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Role of Pre-rRNA Base Pairing and 80S Complex Formation in Subnucleolar Localization of the U3 snoRNP

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In the nucleolus the U3 snoRNA is recruited to the 80S pre-rRNA processing complex in the dense fibrillar component (DFC). The U3 snoRNA is found throughout the nucleolus and has been proposed to move with the preribosomes to the granular component (GC). In contrast, the localization of other RNAs, such as the U8 snoRNA, is restricted to the DFC. Here we show that the incorporation of the U3 snoRNA into the 80S processing complex is not dependent on pre-rRNA base pairing sequences but requires the B/C motif, a U3-specific protein-binding element. We also show that the binding of Mpp10 to the 80S U3 complex is dependent on sequences within the U3 snoRNA that base pair with the pre-rRNA adjacent to the initial cleavage site. Furthermore, mutations that inhibit 80S complex formation and/or the association of Mpp10 result in retention of the U3 snoRNA in the DFC. From this we propose that the GC localization of the U3 snoRNA is a direct result of its active involvement in the initial steps of ribosome biogenesis.

The processing of eukaryotic pre-rRNA involves a series of endo- and exonucleolytic cleavages as well as a significant number of covalent, posttranscriptional modifications. Both the cleavage and modification events require small nucleolar RNAs (snoRNAs). The two major classes of snoRNA function as guide RNAs by base pairing with specific sites of modification in the substrate. The H/ACA snoRNAs function in the site-specific formation of pseudouridine, while the box C/D snoRNAs direct the 2′-O methylation of rRNA and certain snRNAs (reviewed in references 1 and 20). A subset of the box C/D snoRNAs that includes U3, U8, and U14 is essential for pre-rRNA cleavage events (17, 19, 26, 31, 33). These snoRNAs are proposed to function as molecular chaperones that use extensive rRNA complementary regions to orchestrate the folding and cleavage of the precursor transcript.

The U3 snoRNA has two distinct functional domains (Fig. 1A). The 5′ domain contains the sequence elements that are important for base pairing with the pre-rRNA (GAC box, box A, box A′, 5′ hinge, and 3′ hinge) (reviewed in reference 39). Box A base pairs with a region near the 5′ terminus of the 18S rRNA and in doing so regulates the formation of an evolutionarily conserved pseudoknot structure (16, 35). The 5′ hinge and 3′ hinge sequences are complementary to regions of the 5′ external transcribed spacer (5′ ETS) (6). The 3′ hinge sequence has the potential to base pair with the pre-rRNA adjacent to the primary processing site. Interestingly, the 3′ hinge region is more important for pre-rRNA processing in *Xenopus laevis* oocytes (6), while rRNA processing in *Saccharomyces*

cerevisiae is more dependent on the 5′ hinge sequence (3). The 3′ domain of the U3 snoRNA contains the evolutionarily conserved and structurally related box C′/D motif (the C/D motif in other box C/D snoRNAs) and the U3-specific box B/C motif. The box C/D motif is essential for nucleolar localization, RNA stability, and 5′ cap hypermethylation. In contrast, the U3-specific B/C motif is not required for U3 biogenesis but is essential for U3 function (reviewed in reference 39).

The U3 snoRNP is found in both a small 12S monoparticle as well as in a larger, ~80S complex (5, 13, 40). Work in yeast has shown that the U3 monoparticle is comprised of the core box C/D snoRNP proteins Snu13p (15.5K), Nop56p, Nop58p, Nop1p (fibrillarin), and the U3-specific Rrp9p (hU3-55K in human) (45). Numerous U3-associated proteins have been identified, and recent purification of the yeast ~80S small subunit processome (8, 12, 34) characterized a plethora of proteins that are present, along with the U3 snoRNP in a pre-rRNA processing complex. It is believed that many of these proteins associate, either directly or indirectly, with the U3 snoRNP only in the larger pre-rRNA processing complex (5, 13). A model of pre-rRNA processing complex assembly has recently been proposed in which the U3 snoRNP first binds the pre-rRNA and then this event triggers the recruitment of the remaining processing factors (23). However, direct evidence for this model is lacking, and little is known about the recruitment and binding of these proteins to the pre-rRNA processing complex.

The box C′/D motif is essential for the assembly of the core U3 snoRNP complex. Binding of 15.5K to the box C/D (C′/D) motif initiates the hierarchical assembly of NOP56, NOP58, and fibrillarin (44). Complete assembly of the core box C/D snoRNP has been demonstrated to be essential for nucleolar localization (43, 44). It has recently been shown that 15.5K also binds directly to the U3-specific box B/C motif (14, 45). The binding of 15.5K is required, along with specific flanking RNA
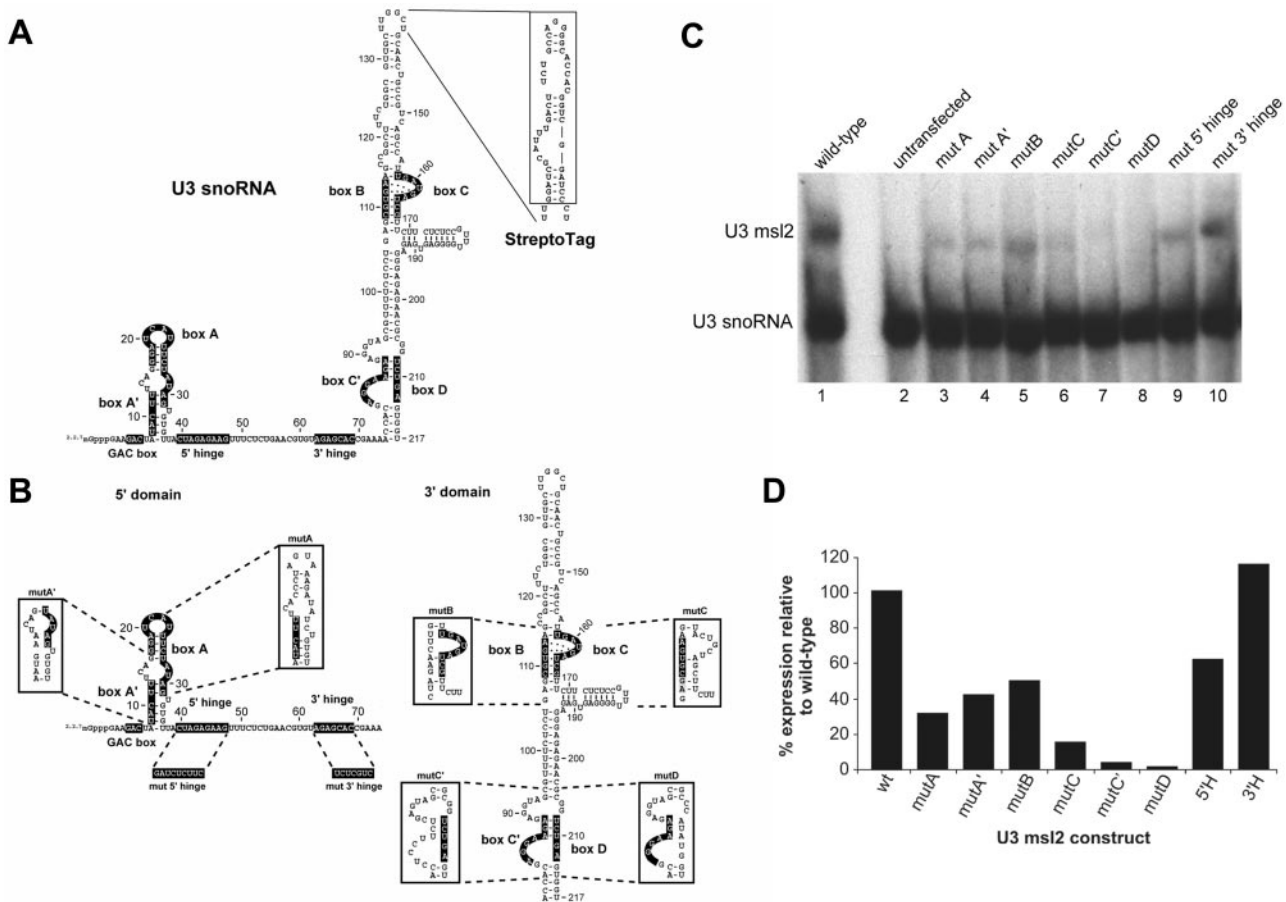
FIG. 1. The box C′/D motif is essential for stable U3 snoRNA production. (A) Proposed secondary structure of the human U3 snoRNA. The secondary structure of the box C′/D and box B/C motifs were drawn as described previously (14). The dotted lines in the C′/D and B/C motifs indicate non-Watson-Crick base pairs. The conserved nucleotides (white on black background) in the box C′/D and box B/C motifs and the GAC, A and A′ boxes, as well as the 5′ and 3′ hinge sequences, are indicated. The proposed secondary structure of the StreptoTag sequence and its location in the U3 msl2 construct are shown. (B) Schematic representation of the mutations introduced into either the 5′ or 3′ domain of the U3 msl2 construct. The sequence and structure of each mutation are indicated in a separate box. The conserved sequence elements are marked as described for panel A. The nucleotide numbering corresponds to the full-length U3 snoRNA. (C) HEp-2 cells were transiently transfected with either wild-type (lane 1) or mutant U3 msl2 constructs (lanes 3 to 10). The cells were then cultured for 16 h. Total RNA was extracted from the cells, separated by denaturing polyacrylamide gel electrophoresis, and analyzed by Northern hybridization by using a U3-specific probe. The U3 msl2 construct used is indicated above each lane. The positions of the endogenous U3 snoRNA and transfected U3 msl2 construct are indicated on the left of the panel. (D) Quantitation of msl2 U3 snoRNA expression levels. For each transfection, the relative amount of plasmid recovered from HeLa cells was determined by Southern blotting by using the StreptoTag probe. In each case, the levels of msl2 U3 snoRNA were normalized relative to the amount of DNA transfected into the cells. The transfected construct is indicated on the horizontal axis and the amount of transcript, relative to the wild type, is indicated on the vertical axis. wt, wild-type U3 msl2; 3′H, U3 msl2 mut 3′ hinge; 5′H, U3 msl2 mut 5′ hinge.

