

## The hU3-55K Protein Requires 15.5K Binding to the Box B/C Motif as Well as Flanking RNA Elements for Its Association with the U3 Small Nucleolar RNA *in Vitro*\*

Received for publication, July 3, 2002, and in revised form, October 13, 2002  
Published, JBC Papers in Press, October 14, 2002, DOI 10.1074/jbc.M206631200

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**The 15.5K protein directly binds to the 5' stem-loop of the U4 small nuclear RNA, the small nucleolar (sno) RNA box C/D motif, and the U3 snoRNA-specific box B/C motif. The box B/C motif has also been shown to be essential for the association of the U3 small nucleolar ribonucleoprotein-specific protein hU3-55K. We therefore set out to determine how 15.5K and hU3-55K recognize the box B/C motif. By using an *in vitro* assembly assay, we show that hU3-55K effectively binds a sub-fragment of the U3 snoRNA surrounding the B/C motif that we have named the U3BC RNA. The association of hU3-55K with the U3BC RNA is dependent on the binding of 15.5K to the box B/C motif. The association of hU3-55K with the U3BC RNA was found to be also dependent on a conserved RNA structure that flanks the box B/C motif. Furthermore, we show that hU3-55K, a WD 40 repeat containing protein, directly cross-links to the U3BC RNA. Our data support a new structural model of the box B/C region of the U3 snoRNA in which the box B/C motif is base-paired to form a structure highly similar to that of both the U4 5' stem-loop and the box C/D motif.**

The U3 small nucleolar RNA (snoRNA)<sup>1</sup> is essential for the processing of the small subunit ribosomal RNA (rRNA) in both vertebrates and fungi (1–3). This box C/D-type snoRNA functions by base pairing with the 5'-external transcribed spacer (ETS) of the pre-rRNA and the 5'-terminal region of the 18 S rRNA sequence. The latter interaction blocks the formation of a universally conserved central pseudoknot; a long range interaction essential for the formation and function of the small subunit rRNA (1, 4). Based on these observations, the U3 snoRNP has been proposed to function as an RNA chaperone in the assembly of the small subunit of the ribosome.

The U3 snoRNP contains the core proteins common to all box C/D snoRNPs, namely fibrillarin (Nop1p in yeast), NOP56,

NOP58 (Nop5p), and 15.5K (Snu13p in yeast) (5–12). These proteins are essential for the biogenesis, stability, and nucleolar localization of the box C/D snoRNPs. In addition the U3 snoRNA has been shown to be associated with several U3-specific proteins in yeast, namely Rrp9p (hU3-55K in human), Imp3p, Imp4p, Mpp10p, Dhr1p, Rcl1p, Sof1p, and Lcp5p (13–19, 22). However, the purified yeast U3 snoRNP mono-particle was shown to contain only one U3-specific protein, namely Rrp9p (11), suggesting that many of the other U3 snoRNA-associated proteins may be part of a larger processing complex (13, 15, 20, 21). Genetic studies in yeast have shown that all of the U3 snoRNP-specific proteins are essential for U3 snoRNP function but not for its biogenesis (13–19, 22).

The 3' domain of the U3 snoRNA can be folded into a stem-rich structure that contains four conserved RNA sequence elements, namely boxes C', B, C, and D. Boxes C' and D are found opposite each other at the base of the 3' domain and form the common core box C/D motif (previously termed the box C'/D motif but referred to here as the box C/D motif for clarity) (23–25). The box C/D motif is required for the biogenesis of the box C/D snoRNAs and has been shown to be essential for nucleolar localization, cap hypermethylation, and accumulation/stability of the transcript (reviewed in Ref. 26). This motif is also responsible for the association of the core box C/D proteins (8, 11, 27–30). The U3 snoRNA also contains a U3-specific RNA element, termed the box B/C motif. This RNA element is a protein-binding site that is essential for the association of Rrp9p in yeast and U3-55K in vertebrates (19, 31). However, unlike the box C/D motif, the box B/C motif is not essential for biogenesis of the U3 snoRNA (32, 33).

The 15.5K protein was initially identified as a U4/U6.U5 tri-snRNP component that directly binds to the 5' stem-loop structure of the U4 snRNA (34). We have shown recently (11) that the 15.5K protein is associated with the box C/D snoRNAs *in vivo* and that it directly binds to both the box C/D motif and the U3 snoRNA-specific box B/C motif *in vitro*. Both the box C/D and the box B/C motifs are capable of forming stem-internal loop-stem structures that are strikingly similar to the U4 5' stem-loop. The discovery that 15.5K can bind three similar RNA motifs raises the interesting question of how 15.5K functions in the assembly of these distinct RNP complexes. In this study we have analyzed the binding of 15.5K and hU3-55K to the box B/C motif. These two proteins have been shown to require the B/C motif in the U3 snoRNA for their association (11, 19, 31). To determine how these two proteins associate with the U3 snoRNA, we made use of a HeLa nuclear extract assembly system. Our results show that the interaction between hU3-55K and the U3 snoRNA is dependent on 15.5K binding to the box B/C motif. Moreover, we show that hU3-55K

\* This work was supported by Deutsche Forschungsgemeinschaft Grants Lu294/12-1 and Fonds der Chemischen Industrie (to R. L.), the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (NWO-CW) Grant 97-033 (to W.J.v.V.), and by an EMBO Short Term Fellowship ASTF 9781 (to S. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: snoRNA, small nucleolar RNA; snoRNP, small nucleolar ribonucleoprotein; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein; GST, glutathione S-transferase.

requires not only the box B/C motif but also sequences flanking this RNA element for its association with the U3 snoRNA. Our data suggest that hU3-55K recognizes structural, rather than sequence-specific, RNA elements within the 15.5K-bound U3 box B/C snoRNA complex.

