp31p and the box C/D proteins Nop56p and Nop58p are homologous and belong to the Nop5p/Nop58p family of proteins (Gautier et al., 1997). The homology between these proteins is observed in a central conserved region of the polypeptides (amino acids 53 through 342 of Prp31p) in which Prp31p exhibits 47% homology (20% identity) with the corresponding regions of Nop56p and Nop58p. Thus, the similarity between the U4 snRNP and the box C/D snoRNPs even extends beyond the common stem-internal loop-stem RNA structure and the binding of Snu13p/15.5kD.

Snu13p/15.5kD binds three distinct RNA-motifs within two different classes of RNP. This raises the interesting question, how do the functionally distinct RNPs assemble around the individual 15.5kD-RNA complexes? There are differences between the three Snu13p/15.5kD binding sites that are especially apparent in nucleotide 1 of the internal loop and in stem II (Figure 5). However, these are only minor differences, and the binding of additional proteins may be dependent on the nature of the flanking sequences, which are different in each RNA. It will, therefore, be extremely interesting to investigate the structural requirements for the assembly of the additional proteins to their cognate core RNP structures.

In addition to the homology at the structural level, the box C/D snoRNPs and the U4 snRNP also show a remarkable similarity at the functional level. One feature all of the different RNAs that bind Snu13p/15.5kD have in common is that the majority function through an extensive base-pairing interaction ($>$ 10 base pairs) with their substrate RNA. The U4 snRNA is proposed to function as a chaperone for the U6 snRNA in pre-mRNA splicing, while the U3 and U8 snoRNAs function as chaperones to guide the assembly and processing of the ribosome (see Introduction). In addition, the majority of the box C/D snoRNAs base pair with the substrate RNA to direct 2′-*O*-methylation. These methylation guide snoRNAs have also been proposed to function as chaperones via their extensive base-pairing interactions with the pre-rRNA.

The U4 snRNA and the box C/D snoRNPs show significant similarities in structure, composition, and function. Although this could indicate convergent evolution, we favor the notion that they share a common ancestral snRNP. It is likely that this complex functioned as a chaperone in guiding RNA folding and perhaps, in some cases, developed the ability to methylate the substrate RNA. The 5′ stem-loop complex of the U4 snRNP may have evolved from a primitive box C/D snoRNP. Archaea possess box C/D snoRNAs but do not contain pre-mRNA spliceosome components, suggesting that the box C/D snoRNPs existed before the U4 snRNP (Gaspin et al., 2000). Archaea also contain a protein homolog of fibrillarin and a protein homologous to both Nop56p and Nop58p (Lafontaine and Tollervey, 1998). However, the Archaeal protein most homologous to Snu13p/15.5kD is the ribosomal protein L7a (also known as S6) (Kimura et al., 1987). It will be interesting to investigate whether

the Archaeal L7a protein is present in both the ribosome and snoRNPs.

Snu13p/15.5kD is the first protein shown to be a common component of the spliceosome and rRNA processing machinery. Genetic depletion of Snu13p leads first to a reduction in the levels of box C/D snoRNAs that is followed soon after by a drastic reduction in the bulk of cellular RNA. The effect the depletion of Snu13p has on the levels of the bulk of cellular RNAs could result from a block in either RNA processing, transcription, and/or a stimulation of RNA degradation. We are, however, at present unable to distinguish between these possibilities. Our results thus raise the interesting possibility that Snu13p may be involved in the regulation of several RNA metabolic pathways. In summary, the work presented in this manuscript provides an important and exciting new insight into the structure and function of the box C/D snoRNPs and raises several intriguing questions regarding the evolution of snoRNPs as well as the global regulation of RNA processing.

Experimental Procedures

DNA Oligonucleoides

1. ATGGCCGAACCGGATCCGTCGACTAAGGATTGCGG; 2. GATC CACAGCATTGTACCCAGAGTCTGTTCCCAGACATTGCACCT GGCGCTGTA; 3. GATCTACAGCGCCAGGTGCAATGTCTGGGAA CAGACTCTGGGTACAATGCTGTG; 4. CGGGATCCTTAAGCGTAG TCTGGGACGTCGTATGGGTAACCACCCTCCACGAAGGTGCCTTT CAT; 5. GGAAGATCTAAAAGCAGATAAAAATGGCAGTTCCCGA GACCCGC; 6. GGGGGGGGTACCGGCAGCTTCTGAATCACT; 7. GCGCGGGAGCTCCAATAAATCTCCTACGAA; 8. ATAATCCGG AACATCATACGGATAAGCGTAGTCTGGGACGTCGTATGGGTAAA TTAATAAAGTTTCAATCTTGTCCTTGAC; 9. CCGTATGATGTT CCGGATTATGCCATGCCACCCTTATGACGTACCTGACTACGCAT AAGTAGATTGGATTATATCTATCAACATAG; 10. GCAAGGAA CTTGAATAAACCAGATTTCATACTATAAAAACTATATTAAAAGG GCGAATTGGAGCTCCAC; 11. GTTAATGCAGCATCGGCTAAT GGGAAAGCCTTTGGGTTTGGGGCAGACATCGGGGGATCCACT AGTTCTAGA; 12. GCAAGGAACTTGAATAAACCAGATTTCAT ACTATAAAAACTATATTAAAACAGCTGAAGCTTCGTGGATCTG; 13. TAGTATATAGATGGTACAAAAACTATGTTGATAGATAT AATGGAATCTACGCATAGGCCACTAGTGGATCTG; 14. GTG AATGATGAATTTAATTCTTTGGTCC; 15. CGTCAGATACTGTGAT AGTCAACC; 16. TGGACGCAACCGGAATCGAACCG; 17. ACCCACT ACACTACTCGGTCAGGC; 18. CACATTACGTATCGCATTTCG CTGCG; 19. GCTTATACTTAGACATGCATGGC; 20. TCCGGTT GGTTTCTTTTCCTCGGC.