elements, for the association of hU3-55K with the human U3 snoRNA in vitro (14). In addition, sequences in and around the 5′ ETS base pairing region in the yeast U3 snoRNA have been shown to be essential for the recruitment of Mpp10p (48).

In higher eukaryotes, the nucleolus contains three subcompartments, namely, the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). The pre-rRNA processing intermediates migrate through the nucleolar compartments, in vectorial fashion, during ribosome biogenesis. The rDNA is found in the FC, and transcription of the pre-rRNA is proposed to take place on the border between the FC and DFC. The initial precursor is found in the DFC, where the rRNA is proposed to undergo the initial cleavages in the 5′ and 3′ ETS sequences. The later stages of ribosome biogenesis are then proposed to take place in the GC (10, 22).

The U8 box C/D snoRNA, as well as fibrillarin and presumably the majority of box C/D snoRNPs, is found in the DFC, which is consistent with its role in the early stages of pre-rRNA processing (4, 10, 28). However, the U3 snoRNA has been found in both the DFC and GC, consistent with a role in both the early and later stages of ribosome biogenesis (reviewed in reference 10). Indeed, a model has been proposed in which U3 snoRNP cycles between different nucleolar compartments during ribosome biogenesis (10). However, there is as yet no direct evidence supporting this hypothesis.

Although the recent proteomic studies in yeast have largely elucidated the protein composition of the U3-containing complexes, little is known about how the U3 snoRNA is incorporated into preribosomal particles and what directs the localization of U3 to its functional destination(s) within the

nucleolus. Furthermore, the majority of the work analyzing pre-rRNA processing and the U3 snoRNP was performed in yeast, and little is known about the formation of large pre-rRNA processing complexes in mammalian systems. We therefore analyzed conserved sequences in the human U3 snoRNA for their role in core box C/D snoRNP protein binding, the association of U3-specific proteins, and higher-order complex formation, as well as nucleolar and subnucleolar localization. Our results suggest that the box C′/D motif is essential for snoRNA accumulation and core protein binding as well as nucleolar localization. Furthermore, the box B/C motif, which is required for the binding of the U3-specific protein hU3-55K, is also required for the formation of the U3-containing 80S complexes. In addition, we show that 80S complex formation and pre-rRNA base pairing sequences are essential for the GC localization of the U3 snoRNA.

MATERIALS AND METHODS

DNA oligonucleotides. The following numbered oligonucleotides were used: 1, ATGCGAGATCTCTGCAGCTTTGAACTCAG; 2, ATGCGAGATCTCCCG AGATCGCGCCACTG; 3, GGCGTTGCTTGGATCGCATTTGGACTTCTG CCCAGGGTGGCACCACGGTCGGATCCCTGCAACTGCCGTCA; 4, TTA AGACTATACTTTCAGCCTAGTACCATATAGTGTGTTACTAGAG; 5, AGGAGGCCGTTAAGACTAATGAAACAGGGATCATTTCTATAG; 6, TC ATTTCTATAGTGTGTTAGATCTCTTCTTTCTCTGAACGTGTAG; 7, GT TTCTCTGAACGTGTTCTCGTCCGAAAACCACGAGGAAG; 8, AGGTAG CGTTTTCTCCTCTAGAACTTGCCGGCTTTCTGGCGTTG; 9, CAACGCC AGAAAGCCGGCAAGTTCTAGAGGAGAAAACGCTACCT; 10, ACTGCC GTCAGCCATACTGCTAGCTTCTTCTCTCCGTATTGG; 11, CCAATACG GAGAGAAGAAGCTAGCAGTATGGCTGACGGCAGT; 12, AGCACCGA AAACCACGTCCTTCTCGAGTAGCGTTTTCTCCTG; 13, CAGGAGAAAA CGCTACTCGAGAAGGACGTGGTTTTCGGTGCT; 14, AGAGGGAGAG AACGCCCATATGGTGGTTTTTCCTTCATG; 15, CATGAAGGAAAAACC ACCATATGGGCGTTCTCTCCCTCT; 16, Cy3-GCAGGGATCCGACCGTG GTGCCACCCTGGGCAGAAGTCAAATGCGATCCAAGC; 17, FITC-CGG TGCTCTACACGTTCAGAGAAACTTCTCTAGTAACACACTATAGAAAT GATCCCTGAA; 18, C*GCTCTACACGTTCAGAGAAACTTCTCTAGTAA CACACTATAGAAATGATCCC; 19, C*GTTCTAATCTGCCCTCCGGAAG GAGGAAACAGGAAACAGGTAAGGATTAT CCCACCC*; 20, C*GCACC GGGGAGTCGGGACGCTCGGACGCGCGAGAGAACAG CAC*; 21, C*TCC TTCCGAGGCAGAGCGCCTCCGAAGTCAACCCACACATC*. C* denotes 5-allyl-cytidine.