#### EXPERIMENTAL PROCEDURES

**Generation of U3 snoRNA Constructs**—The U3 construct pSP6U3 (28) was kindly provided by Stuart Maxwell. Constructs encoding human U3 snoRNA mutants were generated by PCR using pSP6U3 as a substrate, with the oligonucleotides listed in Table I. The PCR products, which contain a T7 promoter upstream of the U3 sequence, were cloned into the *EcoRI* and *BamHI* sites of pUC19. Primer sets for each mutant were as follows: U3BC, oligonucleotides 2 and 3; U3BC $\Delta$ stem V, 2 and 6; U3BC $\Delta$ stem IV+V, 7 and 8; U3BC $\Delta$ stem VI, 2 and 9; U3BC $\Delta$ stem I+VI, 10 and 11; U3BCmutB, 3 and 4; U3BCmutC, 2 and 5; U3BCstemC, 24 and 3; U3BCstemG, 2 and 25; U3BCstemCG, 24 and 25; U3BCstemCCG, 26 and 3; U3BCstemCGG, 2 and 27; U3BCstemCCGCGG, 26 and 27; U3BCmutUU-GC, 22 and 23; U3BCmutloopA-GG, 2 and 28. The U3 snoRNA mutants U3BCmutstem III and U3BCmutAU-AA were generated using the pUC19-U3BC $\Delta$ stem VI construct as template with oligonucleotides 14 and 15, and 1 and 12, respectively. U3BCmutGG-AA and U3BCmutloop-stem were generated via a two-step PCR method using pUC19-U3BC $\Delta$ stem VI as template. In the first round of PCR, oligonucleotides 16 and 19 and 13 and 19 were used for U3BCmutGG-AA and U3BCmutloop-stem, respectively. PCR products were gel-purified and used as primers for a second PCR amplification in combination with oligonucleotide 1. To generate the U3BCmutpyrloop and U3BCmutstem IV constructs, oligonucleotides 17 and 19 were used as a templates, respectively, and amplified using primers 1 and 19 in a PCR. RNA encoded by U3CD PCR product, generated using oligonucleotides 21 and 22, contains a tetraloop (UUCG) in order to join the two sides of the U3 snoRNA. This PCR product was cloned into the *KpnI* and *EcoRI* sites of pUC19. The integrity of each construct was verified by DNA sequencing.

**In Vitro Transcription**—For *in vitro* transcription all pUC19-U3BC constructs were linearized with *BamHI*, whereas pUC19-U3CD was cleaved with *KpnI*. All transcriptions were performed with T7 RNA polymerase in the presence of [<sup>32</sup>P]UTP (Amersham Biosciences) as described previously (17). For cross-linking experiments 4-thio-UTP-labeled RNAs were generated via *in vitro* transcription in the presence of [<sup>32</sup>P]CTP (Amersham Biosciences) using a final concentration of 1 mM UTP and 1 mM 4-thio-UTP. The 4-thio-UTP was a generous gift from Heiko Manninga. The m<sup>7</sup>G-capped U3BC and mutB transcripts were generated as described previously (34).

**Construction and In Vitro Translation of Recombinant Proteins**—The hU3-55K $\Delta$ 1–136 deletion mutation was generated by PCR using pCIneo VSVhU3-55K as a substrate (17) with oligonucleotides 55K $\Delta$ 1–136For and 55Krev (Table I). The remaining pCIneo VSVhU3-55K deletion mutants were described previously (17, 31). <sup>35</sup>S-Labeled proteins were generated using the TNT reticulate lysate-coupled transcription/translation kit (Promega) according to the manufacturer's directions.

**In Vitro Reconstitution Experiments**—The hU3-55K antibodies were generated by immunizing rabbits with a peptide derived from the hU3-55K sequence (EELEEETAQEKKLRLLAK). The resulting antiserum specifically recognizes hU3-55K in Western blots and co-immunoprecipitates only the U3 snoRNA from HeLa total cell extract (data not shown). HeLa nuclear extract was prepared essentially as described (34). For each 20- $\mu$ l reconstitution experiment, 10  $\mu$ l of HeLa nuclear extract, 10  $\mu$ g of tRNA, and 50 fmol of <sup>32</sup>P-labeled RNA were incubated in a buffer containing 20 mM HEPES-KOH (pH 7.9), 150 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.1% (v/v) Triton X-100, 0.5 mM dithiothreitol, and 10% (v/v) glycerol for 30 min at 30 °C. In order to control for the stability of the RNA in nuclear extract, RNA was extracted from 5% of the assembly reaction. The reconstituted complex was then immunoprecipitated using anti-hU3-55K antibodies coupled to protein A-Sepharose in 300  $\mu$ l of IPP150 (20 mM Tris (pH 7.5), 150 mM NaCl, 0.2% Triton X-100). The bound material was washed four times in IPP150, and the co-immunoprecipitated and control RNAs were isolated via phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The recovered RNAs were resolved on an 8% polyacrylamide, 7 M urea gel.

Competition experiments using U4 SL1 and U4 SL17 RNA oligonucleotides (30, 34) were performed by preincubating nuclear extracts with either 100, 200, 400, or 800 pmol of the competitor for 30 min at 30 °C. U3BC RNA was added (100 fmol), and the mixture was incubated for another 30 min at 30 °C. For the add-back experiment shown in Fig.

6C, the U3BCwt RNA was first preincubated with recombinant 15.5K protein (2.5–40 pmol) for 1 h on ice in Buffer A (20 mM HEPES-KOH (pH 7.9), 150 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA (pH 8.0), 0.5 mM dithiothreitol, 0.1% Triton X-100).

The *in vitro* reconstitution experiment shown in Fig. 2D was performed as described previously (34). Basically, 6 pmol of m<sup>7</sup>G-capped U3BC or m<sup>7</sup>G-capped mutB RNA was incubated with <sup>35</sup>S-hU3-55K *in vitro* translate and increasing concentrations of recombinant 15.5K protein (0.3, 0.6, and 1.2 pmol) in Buffer A in a final volume of 20  $\mu$ l. After 1 h of incubation on ice, 240  $\mu$ l of Buffer A was added to the reaction, and the reconstituted complex was immunoprecipitated using the H20 monoclonal antibody (specific for m<sup>2,2,7</sup>G and m<sup>7</sup>G cap structures (35)). The bound material then separated on a 12% SDS-polyacrylamide gel.

**GST Pull-down Assays**—Recombinant GST15.5K was expressed and purified as described previously (34). Binding studies with mutant U3BC RNAs were performed using 1  $\mu$ g of GST15.5K with 50 fmol of <sup>32</sup>P-labeled RNA and 1  $\mu$ g of yeast total RNA in Buffer A in a final reaction volume of 10  $\mu$ l. After 1 h of incubation on ice, 240  $\mu$ l of Buffer B (20 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA (pH 8.0), 0.2% Triton X-100, 150 mM KCl) and 10  $\mu$ l of glutathione-Sepharose beads (Amersham Biosciences) were added, and the samples were rotated for 2 h at 4 °C. The beads were washed four times with Buffer B. Co-precipitated RNAs were isolated by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. RNAs were resolved on 8% polyacrylamide, 7 M urea gels.

In order to analyze the binding of mutant hU3-55K *in vitro* translates, 10 ng of GST15.5K was incubated with 0.6 pmol of U3BC transcript and the radiolabeled protein in a final reaction volume of 20  $\mu$ l of Buffer A. After 1 h of incubation on ice, reconstituted complexes were bound to GST beads, washed four times in Buffer B, and the proteins separated on a 12% SDS polyacrylamide gel.