Plasmid Construction

A BamHI site was inserted into the U3 coding sequence (between nucleotides 158 and 159) of M13mp9::T7snR17A using oligo 1 (Ségault et al., 1992). The U1A protein binding site (van Gelder et al., 1993) was then cloned into this BamHI site to produce M13mp9::T7snR17A-bU1A (oligos 2 and 3). The SalI/EcoRI fragment from this plasmid was cloned into pASZ11::pU3A (Méreau et al., 1997) to create pASZ11::U3A-bU1A.

The first 306 base pairs of the human U1A coding sequence (with glycine spacer and HA tag) were PCR amplified from a plasmid containing the complete U1A ORF (kindly provided by A. Bergland), using oligos 4 and 5 and cloned into the BamHI site of p413TEF (*HIS3*, CEN, ARS) to create the p413TEF::U1A-HA (Mumberg et al., 1995).

The *SNU13* gene was amplified from yeast genomic DNA using oligos 6 and 7 and cloned into pRS316 to create pRS316SNU13. Three copies of the HA epitope tag were added to the C terminus of the *SNU13* coding sequence as described previously using oligos 6 and 8, and 7 and 9 (Watkins et al., 1998a).

Yeast Manipulation

Standard methods were used for the growth and maintenance of yeast (Guthrie and Fink, 1991). YSNU13GAL (Mata, trp1-Δ63; his3-Δ200; ura3–52; lys2–801; ade2–101; leu2-Δ1; *HIS3::GAL1::SNU13*) was created by transforming strain YPH499 with a HIS3-GAL1 cassette (oligos 10 and 11). The growth curve of strains YSNU13GAL and YPH499 was performed as and analyzed as described previously (Watkins et al., 1998a) and RNA prepared as described by Wise (1991). Western blot analysis using anti-15.5kD antibodies was performed as described previously (Nottrott et al., 1999). The additional probe for U18 was generated by PCR from yeast genomic DNA using oligos 14 and 15. Primer extension was performed as described previously (Nottrott et al., 1999). Primers 16, 17, 18, 19, and 20 were used to detect $tRNA^{Ala}$, 5S rRNA, 5.8S rRNA, 18S rRNA, and 25S rRNA, respectively.

The *SNU13* null allele was created as described by Wach et al. (1994) using oligos 12 and 13 and yeast strain W303 (Mat α, ade2–1; his3–11; leu2–3; 112 trp1–1; ura3–1; can1–100) to form YΔSNU13. Tetrads derived from this strain showed a 2:2 segregation for growth, demonstrating that Snu13p is essential for viability (data not shown). This strain was transformed with pRS316SNU13HA and sporulated to generate the strain YSNU13HA after tetrad dissection. The yeast strain YNop1-protA, expressing protein A-tagged Nop1p, was obtained by transforming the yeast strain BJ2168 with the plasmid pUN100Nop1-ProtA (Gautier et al., 1997).

Purification of Yeast U3 snoRNP

Yeast strain JH84 (Matα snr17a Gald: *URA*3 snR17b::*LEU*2 his3 ade 2 can1) (Hughes, 1996) was transformed with the plasmids pASZ11::U3A-bU1A and p423TEF::U1A-HA to create the strain YBCVS1. Capped snRNPs were isolated according to Fabrizio et al. (1994) from this yeast strain, passed over a protein A Sepharose anti-HA antibody column, and the tagged U3 snoRNP eluted using 1 mg/ml HA peptide. The RNA was separated on a 10% polyacrylamide/7 M urea gel and individual RNAs visualized by silver staining. The proteins were separated on a 12% SDS polyacrylamide gel and stained with colloidal Coomassie blue (Sigma). Protein bands were excised, in-gel digested with trypsin, and the resulting peptides extracted as described previously (Wilm et al., 1996). For MALDI mass mapping, the thin film technique was applied for target preparation as described previously (Vorm et al., 1994).

Immunoprecipitation of snoRNP Particles and RNA Binding Analysis

Yeast whole cell extracts were prepared from strains YSNU13HA, BJ2168, and YNop1-protA and immunoprecipitated as described previously (Watkins et al., 1998a). Purified HeLa nuclei (Nottrott et al., 1999) were resuspended in NET400 [20 mM HEPES-NaOH (pH 7.9), 400 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 8% glycerol], sonicated three times for 30 s using a Branson microtip at a setting of 1.5, and then centrifuged for 30 min at 16,000 $\times$ g. Anti-Nop58 antibodies were raised and purified according to Lyman et al. (1999). SnRNAs and snoRNAs were in vitro transcribed as described previously (Watkins et al., 1998b). Oligos were obtained from Eurogentech (Belgium). RNA-protein interactions were analyzed by native gel electrophoresis as described previously (Nottrott et al., 1999).

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