Cloning of the human U3 snoRNA gene and construction of mutants. The HU3-1 DNA sequence (49) was amplified from HeLa genomic DNA by using oligonucleotides 1 and 2 and cloned into pCRII TOPO (Invitrogen). The U3 msl2 construct was generated by the amplification of the U3 coding sequence with oligonucleotides 3 and 4. The PCR product was then used as a megaprimer in a second PCR with oligonucleotide 1. The resulting product was cloned into pCR4 TOPO (Invitrogen). The U3 msl2 mutants in box A (mutA), box A′ (mutA′), the 5′ hinge (mut 5′ hinge), and 3′ hinge (mut 3′ hinge) were generated by PCR by using oligonucleotides 4, 5, 6, and 7, respectively, in combination with oligonucleotide 2. The products were then used in a second-round PCR in combination with oligonucleotide 1, and the resulting products were cloned into pCR4 TOPO. The U3 msl2 mutants in box B (mutB), box C (mutC), box C′ (mutC′), and box D (mutD) were generated by site-directed mutagenesis (QuikChange; Stratagene) by using the oligonucleotide pairs 8 and 9, 10 and 11, 12 and 13, and 14 and 15, respectively. The integrity of all constructs was verified by DNA sequencing.

Immunoprecipitation and glycerol gradient analysis of U3 msl2 complexes. HEp-2 monolayer cells were grown to 70% confluency in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. For immunoprecipitation experiments, 2×10^6 HEp-2 cells were transfected with 20 μg of DNA by electroporation with a Gene Pulser II (Bio-Rad). After 18 h, the cells were disrupted in 500 μl of lysis buffer (25 mM Tris-HCl [pH 7.5], 0.2 mM EDTA, 0.2% Triton X-100 [vol/vol], 150 mM NaCl). Insoluble material was removed by centrifugation, and immunoprecipitation experiments were performed as previously described (13). For glycerol gradient analysis, 5×10^6 cells were transfected with 40 μg of DNA. After 16 h of incubation, cells were disrupted by

sonication in 1 ml of gradient buffer (20 mM HEPES-KOH [pH 7.9], 150 mM NaCl, 0.5 mM dithiothreitol). Triton X-100 was added to 0.2% (vol/vol), and insoluble material was removed by centrifugation. The supernatant was loaded on a 10 to 30% glycerol gradient (vol/vol) (gradient buffer containing 0.2% Triton X-100) and centrifuged in a Th674 rotor (Sorvall) for 15 h at 25,000 rpm. RNA was isolated from the gradient fractions and analyzed by Northern hybridization. The U3 msl2 RNA in the gradient fractions (see Fig. 3) was detected by using a ^{32}P-labeled StreptoTag-specific oligonucleotide.

Fluorescence in situ hybridization (FISH). HeLa monolayer cells were grown to 70% confluency in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. HeLa cells (10^5) were transfected with 100 ng of U3 msl2 DNA by using Lipofectamine 2000 (Invitrogen). The cells were cultured for 16 h and subsequently fixed with phosphate-buffered saline containing 4% paraformaldehyde. In situ hybridization was performed as described previously (38). The U3 msl2 RNAs were visualized by using oligonucleotide 16. Oligonucleotide 17 was used to detect the U3 snoRNA (see Fig. 5). Synthetic DNA oligonucleotides 18, 19, 20, and 21 contain one or more 5-alyl-cytidine groups and were used to detect U3 (see Fig. 4), U8, upstream (US) 5′ ETS, and downstream (DS) 5′ ETS, respectively. The oligonucleotides were coupled to fluorophores containing N-hydroxysuccinimide as described by the manufacturers (Amersham Pharmacia Biotech and Molecular Probes).

RESULTS

In vivo expression of the human U3 snoRNA is dependent on intact boxes C′ and D. The initial goal of this study was to set up an in vivo system to study the role evolutionarily conserved elements in the human U3 snoRNA play in the formation and subcellular localization of the U3 snoRNP in vivo. For this purpose we generated an expression construct that contains the minimal requirements for efficient and accurate expression of the human U3 snoRNA in vivo (see Materials and Methods) (49). In order to be able to distinguish between the RNA derived from our construct and the endogenous U3, we cloned the streptomycin binding aptamer sequence (Strepto-Tag) (2) between nucleotides U135 and C138 of the U3 snoRNA coding sequence (Fig. 1A, U3 msl2). A similar tag sequence was successfully introduced into this region of the yeast U3 without disrupting the in vivo function of the snoRNA (45). HEp-2 cells were transiently transfected with this construct and subsequently cultured for 16 h. RNA prepared from cell extracts was analyzed by Northern blot hybridization by using a U3 snoRNA-specific probe. Figure 1C shows that the U3 msl2 RNA was indeed expressed and could readily be distinguished from the endogenous U3 snoRNA based on its slower migration behavior.

The U3 snoRNA contains a series of evolutionarily conserved sequence elements that are essential for the biogenesis and/or function of this RNA (see introduction). Mutants were therefore generated in the U3 msl2 construct in order to address the role of these conserved motifs in the biogenesis and localization of the human U3 snoRNA (Fig. 1B). In each mutant the complete element was replaced by an unrelated sequence. A similar approach has been used in the analysis of U3 snoRNA in other organisms (30, 32, 36, 37, 42, 48).

We first investigated the effect of these mutations on the expression of the U3 msl2 RNA. HEp-2 cells were transiently transfected with the mutant constructs, and 16 h later total RNA was isolated and analyzed by Northern hybridization. As seen in Fig. 1C, no U3 msl2 RNA was detected in the box C′ and D mutations (lanes 7 and 8). In contrast, detectable levels of U3 msl2 RNA were observed in each of the other mutations. We analyzed the level of plasmid present in the cells by Southern blotting by using a probe specific for the StreptoTag (data

not shown) to compare the transfection efficiency of each construct. Transfection efficiencies varied from 63% (mut 5′ hinge) to 108% (mutB) relative to the wild-type construct. RNA levels were quantitated and normalized to the amount of transfected plasmid to provide accurate analysis of the expression levels of each construct (Fig. 1D). These results are consistent with earlier work in which the box C/D (C′/D in U3) motif was shown to be essential for the stability and accumulation of box C/D snoRNAs (32). However, mutations mutC, mutB, mutA, mutA′, and mut 5′ hinge were all expressed at lower levels than the wild-type RNA, suggesting that other regions within the human U3 snoRNA may also function in the accumulation of the transcript. Interestingly, mutations in the 5′ end of the yeast U3 snoRNA also result in reduced expression levels (48), suggesting that this effect is not specific to the human U3 snoRNA.

Identification of the sequences necessary for the association of the common box C/D proteins and the U3-specific proteins hU3-55K and hMpp10 with the U3 snoRNA. The U3 snoRNP is associated with a diverse number of proteins. Using antibodies specific for the common core box C/D proteins, as well as the U3-specific human (h) proteins hMpp10 and hU3-55K (13, 44, 47), we analyzed the association of these proteins with the wild-type and mutant U3 msl2 RNAs expressed in HEp-2 cells. Transfection experiments were performed as outlined above, and after 16 h of incubation extracts were prepared, immunoprecipitations were performed, and the coprecipitated RNAs were analyzed by Northern blot hybridization (Fig. 2A and B). For quantitation, the ratio of msl2 U3 snoRNA immunoprecipitated with each antiserum (pellet/total) to that of endogenous wild-type U3 immunoprecipitated with the same antibodies from the same extract (pellet/total) was calculated (Fig. 2C).