**UV Cross-linking Experiments**—For UV cross-linking experiments, 500 fmol of modified RNA was used in a standard reconstitution reaction with a final volume of 50  $\mu$ l. Samples were then subjected to UV light (365 nm) for 15 min on ice. For direct analysis of proteins cross-linked to the RNA (Fig. 6A), 15  $\mu$ g of RNase T1 was added to the samples followed by a 1-h incubation at 50 °C, and the resultant cross-links were analyzed by SDS-PAGE. For immunoprecipitation, reconstitution reactions were first dissociated by the addition of SDS to 1% (w/v) and incubated at 75 °C for 15 min. Triton X-100 was then added to a final concentration of 5% (v/v) and the reaction diluted to 350  $\mu$ l with IPP150. Immunoprecipitations were then performed as described earlier. Precipitated complexes were subsequently treated with proteinase K and the extracted RNAs were resolved on an 8% polyacrylamide, 7 M urea gel.

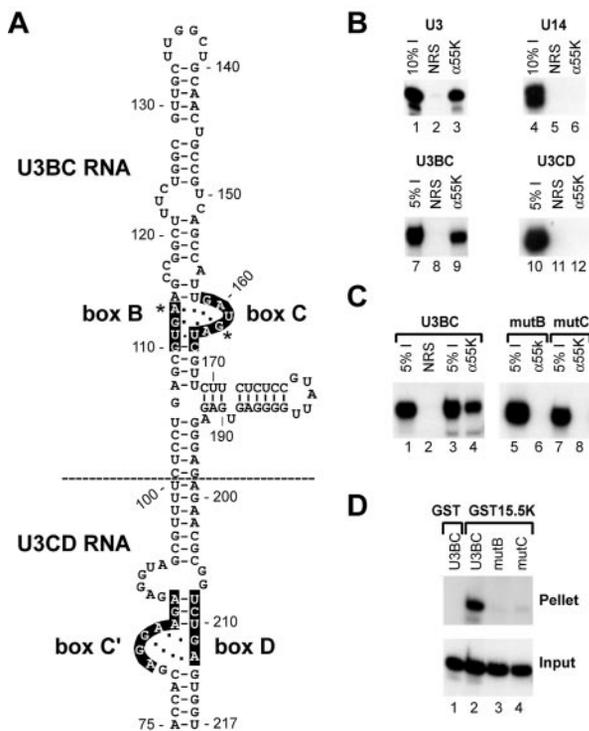
#### RESULTS

**The Box B/C Is Essential for the Binding of Both 15.5K and hU3-55K to the B/C Fragment of the U3 snoRNA**—The 15.5K and hU3-55K proteins have both been proposed to bind to the conserved box B/C region of the U3 snoRNA (11, 31). We therefore used an *in vitro* reconstitution assay, using HeLa nuclear extract, in order to analyze the binding of these two proteins to the U3 snoRNA. This system has been used previously to study the assembly of the box C/D and H/ACA snoRNPs (8, 29, 30, 36). In order to analyze assembly, <sup>32</sup>P-labeled U3 snoRNA transcripts were incubated in nuclear extract, and the association of hU3-55K was determined by immunoprecipitation with anti-hU3-55K antibodies. The co-precipitated RNA was then analyzed by PAGE. In order to show that each of the RNAs used in this investigation was not degraded during our experiments, after incubation in nuclear extract 5% of the material was isolated and also analyzed (Fig. 1B, 5% I).

The U3 snoRNA was co-precipitated with anti-hU3-55K antibodies (Fig. 1B, lanes 1 and 3) but not with the normal rabbit serum (lane 2). Under the same conditions no U14 snoRNA was co-precipitated with anti-hU3-55K antibodies demonstrating the specificity of our assay (Fig. 1B, lane 6) as hU3-55K is a U3-specific protein (17, 31). Previous studies (31) have mapped the sequences required for hU3-55K association to a region of the U3 snoRNA that consists of the box B/C motif and its flanking stems. We could also show that an RNA comprising

TABLE I  
Oligonucleotides used in this study

Oligonucleotide	Sequence
1) U3BCT7Forw	5'-GCGGAATTCTAATAGGACTC-3'
2) U3BCForw	5'-GCGGAATTTCTAATACGACTCACTATAGGGCCCCGGGCTCCTGAGCGTGAAGCCG-3'
3) U3BCRev	5'-GCGGATCCCTCCCTCTCACTCCCAAT-3'
4) U3BCmutB	5'-GCGGAATTTCTAATACGACTCACTATAGGGCCCCGGGCTCCTGAGCGTGAAGCCG-3'
5) U3BCmutC	5'-GCGGATCCCTCCCTCTCACTCCCAATACGGAGAG-3'
6) U3BCForDstem V	5'-GCGGAATTTCTAATACGACTCACTATAGGGCCCCGGGCTCCTGAGCGTGAAGCCGTTCTGTAGCCCATTTGATGATCGTTCTT-3'
7) U3BCForDstem IV+V	5'-GCGGAATTTCTAATACGACTCACTATAGGGCCCCGGGCTCCTGAGCGTGAAGCCGCAATTTGCGGATTTGAGAGGGGAG-3'
8) U3BCRevDstem IV+V	5'-GCGGATCCCTCCCTCTCACTCCCAATACGGAGAG-3'
9) U3BCStemII Rev	5'-GCGGATCCCTCCCTAAACGATCATCAATGGCTGACGG
10) U3BCForDstem I+VI	5'-GCGGAATTTCTAATACGACTCACTATAGGGCCCCGGGAGCGGTGAAGCCGCTTTCTG-3'
11) U3BCRevDstem I+VI	5'-GCGGATCCCAACGATCACTCAATGGCTGA-3'
12) U3BCmutAU-AA Rev	5'-GCGGATCCCTCCCTAAACGATCACTATAGGGCTGACGGCAGTTGCA-3'
13) U3BCmutloop-stem For	5'-GAAGCGGGCTGACGGCGTTGGCTGCACAAAGCCGGCTGACGGCGTTGCTTGGCTGC AAC-3'
14) U3BCmutistem III For2	5'-GCGGAATTTCTAATACGACTCACTATAGGGCCCCGGGCTCCTGAGCGTGAAGCCGCTTCTGGCCGTTGC3-3'
15) U3BCmutistem III Rev	5'-GCGGATCCCTCCCTAAACGATCATCGATGCTGACGGCAGTTGCA-3'
16) U3BCmutIG-AAFor	5'-GAGCTGAAGCCCACTTTTCGGCGTTGCTTGGCTGC-3'
17) U3BCmutistem IV	5'-CTCCTGAGCGTGAAGCGCGTTCCTGCTGGCTGCAACTGCCGTCACGCAITTTGATGATCGTTAGGGAG-3'
18) U3BCmutypyloop	5'-CTCCTGAGCGTGAAGCGCGTAAAGTGGCGTTGCTTGGCTGCAACTGCCGAGAGCCCAITTTGATGATCGTTAGGGAG-3'
19) U3BCDstem VI Rev	5'-GCGGATCCCTCCCTAAACGATCATCAATGG-3'
20) U3C'DFor	5'-GCGGAATTTCTAATACGACTCACTATAGGGACCACGAGGAAAGAGAGGTAGCCGTTTTTTTCGAGAACCGCGGCTCTGAGTGGT-3'
21) U3C'DRev	5'-GCGGATCCCACTAGAGCGGCTTCT-3'
22) U3BCmutUU-GC For	5'-GCGGAATTTCTAATACGACTCACTATAGGGCCCCGGGCTCCTGAGCGGGAAAGCCGGCTTTTCTTGGCGT-3'
23) U3BCmutUU-GC Rev	5'-GCGGATCCCTCCCTCTCACTCCCAATACGGAGAGAAAGCGGTCACTCAATGGC-3'
24) U3BCstemC	5'-GCGGAATTTCTAATACGACTCACTATAGGGCCCCGGGCTCCTGAGCGTGAAGCCGCTTCTGGCC-3'
25) U3BCstemG	5'-GCGGATCCCTCCCTCTCACTCCCAATACGGAGAGAAACCTCATCAATGGC-3'
26) U3BCstemCCG	5'-GCGGAATTTCTAATACGACTCACTATAGGGCCCCGGGCTCCTGAGCGTGAAGCCGCTTCTGGCC-3'
27) U3BCstemCGG	5'-GCGGATCCCTCCCTCTCACTCCCAATACGGAGAGAAAGCGGTCACTCAATGGC-3'
28) U3BCmutloopA-GG	5'-GCGGATCCCTCCCTAAACGATCACTAAGCGGCTGACGGCAFTTGA-3'
29) 55Kdelta1-136For	5'-GCGGAATTTCTAATACGACTCACTATAGGGAGCCTCGAGGCCACCATGTCAGTGCACATTCGGGTTTTTAC-3'
30) 55KRev	5'-GCGGATCCCTCCCTGAGGAAACCAGCAGCTGGGGGTA-3'



**FIG. 1. The box BC fragment of the U3 snoRNA associates with 15.5K and hU3-55K *in vitro*.** A, sequence and proposed secondary structure of the U3 box B/C and C/D motifs in the 3' domain of the U3 snoRNA. This model includes the new structural organization of the box B/C motif (43). The secondary structure of the box C/D motif (containing the C' and D sequences) was drawn as described previously (25, 43). The highly conserved nucleotides in either the box C/D or box B/C motifs are depicted in white on a black background. The dotted line indicates non-Watson-Crick base pair interactions predicted from the crystal structure of the U4 5' stem-loop bound by 15.5K (37). The dashed line dissecting the 3' domain of the U3 snoRNA indicates how this region of the RNA was sub-divided to generate the BC (upper part) and CD (lower part) fragments of the U3 snoRNA. In the U3CD RNA, the BC fragment is replaced by the tetra-loop sequence UUGA. The asterisks in the U3BC fragment mark the nucleotides mutated in box C and box B in order to generate mutC and mutB, respectively. B, the U3BC RNA is sufficient for the association of hU3-55K. *In vitro* reconstitution assays were performed using <sup>32</sup>P-radiolabeled RNAs incubated in HeLa nuclear extract. RNP complexes formed during this reaction were then immunoprecipitated, and the co-purifying RNAs were analyzed on an 8% polyacrylamide, 7 M urea gel. The RNA used is indicated above each panel. NRS, normal rabbit serum; α55K, anti-hU3-55K antibodies; 5% I, 5% of the input material after incubation in nuclear extract; 10% I, 10% of the input material after incubation in nuclear extract. C, binding of hU3-55K to the U3BC RNA is dependent on an intact box B and box C. *In vitro* reconstitution experiments using HeLa nuclear extract were performed as described in B using the mutB and mutC RNAs. The RNA used is indicated at the top, and the antibodies used are as described in B. D, binding of 15.5K to the U3BC RNA is dependent on an intact box B and box C. GST pull-down experiments, using either recombinant GST or GST15.5K, with the U3BC, mutB, and mutC RNAs, were performed as described under "Experimental Procedures." The bound RNAs were then resolved on an 8% polyacrylamide, 7 M urea gel (indicated by Pellet). The use of GST or GST15.5K is indicated. The RNA used is indicated above each lane. Input represents 10% of the starting material.

just the B/C motif region of the U3 snoRNA (Fig. 1A, U3BC RNA) was effectively co-precipitated by anti-hU3-55K antibodies after incubation in nuclear extract (Fig. 1B, lane 9). In contrast, the U3CD region of the U3 snoRNA (Fig. 1A) was not precipitated by the anti-hU3-55K antibodies. This confirmed that all of the essential sequences necessary for hU3-55K binding are contained within the box B/C region of the U3 snoRNA. The U3BC RNA was therefore used as the substrate RNA in our investigations as the analysis of protein binding to this fragment most likely reflects what is observed with the full-

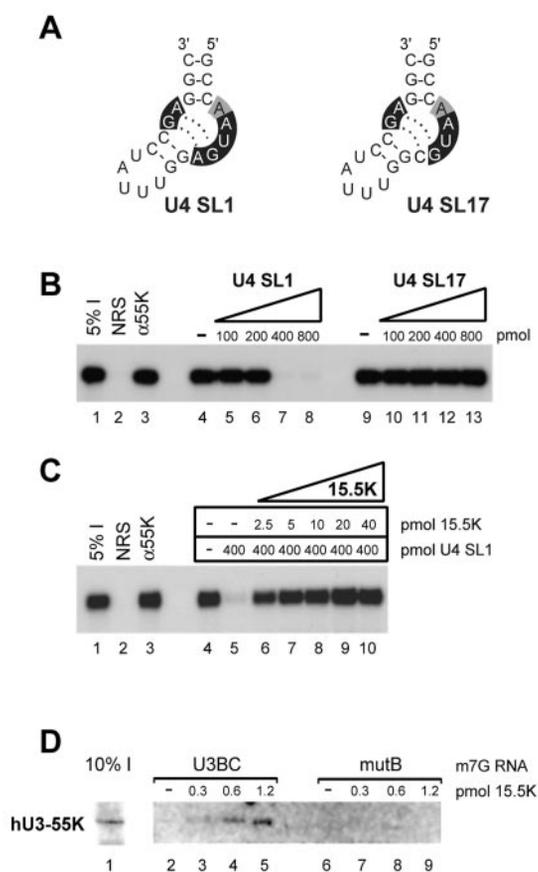
length snoRNA. In addition, the physical separation of the two 15.5K-binding sites facilitates the direct analysis of 15.5K binding to the box BC motif in the absence of the box C/D motif.