The wild-type U3 msl2 RNA was coprecipitated by each of the antibodies tested with a similar efficiency to the endogenous U3 snoRNA but, importantly, not when beads alone were used (Fig. 2A and B, lanes 1, and Fig. 2C). Therefore, the introduction of the StreptoTag into the U3 snoRNA has little or no effect on the association of the proteins assayed in these experiments. The association of the common box C/D snoRNP proteins (NOP56, NOP58, and fibrillarin) was not blocked by mutations in the 5′ domain or in the box B/C motif (Fig. 2A and B, lanes 2 to 7, and Fig. 2C). In each case the association of each of these proteins with the msl2 RNAs appears as good as or slightly better than with the endogenous U3 snoRNA. This is in agreement with a model in which the box C′/D motif (C/D motif in other box C/D snoRNPs) is sufficient for the assembly of the core box C/D snoRNP complex. However, one surprise was the reduced (approximately threefold) association of NOP56 with the mutC transcript. Our earlier data predicted that NOP56 did not bind the box B/C motif. While we cannot rule out the possibility that this protein contacts box C, we believe that this mutation may perhaps, either directly or indirectly, destabilize the association of NOP56.

We next asked whether any of the mutations affected the association of the U3-specific proteins hMpp10 and hU3-55K (Fig. 2). Mutation of box C resulted in the loss of detectable binding of hMpp10 as well as hU3-55K, while the box B mutation resulted in the reduced association of these two proteins (Fig. 2A and B, lanes 6 and 7, and Fig. 2C). This finding is

consistent with the proposed role of the box B/C motif in the recruitment of the U3-specific proteins (14). The reduced binding observed for hMpp10 and hU3-55K with mutB may be explained by the fact that this RNA may still be capable of binding the 15.5K protein (see Discussion). Interestingly, the association of hMpp10 with the tagged U3 snoRNAs was severely reduced by the 3′ hinge mutation while significantly lower with mutations in the 5′ hinge and A′ box (Fig. 2B, lanes 3 to 5, and Fig. 2C). The 3′ hinge region, which can potentially base pair with the 5′ ETS, has been demonstrated to be essential for the primary cleavage event in *X. laevis* oocytes (6). Interestingly, this mutation did not significantly affect the association of any of the other tested proteins. Taken together, these data show that the C′/D motif is essential for the recruitment of the core box C/D proteins and that the box B/C motif is essential for U3-specific protein binding, while the hinge region and box A′ are important for the association of hMpp10.

Formation of 80S U3 snoRNP complexes requires the box B/C motif but not the pre-rRNA interaction sequences. The U3 snoRNA was demonstrated, by density gradient centrifugation, to sediment in a 12S monoparticle and in a larger 80S pre-rRNA processing complex (13, 40). We were, therefore, interested in defining the sequence elements within the U3 snoRNA necessary for 80S U3 complex formation. To achieve this, HEp-2 cells were transiently transfected with the U3 msl2 snoRNA wild-type and mutant constructs. After 16 h of incubation, total cell lysates were prepared and fractionated by 10 to 30% glycerol gradient centrifugation. RNA was isolated from the gradient fractions and analyzed by Northern blot hybridization by using either a probe specific to the StreptoTag sequence for the detection of U3 msl2 RNA or a U3 snoRNA-specific probe for the detection of endogenous U3 snoRNA (Fig. 3A). Consistent with the fact that the StreptoTag sequence does not appear to affect the binding of U3-associated proteins, the U3 msl2 RNA efficiently formed the 80S U3 complex. Indeed, the relative amount of 80S complex formed was approximately the same for both the endogenous and the msl2 RNA. We next analyzed the ability of each of the msl2 mutant RNAs to be incorporated into 80S complexes (Fig. 3A). Using the endogenous U3 snoRNP as a comparison, we calculated the relative efficiency of 80S complex formation for each mutant RNA (Fig. 3B). This revealed that mutations in the 5′ domain of the U3 snoRNA did not significantly reduce the formation of 80S U3 complexes. Indeed, the 5′ and 3′ hinge RNAs appeared to be slightly more efficiently incorporated into higher-order complexes than the wild-type U3 msl2 RNA (Fig. 3B). In contrast, mutation of box C severely reduced the association of the U3 msl2 RNA with the 80S complex. In addition, mutB RNA was reproducibly less efficiently associated with 80S complexes than the wild-type U3 msl2 RNA (Fig. 3B). This is consistent with the reduced association of hMpp10 and hU3-55K with the mutB RNA. Therefore, these data show that an intact box B/C motif is necessary for the efficient assembly of the 80S U3 complex. In contrast, individual sequences proposed to base pair with the pre-rRNA are not required for this event.