We have shown previously that the GA dinucleotides present in the U4 5' stem-loop, box C/D motif, and the box B/C motif are essential for the binding of 15.5K *in vitro* (11, 34). Therefore, we next addressed the role these highly conserved nucleotides play in hU3-55K binding. Constructs were generated with point mutations in either box B or box C of the U3BC RNA (Fig. 1A; nucleotides marked by an asterisk). Mutation of either A<sup>113</sup> (mutB) or G<sup>162</sup> (mutC) (numbers refer to the position in the intact U3 snoRNA) in the U3BC RNA abolished the binding of hU3-55K in HeLa nuclear extract (Fig. 1C, lanes 6, and 8). However, neither mutation affected the stability of the RNA in nuclear extract (Fig. 1C, lanes 5 and 7).

We next wanted to address whether the 15.5K protein specifically binds to the U3BC RNA in HeLa nuclear extract. However, anti-15.5K antibodies co-precipitated only very low amounts of the U3BC RNA in this assay (data not shown). The analysis of the interaction of 15.5K with the RNA by immunoprecipitation was therefore not possible. The 15.5K antibodies also fail to immunoprecipitate the U4/U6.U5 tri-snRNP suggesting that in certain complexes this protein is not accessible for antibody binding (34). As the anti-15.5K antibodies could not be readily used to study the binding of 15.5K to the U3BC RNA, an alternative means of analyzing 15.5K binding to the box B/C motif was required. We therefore analyzed the ability of recombinant GST-tagged 15.5K protein to bind the mutant and wild-type RNAs. The RNA bound to GST15.5K was co-precipitated using glutathione-Sepharose beads. The results show that GST15.5K efficiently bound to the U3BC RNA but not to either of the mutant transcripts (Fig. 1D, lanes 2–4). When the GST tag alone was used, no wild-type RNA was co-precipitated (Fig. 1D, lane 1). These results show that mutations that inhibit 15.5K binding to the box B/C motif also block the association of hU3-55K. However, a more direct analysis of the role 15.5K plays in hU3-55K binding was required before any further conclusions could be made about the assembly pathway of this RNP complex.

**Binding of 15.5K to the B/C Motif Is Essential for the Association of hU3-55K with the U3 snoRNA**—The fact that the 15.5K protein can bind the B/C motif in the absence of hU3-55K suggests that this protein may indeed be the primary RNA-binding protein. Therefore, we next addressed whether binding of the 15.5K protein to the box B/C motif was an essential prerequisite for the association of hU3-55K. In order to investigate this, competition experiments were performed with a chemically synthesized oligonucleotide corresponding to the 5' stem-loop of the U4 snRNA (Fig. 2A, U4 SL1) that specifically binds 15.5K (34). The nuclear extract was preincubated with U4 SL1 oligonucleotide, in order to sequester 15.5K binding, prior to the addition of U3BC RNA, and then assayed for hU3-55K binding.

As seen in Fig. 2B, the addition of 400 pmol of U4SL1 oligonucleotide inhibited the co-precipitation of the U3BC RNA with anti-hU3-55K antibodies (Fig. 2B, lane 7). As a control, oligonucleotide U4 SL17 (Fig. 2A), containing a point mutation that abolishes 15.5K binding (34), was also used. The addition of up to 800 pmol of the U4 SL17 RNA oligonucleotide had no effect on the association of hU3-55K with the U3BC RNA (Fig. 2B, lane 13). Therefore, inhibiting the binding of 15.5K to the box B/C motif effectively blocked the association of hU3-55K with the U3BC RNA transcript. We next asked whether we could restore hU3-55K binding to the U3BC RNA, in the 15.5K-sequestered extract, by the addition of recombinant 15.5K. The 15.5K protein was first pre-bound to U3BC RNA and then



**FIG. 2. The 15.5K protein is required for the binding of hU3-55K to the U3 snoRNA.** *A*, sequence and structure of the two U4 5' stem-loop oligonucleotides used to block 15.5K binding. The conserved internal loop nucleotides are shown in white on a black background. U4 SL1 is the wild-type sequence, whereas U4 SL17 contains a point mutation in the internal loop that inhibits 15.5K binding (34). *B*, an excess of a U4 5' stem-loop oligonucleotide can specifically block the assembly of the box C/D snoRNP. Radiolabeled U3BC RNA was incubated in HeLa nuclear extract that had been pre-incubated with increasing amounts of either U4 SL1 or U4 SL17 RNA oligonucleotides. The binding of hU3-55K was then assayed by immunoprecipitation with anti-hU3-55K antibodies. Bound RNA was recovered and then separated on an 8% polyacrylamide, 7 M urea gel. The amount of oligonucleotide used was as follows: no oligonucleotide in lanes 1–4 and 9; 100 pmol in lanes 5 and 10; 200 pmol in lanes 6 and 11; 400 pmol in lanes 7 and 12; 800 pmol in lanes 8 and 13. *NRS*, normal rabbit serum; *α55K*, positive immunoprecipitation using anti-hU3-55K antibodies in the absence of U4 oligonucleotides; *5% I*, 5% of the input material after incubation in nuclear extract. The identity of the RNA oligonucleotide used is indicated at the top of the panel. *C*, rescue of U4 SL1-oligonucleotide blocked extract by the addition of recombinant 15.5K. HeLa nuclear extract was pre-incubated with either 400 pmol of U4 SL1 RNA oligonucleotide (lane 5) or buffer (lanes 2–4). Radiolabeled U3BC RNA was subsequently added in the absence or presence of increasing amounts of recombinant 15.5K (lanes 6–10). The inclusion of the U4 SL1 oligonucleotide and the amount of recombinant 15.5K used is indicated above each lane. The binding of hU3-55K was determined as described in *B*. *D*, the 15.5K protein is required for the binding of *in vitro* translated hU3-55K to the U3BC RNA. <sup>35</sup>S-Labeled-hU3-55K was incubated with 6 pmol of wild-type m<sup>7</sup>G-capped U3BC RNA (lanes 2–5) or 6 pmol of m<sup>7</sup>G-capped U3BC mutB RNA (lanes 6–9) in the presence or absence of recombinant 15.5K. Bound radiolabeled proteins were precipitated with anti-cap antibodies (H20) and analyzed on an SDS-13% polyacrylamide gel. The amount of recombinant 15.5K used is indicated above each lane. *10%I*, 10% of the input *in vitro* translated hU3-55K protein.

added to the oligonucleotide-inhibited extract. As seen in Fig. 2C (lanes 6–10) the addition of recombinant 15.5K effectively restored hU3-55K binding. This therefore clearly demonstrates that 15.5K binding to the box B/C RNA is an essential step in the hierarchical assembly of the U3 box B/C RNP complex.

It was reported previously (31) that hU3-55K, translated *in vitro* in wheat germ extract, binds to the U3 snoRNA *in vitro* without the addition of exogenous proteins. We therefore performed similar experiments in order to determine whether our results are in contradiction to this earlier publication. *In vitro* translated hU3-55K was incubated with m<sup>7</sup>G-capped U3BC RNA in either the absence or presence of recombinant 15.5K. The complexes were then isolated using the H20 antibody, which specifically recognizes the m<sup>2,2,7</sup>G and m<sup>7</sup>G cap structures. The bound protein was then analyzed on an SDS-12% polyacrylamide gel. Efficient binding of hU3-55K to the U3BC RNA was only observed in the presence of 15.5K (Fig. 2D). Importantly, this binding was not observed when the U3BC mutB transcript was used. Therefore, this confirms that 15.5K binding to the snoRNA is essential for the subsequent association of hU3-55K. The hU3-55K protein used in the previously reported experiments was translated *in vitro* in wheat germ extract, whereas the protein used in these experiments was produced in reticulocyte lysate. It is therefore possible that factors in the wheat germ extract, such as endogenous 15.5K, mediate hU3-55K binding to the U3 snoRNA.

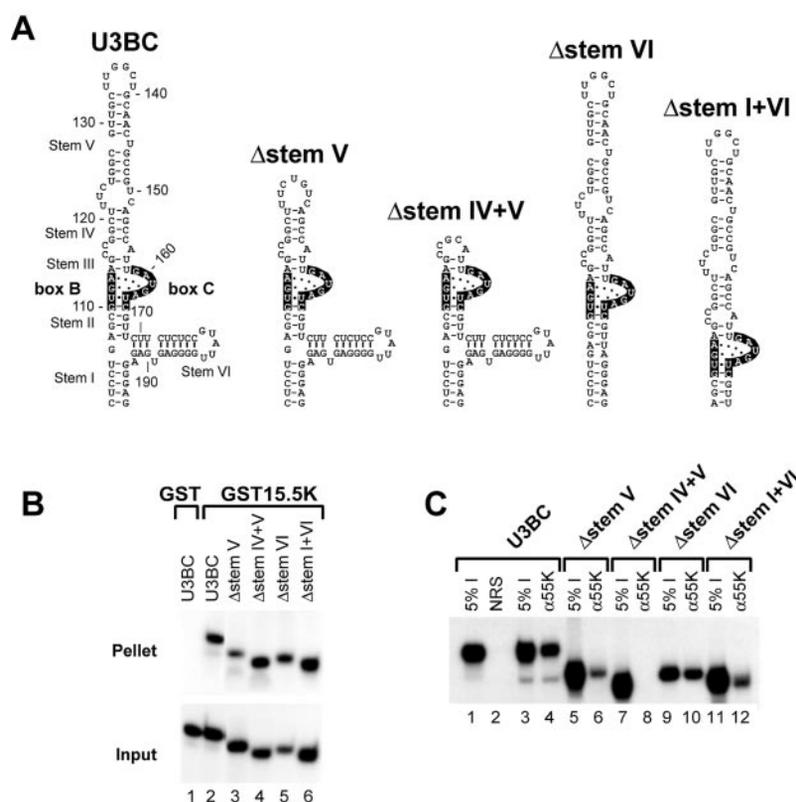
*hU3-55K Requires Additional RNA Elements Flanking the Box B/C Motif for Its Association with the U3 snoRNA*—We next decided to analyze whether RNA sequences flanking the box B/C motif were also necessary for the association of hU3-55K. In order to achieve this, four deletion mutants were generated in the U3BC RNA (Fig. 3A). The ability of 15.5K to bind to the various RNAs was determined by pull-down experiments using GST-tagged 15.5K. As seen in Fig. 3B, each of the deletion mutants bound 15.5K with approximately the same efficiency confirming that the binding of this protein to the U3BC RNA is solely dependent on the central box B/C motif.

The binding of hU3-55K to the mutant transcripts was next analyzed in nuclear extract by immunoprecipitation with anti-hU3-55K antibodies, as described above. Importantly, none of the mutations had any effect on the stability of the RNAs in nuclear extract (Fig. 3C, lanes 1, 3, 5, 7, 9, and 11). Interestingly, the removal of both stems V and IV (U3BCΔstem IV+V) abolished the association of hU3-55K (Fig. 3C, lane 8), whereas deletion of stem I and stem-loop VI (U3BCΔstem I+VI) or deletion of stem V (U3BCΔstem V) only reduced the binding of hU3-55K (Fig. 3C, lanes 6 and 12). In contrast, deletion of stem VI (U3BCΔstem VI) did not affect the association of hU3-55K (Fig. 3C, lane 10). These results show that in addition to the box B/C motif, other regions of the U3BC RNA, most notably stems IV and V, are important for the stable association of hU3-55K with the U3 snoRNA.

*The Association of hU3-55K with the U3BC RNA Requires an Internal Loop Structure Adjacent to the Box B/C Motif*—Since the deletion of stems IV and V abolished the binding of the hU3-55K protein to the U3BC RNA, we decided to determine the sequence and/or structural elements in this region that are essential for hU3-55K binding. Phylogenetic analysis of the U3BC domain from a wide range of eukaryotes revealed no clear sequence conservation in this region of the RNA (Fig. 4A, bracketed area). However, in all cases examined, stem IV is separated from the flanking helices by small internal loop structures. Indeed, the upper loop between stem IV and stem V is pyrimidine-rich (Fig. 4A). We therefore constructed a series of mutants in order to define the nucleotides in this region required for hU3-55K association (Fig. 4B). Since the U3BCΔstem VI mutant bound hU3-55K as well as the full-length U3BC, we decided to use the U3BCΔstem VI as template for this analysis.