Correct subnucleolar localization of the U3 snoRNA is dependent on the box B/C motif and sequences that interact with the 5′ ETS of the pre-rRNA. The U3 snoRNA is found in both the DFC and GC in the nucleolus. It was proposed that the U3
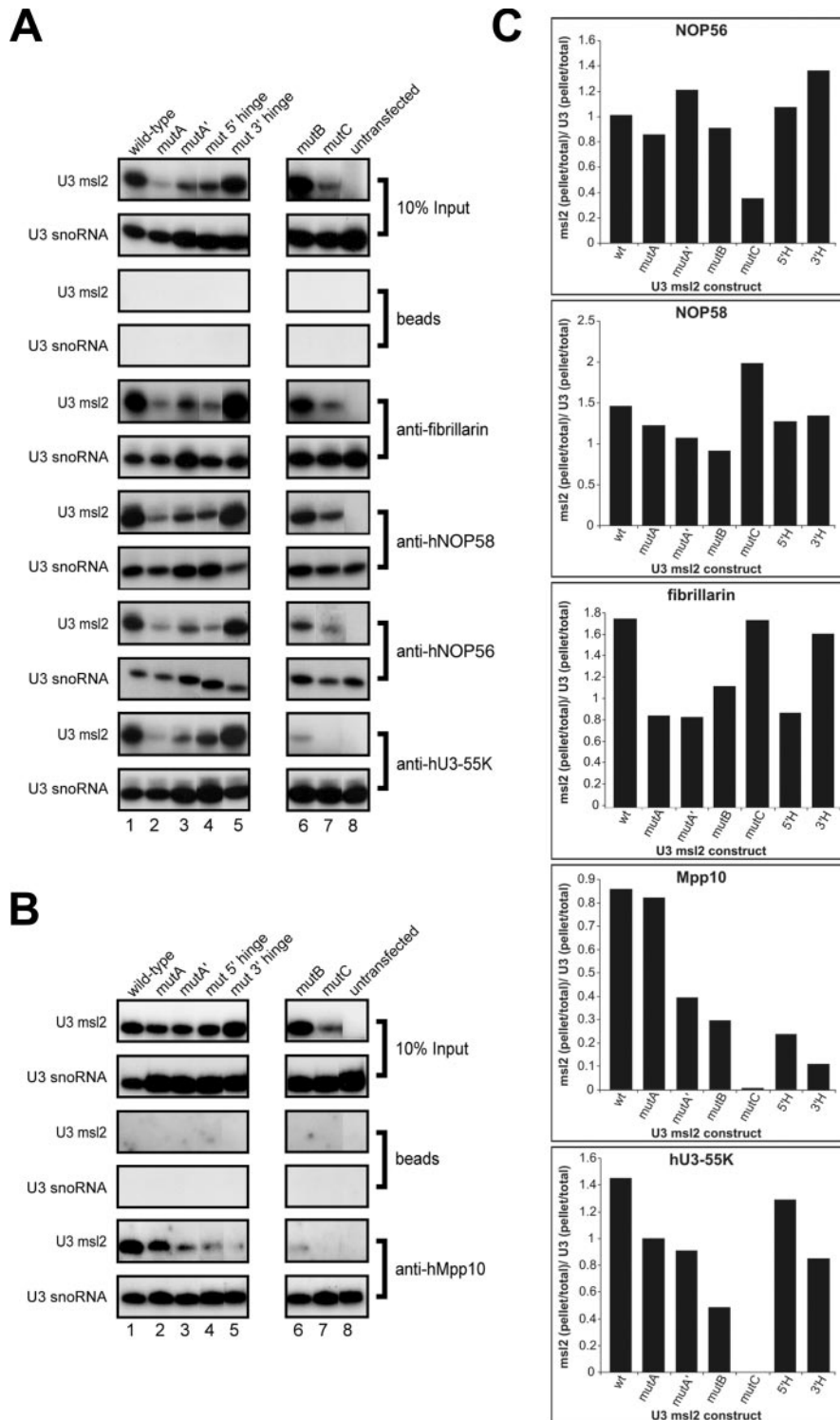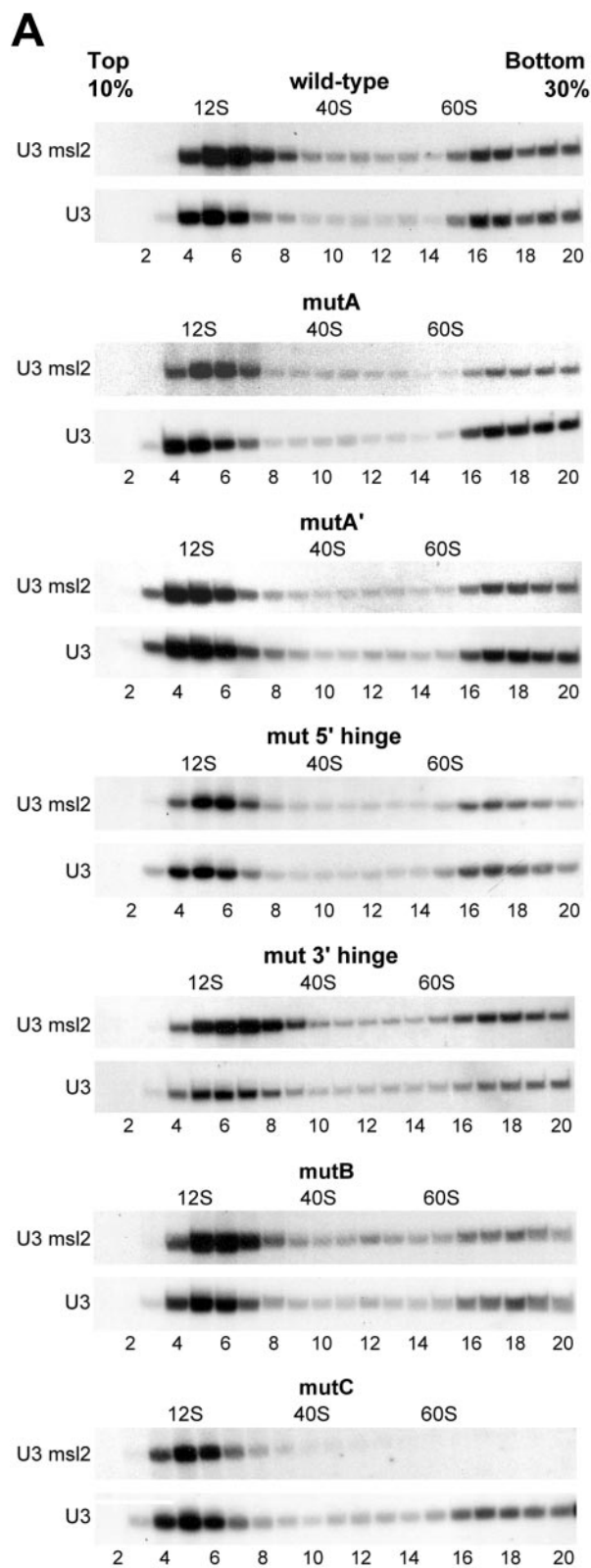
FIG. 2. Association of core box C/D and U3-specific proteins with the mutant U3 msl2 RNAs. (A and B) HEp-2 cells were transiently transfected with either wild-type or mutant U3 msl2 constructs. The snoRNPs were then immunoprecipitated from total cell extracts by using either anti-hU3-55K, antifibrillarin, anti-hNOP56, anti-hNOP58, or anti-hMpp10 antibodies. The copurifying RNAs were analyzed by Northern blot hybridization. The identity of the RNA shown in each panel is indicated on the left. Note that shorter exposures were used for the endogenous U3 snoRNA. The U3 msl2 construct used is indicated above each lane. The antibodies used are indicated on the right. (C) Quantitative analysis of the effects of the mutations on U3 msl2 on protein association. Signal intensities obtained by phosphorimaging were used to calculate the ratio between the relative amount of U3 msl2 and endogenous U3 snoRNA coimmunoprecipitated by each antibody (vertical axis). The transfected construct is indicated on the horizontal axis. wt, wild-type U3 msl2; 3′H, U3 msl2 mut 3′ hinge; 5′H, U3 msl2 mut 5′ hinge; beads, protein A Sepharose beads alone.
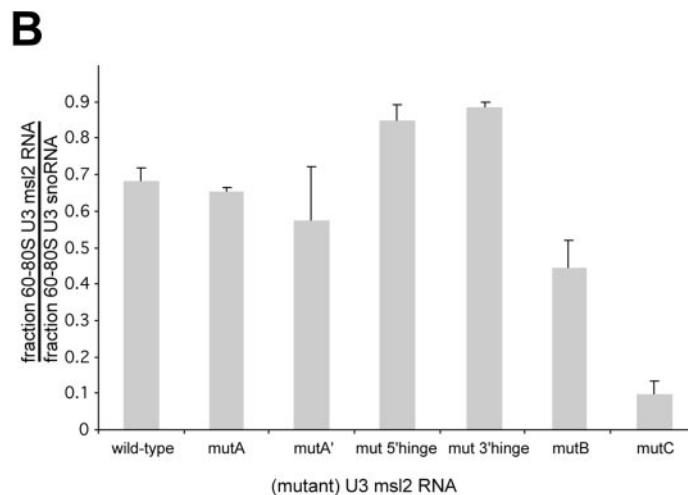
FIG. 3. Effects of the U3 snoRNA mutations on the association with 80S particles. HEp-2 cells were transiently transfected with either wild-type or mutant U3 msl2 constructs. Cell extracts were prepared and separated by glycerol gradient centrifugation. (A) RNA was isolated from each fraction and analyzed by Northern blot hybridization by using either a probe specific for the StreptoTag or a probe specific for the U3 snoRNA. The RNA detected is indicated on the left of each panel. The transfected U3 msl2 construct is indicated above each panel. The peak positions of the 40S and 60S ribosomal subunits as well as of the 12S U1 snRNP, present in the gradient fractions, are indicated at the top of each panel. (B) Quantitative analysis of the effects of the mutations on 80S complex formation. Signal intensities obtained by phosphorimaging were used to calculate the ratio between the relative amount of U3 msl2 and endogenous U3 snoRNA associated with 80S complexes (vertical axis). The transfected construct is indicated on the horizontal axis. The average values derived from two independent experiments are depicted in the graph. Error bars indicate the variation between the two experiments.

snoRNA, which plays an essential role in both the early and later steps of pre-rRNA processing, may move from the DFC to the GC with the pre-rRNA processing complex (10). In contrast, U8 snoRNA is found primarily in the DFC where,

like the majority of the snoRNAs, it participates in the initial rRNA processing steps. While the localization of the U3 and U8 snoRNAs as well as the 5′ ETS sequences has been independently documented, the distribution of these RNAs in the nucleolus has not been directly compared by FISH. Therefore, we first characterized the FISH profiles of the snoRNA and pre-rRNA sequences. A stable HeLa cell line expressing enhanced cyan fluorescent protein (ECFP)-fibrillarin (25) was hybridized with fluorescent probes specific for U3 and U8 snoRNAs (Fig. 4A). Both snoRNAs are almost exclusively found in the nucleolus, and the U8 snoRNA colocalizes with the ECFP-fibrillarin, an established DFC marker (Fig. 4A). In contrast, the U3 snoRNA is dispersed throughout the nucleolus and is abundant in both the DFC and GC.

We next compared the distribution of the U3 snoRNA with the localization of the 5′ ETS sequences. Probes complementary to either the US 5′ ETS or DS 5′ ETS of the primary processing site were used (Fig. 4B). The US 5′ ETS probe, which detects pre-rRNA prior to the primary cleavage event, showed that the primary precursor is restricted to discrete subdomains in the nucleolus, reminiscent of the DFC (Fig. 4C). The DS 5′ ETS probe binds to both the cleaved and uncleaved pre-rRNA. However, as there is significantly more of the pre-rRNA that has undergone the initial cleavage than the initial transcript (15), the signal from the DS 5′ ETS probe
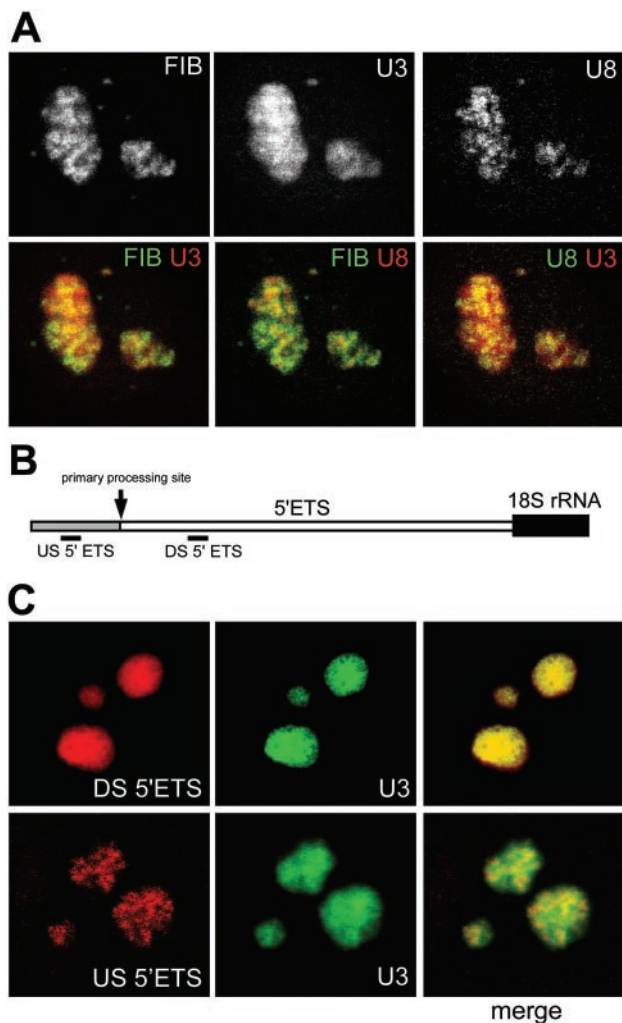
FIG. 4. Subnucleolar localization of the U3 and U8 snoRNAs, snoRNP proteins, and pre-rRNAs. (A) Confocal microscopy was performed by using a stable HeLa cell line expressing ECFP-fibrillarin hybridized with oligonucleotides complementary to U3 and U8 snoRNAs. The localization of fibrillarin and the individual snoRNAs is shown in the upper panels. Pairwise confocal overlays of the three components are shown in the lower panels. The identities of the probes are indicated in each panel. The color of the label represents the pseudocolor of the corresponding image in each overlay. (B) Schematic representation of the 5′ ETS sequence showing the localization of the DS 5′ ETS and US 5′ ETS probes relative to the primary processing site. The mature 18S rRNA is shown in black. (C) Confocal microscopy analysis of HeLa cells hybridized with an oligonucleotide complementary to U3 and an oligonucleotide complementary to either the US 5′ ETS or DS 5′ ETS of the primary cleavage site in the 5′ ETS. Shown are the hybridization patterns of the 5′ ETS probes alone (left), U3 snoRNA localization (middle), and a confocal overlay of both (right).

is likely to represent the distribution of the processed transcript. Strikingly, the signal from the DS 5′ ETS probe was found throughout the nucleolus and perfectly colocalized with the U3 snoRNA (Fig. 4C). The colocalization of the U3 snoRNA with the DS 5′ ETS signal provides further evidence that the U3 snoRNA is indeed involved in pre-rRNA processing in the GC.

We therefore analyzed the importance of the conserved sequence elements within the U3 snoRNA for GC localization.

HeLa cells were transfected with the U3 msl2 wild-type and mutant constructs. After 16 h of incubation, the cells were fixed, and the U3 msl2 RNA was detected by using fluorescent probes specific for the StreptoTag as well as for the endogenous U3 snoRNA. Figure 5A shows that the wild-type U3 msl2 can be readily detected in transfected cells and displays a nucleolar distribution very similar to that observed for the endogenous U3 snoRNA. Importantly, this signal was not observed in nontransfected cells (data not shown). Analysis of the mutant U3 msl2 RNAs revealed that each of the RNAs was found in the nucleolus, which is consistent with the fact that only the box C/D motif (C′/D in U3) is responsible for nucleolar localization. Upon closer examination, we observed that three of the mutations, namely, the mutations in the 3′ hinge, box C, and to a lesser extent box B, had an effect on the subnucleolar localization of the U3 msl2 snoRNA. In each case, the U3 msl2 snoRNA was concentrated in discrete regions of the nucleolus. This finding implies that an intact box B/C motif, and therefore presumably 80S complex formation, is required for correct subnucleolar localization of the U3 snoRNA. Strikingly, the 3′ hinge region, which is required for hMpp10 association and likely important for the interaction with the 5′ ETS, is also essential for the correct localization of U3. The punctate localization pattern seen for mutC and mut 3′ hinge suggests that they are restricted in the DFC of the nucleolus. In order to confirm this, cells transfected with either the mutC or mut 3′ hinge constructs were hybridized with probes specific for the StreptoTag and either U8 snoRNA or US 5′ETS. The mutC and mut 3′ hinge U3 msl2 RNAs, indeed, colocalized with both the U8 snoRNA (Fig. 5B) and US 5′ETS (Fig. 5C). Therefore, these two mutations are found in the DFC. This demonstrates that the box B/C motif and the 3′ hinge region of the U3 snoRNA are essential for GC localization within the nucleolus.