We first analyzed the effect of these mutations on the binding of GST15.5K in pull-down experiments. This demonstrated

**FIG. 3. RNA elements flanking the box B/C motif are involved in the binding of hU3-55K.** **A**, schematic representation of U3BC RNA deletion mutants. The conserved nucleotides of the box B/C motif are represented as in Fig. 1A. **B**, binding of GST15.5K to U3BC deletion mutants. GST pull-down experiments were performed with either recombinant GST or GST15.5K using the RNAs outlined in A. The bound RNAs were then resolved on an 8% polyacrylamide, 7 M urea gel (indicated by *Pellet*). The use of GST or GST15.5K is indicated. The RNA used is indicated *above* each lane. **Input** represents 10% of the material used for binding. **C**, *in vitro* reconstitution experiments performed in HeLa nuclear extract with the U3BC RNA and the deletion mutants. The U3BC as well as the mutant transcripts outlined in A were incubated in HeLa nuclear extract, and the binding of hU3-55K was assayed by immunoprecipitation with anti-hU3-55K antibodies ( $\alpha 55K$ ) with the bound RNAs being separated on an 8% polyacrylamide, 7 M urea gel. The RNAs used are indicated *above* the respective lanes. *NRS*, normal rabbit serum; *5% I*, 5% of the input material after incubation in nuclear extract.



that changing the sequence of the upper internal loop (mutpyrloop), closing this loop to join stems IV and V (mutloop-stem), or altering the sequence of a section of stem IV (mutstem IV) had no noticeable effect on 15.5K binding (Fig. 4C, lanes 3–5). In contrast, disruption of stem IV (mutGG-AA) or disruption of the AU base pair in stem III (mutAU-AA) abolished the binding of 15.5K (Fig. 4C, lanes 6 and 8). This effect is likely due to the destabilization of the secondary structure in this region of the RNA inhibiting the correct folding of the box B/C motif for 15.5K binding. This hypothesis is enforced by the fact that mutations that stabilize the structure of this region of the RNA, *i.e.* by either closing the internal loop joining stem III and IV (mutloopA-GG) or the exchange of a AU base pair in stem III for a GC base pair (mutstem III), significantly enhanced the binding of 15.5K (Fig. 4C, lanes 7 and 9).

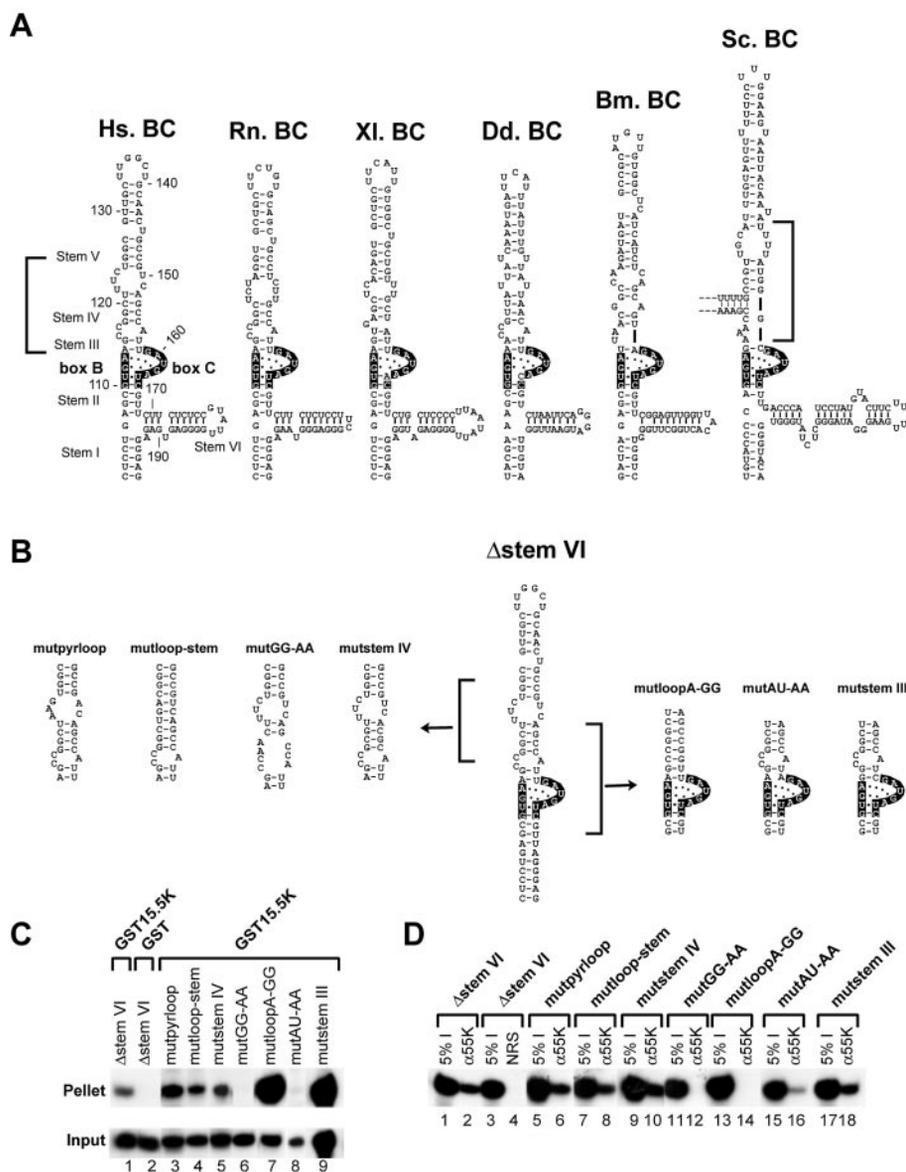
We next analyzed the effect of these mutations on hU3-55K binding in nuclear extract. Importantly, none of the mutations affected the stability of the RNA in nuclear extract (Fig. 4D, lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17). Consistent with the fact that 15.5K binding is essential for the association of the U3-specific protein, mutations that block 15.5K binding by destabilizing stem III (mutAU-AA) or IV (mutGG-AA) also inhibited the association of hU3-55K (Fig. 4D, lanes 12 and 16). Interestingly, the A-GG mutation, which closes the internal loop between stem III and IV, completely blocked the binding of hU3-55K (Fig. 4D, lane 14). This point is even more striking considering the fact that this mutation significantly enhanced the binding of 15.5K (Fig. 4C, lane 7). In contrast, mutations that altered the upper internal loop (mutpyrloop and mutloop-stem) or that changed the sequence of stem IV (mutstem IV) had no visible effect on hU3-55K binding (Fig. 4D, lanes 6, 8, and 10). In addition, the exchange of the AU base pair for a GC base pair in stem III (mutstem III) did not noticeably affect the binding of hU3-55K (Fig. 4D, lane 18). Taken together, these data clearly show that the internal loop structure joining stem III and stem IV is absolutely essential for the binding of hU3-55K and probably helps to provide the structural platform

necessary for the binding of this protein.