DISCUSSION

In this study we have analyzed the biogenesis and subcellular localization of the human U3 snoRNP in vivo. We have shown that the box C′/D motif is sufficient for the accumulation of the U3 RNA and the assembly of the core box C/D snoRNP complex, as well as the nucleolar localization of the U3 snoRNA in cultivated human cells. The box B/C motif and 3′ hinge, which are U3-specific sequence elements, are primarily responsible for the recruitment of the U3-specific proteins. The box B/C motif, a protein-binding site, is required for the formation of the large 80S U3-associated pre-rRNA processing complex. Furthermore, we have shown that sequences that are predicted to base pair with the 5′ ETS and direct the initial pre-rRNA cleavage event are essential for the correct subnucleolar localization of the U3 snoRNA. This suggests that the GC localization of the U3 snoRNA is dependent on its involvement in rRNA processing.

Assembly of the U3 snoRNP monoparticle and nucleolar localization. It has previously been shown in vitro that the initial binding of 15.5K directs the specific recruitment of NOP56, NOP58, and fibrillarin to the box C/D motif (C′/D motif in U3) and hU3-55K to the box B/C motif (14, 27, 44). The individual sequence elements that make up both the C′/D and B/C motifs are essential for the recruitment of 15.5K as
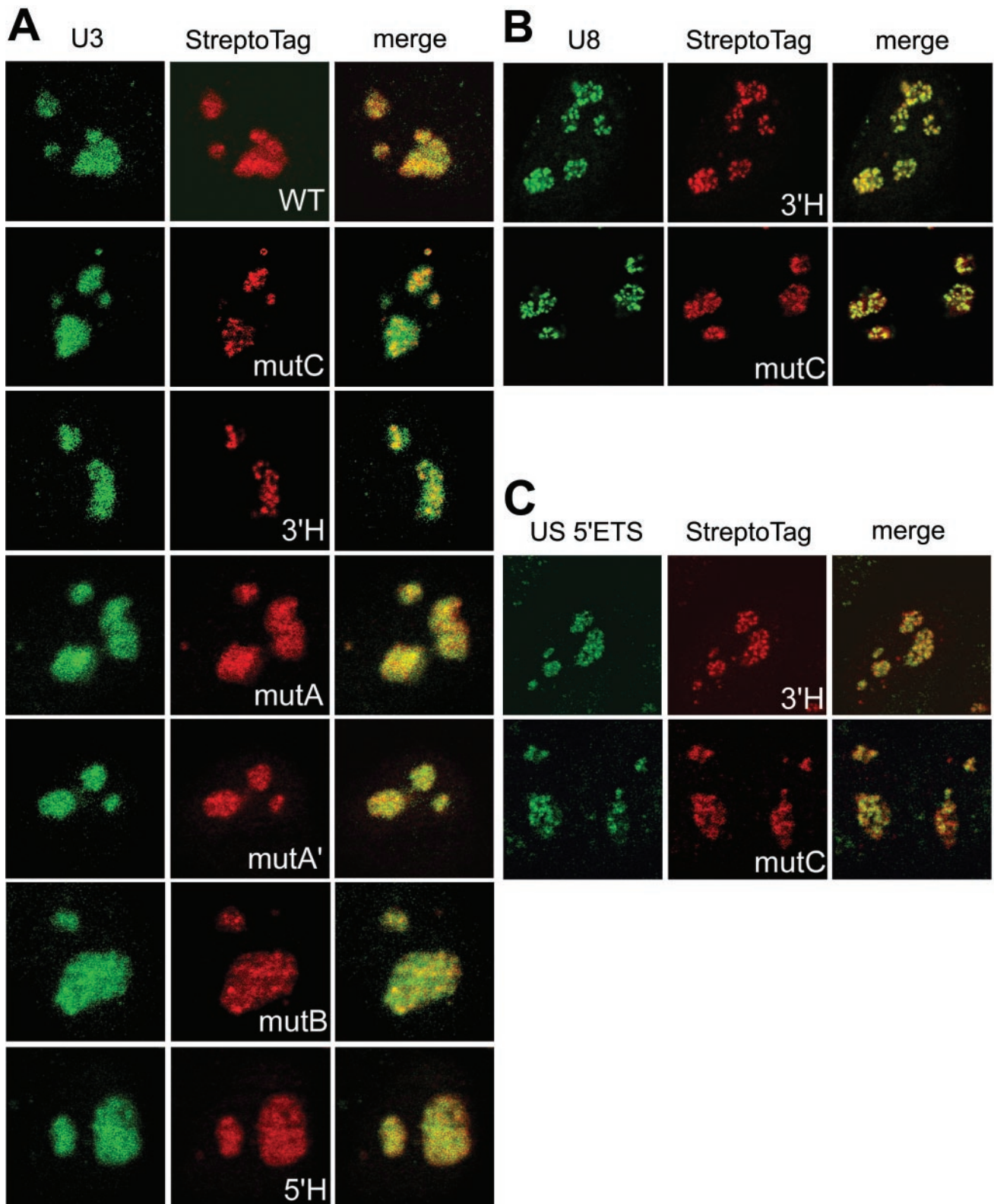
FIG. 5. The box B/C motif and the 3′ hinge sequence are required for the correct subnuclear localization of the U3 snoRNA. (A) Confocal analysis of HeLa cells transfected with either wild-type or mutant U3 msl2 constructs and hybridized with oligonucleotides specific for the StreptoTag sequence and the U3 snoRNA. Shown are the localization of the total U3 snoRNA (left), the StreptoTag staining (middle), and a confocal overlay of both (right). The identity of the U3 msl2 construct is indicated in the middle panels. (B) Fluorescence microscopy of HeLa cells transfected with either mut 3′ hinge or mutC msl2 constructs. Cells were hybridized with oligonucleotides specific for the StreptoTag sequence and the U8 snoRNA. Shown are the localization of the U8 snoRNA (left), the StreptoTag U3 msl2 RNA (middle), and a confocal overlay of both (right). The identity of the U3 msl2 construct is indicated in the middle panel. (C) Fluorescence microscopy of HeLa cells transfected with either mut 3′ hinge or mutC msl2 constructs. Cells were hybridized with oligonucleotides specific for the StreptoTag sequence and the US 5′ETS. Shown are the localization of the US 5′ETS (left), the StreptoTag U3 msl2 RNA (middle), and a confocal overlay of both (right). The identity of the U3 msl2 construct is indicated in the middle panel. WT, wild-type U3 msl2; 3′H, U3 msl2 mut 3′ hinge; 5′H, U3 msl2 mut 5′ hinge.

well as the complex-specific proteins. Work presented in this study is consistent with the view that these two sequence elements direct the assembly of two RNP complexes that are distinct in both protein composition and function. However, mutation of box C resulted in a slightly reduced association of NOP56. While this could indicate that NOP56 binds box C, earlier work indicated that this protein does not bind the box B/C motif, and this mutation may, perhaps through changing the global structure of the U3 snoRNA, indirectly affect protein binding. Box C is essential for hU3-55K binding in vivo; however, mutation of box B only reduced hU3-55K binding. Closer examination of the mutB construct revealed that the mutation unfortunately creates an alternative, potential 15.5K binding site that, while likely quite weak, may support RNP formation. We therefore believe that this result is not contrary to our previous observations.

Mutations in either the B/C motif or the 5′ end of the U3 snoRNA had no effect on the ability of the U3 snoRNA to localize to the nucleolus. Therefore, our data suggest that association of the common box C/D snoRNP proteins with the U3 snoRNA is sufficient for nucleolar localization. Nucleolar targeting may be enhanced by clusters of basic residues in the associated proteins (18, 41). Interestingly, NOP56 and NOP58 contain regions highly enriched in basic amino acids (9) and likely play a role in this process. Unfortunately, due to the fact that mutation of either box C′ or D resulted in the lack of a detectable accumulation of the U3 snoRNA, it is not possible to directly assay the role of these sequence elements in the recruitment of the core proteins and nucleolar localization.

In an earlier study, Narayanan et al. (30) showed that boxes C′ and D are essential for nucleolar localization of U3 snoRNA in *X. laevis* oocytes. In addition, several studies have shown that the C/D motif (C′/D in U3) is essential for nucleolar localization of other box C/D snoRNAs (21, 30, 32, 36, 44). In contrast, Lange et al. (21) reported that box C of the B/C motif and box D of the C′/D (C/D) motif are required for nucleolar localization of the U3 snoRNA in *X. laevis* oocytes. This observation is difficult to explain, as almost all of the data published so far, in both yeast and vertebrate systems, as well as the work described in the present study suggest that the box B/C and C′/D motifs are two separate protein-binding elements. Indeed the C/D (C′/D in U3) motif is the sequence that binds the core box C/D snoRNP proteins, and the formation of the core box C/D complex has been shown to be responsible for nucleolar localization (43, 44).

Assembly of 80S U3 snoRNP complex. The U3 snoRNP is found in both a 12S monoparticle and an 80S particle in extracts derived from HEp-2 cells. The 80S U3 comigrates with pre-rRNA and probably represents a pre-rRNA processing complex (13, 40). In the present study we have provided the first data characterizing the U3 sequence elements required for 80S U3 complex formation. We have shown that the box B/C motif, but none of the individual pre-rRNA and rRNA base pairing sequences, is essential for the formation of the 80S U3 complex. Strikingly, the fact that the box B/C motif is a protein-binding sequence, bound by 15.5K and hU3-55K, suggests that protein-protein interactions are essential for the integration of the 12S monoparticle into the 80S U3 complex. We believe that it is likely that hU3-55K, a WD40 protein, and/or an additional protein(s) interacting with the box B/C motif

provide an essential platform for the assembly of the 80S complex. Our data suggest that individual pre-rRNA base pairing elements are not essential for 80S formation. Furthermore, the formation of this large U3 complex may occur prior to the interaction with the pre-rRNA. However, the interaction between the U3 snoRNP and the pre-rRNA is likely, in part, mediated by protein-protein contacts. Indeed, nucleolin, which binds the 5′ETS, has been shown to interact with the U3 snoRNP and is proposed to function in recruiting the snoRNP to the pre-rRNA processing complex (11). The pre-rRNA and rRNA base pairing sequences in the U3 may serve to help dock the U3 snoRNP onto the pre-rRNA; however, they could also solely function to align the U3 snoRNP and the processing factors with the respective processing sites.

We have also shown that the box B/C motif, along with sequences in the 5′ end of the U3 snoRNA, is required for the stable association of hMpp10. Interestingly, mutation of the hinge region of the yeast U3 snoRNA also inhibited Mpp10p binding in *S. cerevisiae* (46). However, mutations in the yeast U3 box B/C motif only reduced Mpp10 binding. Furthermore, our data also indicate that box A′ may also play a role in hMpp10 association. The 3′ hinge mutation can efficiently form the 80S complex; therefore, the stable association of hMpp10 is not necessary for the formation of the pre-rRNA processing complex. From our mutagenesis studies we cannot distinguish whether hMpp10 binding requires just the pre-rRNA or rRNA base pairing regions in the snoRNA or whether this protein binds once the U3 snoRNP complex has docked correctly onto the pre-rRNA. It has been previously reported that hMpp10 is associated with the two RNA binding proteins hImp3 and hImp4 in yeast and mammals (13, 24). Indeed, it was shown that the association of yeast Mpp10p with the 80S processing complex is dependent on the RNA binding protein Imp3p (46). It is, therefore, interesting to speculate that the association of the hImp3/hMpp10/hImp4 heteromer with the 80S U3 complex may be directed by the binding of one or both of the RNA binding proteins to the intermolecular duplex generated by the base pairing of the 5′ ETS to the U3 snoRNA.

It was recently suggested that the U3 snoRNP first binds the pre-rRNA and that this interaction serves as a platform for the assembly of the large pre-rRNA processing complex (23). Our data clearly show that hMpp10 binding is not required for 80S complex formation and could occur at a later stage in pre-rRNA processing. Furthermore, it would be of interest to correlate the effects of U3 mutations on 80S complex formation and GC localization with the ability of the snoRNA to associate with the pre-rRNA. Unfortunately, this was not possible with our tagged U3 system as the StreptoTag sequence, in the context of the msl2 U3 snoRNA, was not recognized by streptomycin. Therefore, until we can show the role of each mutation on U3 snoRNA–pre-rRNA interaction, we are unable to clarify whether the model proposed by Leary et al. (23) is correct. We are, therefore, in the process of developing such a system with which we can analyze the association of the U3 snoRNA with the pre-rRNA.

Subnucleolar localization of the U3 snoRNA. The direct demonstration of the colocalization of the U3 snoRNA in the GC with the pre-rRNA that has been cleaved at the primary processing site prompted us to determine which U3-specific

sequences are responsible for GC localization. FISH analysis of the U3 msl2 mutants expressed in HeLa cells revealed that the B/C motif and the 3′ hinge region are essential for GC localization. Therefore, the formation of the 80S complex and the potential interaction with the pre-rRNA are essential for the correct localization of U3. Indeed, the sequences required for GC localization are similar to those required for hMpp10 binding. It is not possible to determine whether GC localization is a direct result of hMpp10 binding. Indeed, it is possible that hMpp10, functioning as either a localization or retention factor, is directly responsible for GC localization. However, it is likely that hMpp10 along with hImp3 and/or hImp4 either directly or indirectly recognizes the 5′ ETS-3′ hinge base pairing interaction and that the binding of this protein to the 80S complex is likely required for the initial 5′ ETS cleavage reaction. Consistent with this idea, all sequences that base pair with the 18S rRNA, which we assume in human cells are required for the latter stages of pre-rRNA processing, are not essential for 80S complex formation, hMpp10 binding, and GC localization. However, while we have provided compelling evidence that U3 GC localization occurs as part of the pre-rRNA processing complex, we cannot directly show that the U3 snoRNA found in the GC is still associated with the preribosomes. Our data are consistent with the present view that a single U3 snoRNP interacts with each pre-rRNA and is essential for the primary cleavage as well as the processing at the 5′ end of 18S rRNA (7, 23, 29, 46). However, it is also conceivable that distinct U3 snoRNPs could be required for maturation steps occurring at two distinct locations. We are therefore now focusing on analyzing the association of the pre-rRNA with the U3 snoRNA and the msl2 mutants. In addition, work is at present under way to determine the role of hMpp10, hImp3, and hImp4 in the localization of the U3 snoRNA and the pre-rRNA to the GC.

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