*The Structure but Not the Sequence of Stem II in the U3BC RNA Is Essential for the Association of Both hU3-55K and 15.5K*—The crystal structure of the U4 5' stem-loop bound to 15.5K demonstrated that this protein primarily contacts the internal loop and makes minimal contact to the two stem structures (37). Our data suggest that 15.5K binds the box B/C motif in a manner analogous to the U4 snRNA and primarily contacts the internal-loop nucleotides (11, 34, 37). The nucleotides of stem II in the U3BC RNA that are part of the conserved box B/C motif sequence are likely not bound by 15.5K and could therefore provide a sequence-specific binding site for U3-specific proteins such as hU3-55K. In order to confirm the role of stem II in 15.5K binding, as well as to determine the role of the conserved nucleotides in U3 snoRNP assembly, a series of mutations were generated in stem II (Fig. 5A) and analyzed for 15.5K and hU3-55K binding as described above.

We first analyzed the effect of these mutations on the binding of GST15.5K. As can be seen in Fig. 5B, all of the mutations that disrupted the base pairing in stem II, whether conserved or non-conserved nucleotides, either severely reduced or abolished 15.5K binding to the U3BC RNA (Fig. 5B, lanes 3, 4, 7, and 8). In contrast, all mutations that altered the sequence of stem II but retained the base-pairing potential (Fig. 5B, *stemCG* and *stemCCGCGG*, lanes 5 and 9, respectively) bound 15.5K as well as the wild-type RNA. This strongly supports the fact that stem II exists in the U3 snoRNA and confirms its involvement in the formation of the stem-internal loop-stem structure necessary for 15.5K binding. Interestingly, substitution of the UU pair with a GC base pair (mutUU-GC) reduced the association of 15.5K at least 2-fold (Fig. 5B, lane 6). This is somewhat surprising considering that a GC base pair is present at the equivalent position in the U4 5' stem-loop. However, it is entirely possible that some differences exist in the binding of 15.5K between the box B/C motif and the U4 5' stem-loop and that in the context of the U3 snoRNA the UU pair is more favorable for protein binding.

**FIG. 4. A short internal loop between stem III and stem IV of the U3BC RNA is essential for hU3-55K binding to the U3 snoRNA.** *A*, the proposed secondary structure of U3BC RNA fragments derived from the *Homo sapiens* (*Hs. BC*), *Rattus norvegicus* (*Rn. BC*), *Xenopus laevis* (*Xl. BC*), *Dictyostelium discoideum* (*Dd. BC*), *Bombyx mori* (*Bm. BC*), and *Saccharomyces cerevisiae* (*Sc. BC*). The conserved nucleotides of the box B/C motif are represented as in Fig. 1A. The bracket indicates the conserved stem structures found above the box B/C motif that are essential for the binding of hU3-55K to human U3 snoRNA. *B*, schematic representation of the mutations generated in the upper region of the U3BC RNA mutant U3BC $\Delta$ stem VI. The mutations and their likely effect on the secondary structure of the RNA are depicted. The conserved nucleotides of the box B/C motif are represented as in Fig. 1A. *C*, binding of GST15.5K to mutations in U3BC $\Delta$ stem VI. GST pull-down experiments were performed with either recombinant GST or GST15.5K using the RNAs outlined in *B*. The bound RNAs were then resolved on an 8% polyacrylamide, 7 M urea gel (indicated by *Pellet*). The use of GST or GST15.5K is indicated. The RNA used is indicated *above* each lane. *Input* represents 10% of the starting material. *D*, *in vitro* reconstitution experiments performed in HeLa nuclear extract with the U3BC $\Delta$ stem VI RNA and mutants. The U3BC $\Delta$ stem VI as well as the mutant transcripts outlined in *B* were incubated in HeLa nuclear extract, and the binding of hU3-55K was assayed by immunoprecipitation with the bound RNAs being separated on an 8% polyacrylamide, 7 M urea gel. The RNA mutants used are indicated *above* the respective lanes. *NRS*, normal rabbit serum;  $\alpha$ 55K, anti-hU3-55K antibodies; 5% *I*, 5% of the input material after incubation in nuclear extract.

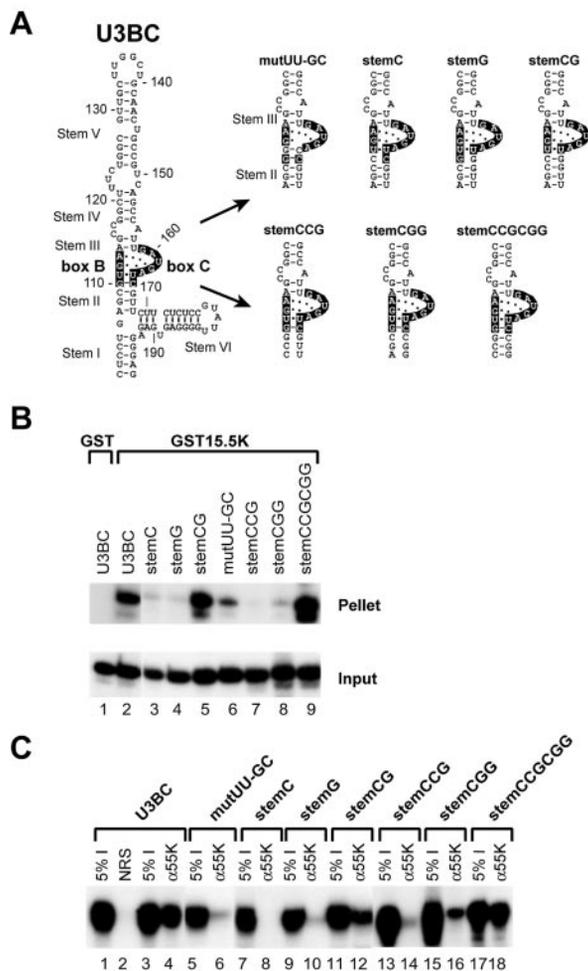


We next analyzed the effect of these mutations on the binding of hU3-55K in nuclear extract. Consistent with previous data, none of the mutations affected RNA stability in nuclear extract (Fig. 5C, lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17). As seen in Fig. 5C, mutations that inhibiting 15.5K binding, either by disrupting stem II or mutating the potential UU base pair, also inhibit the association of hU3-55K to an equivalent degree (Fig. 5C, lanes 8, 10, 14, and 16). In contrast, mutations that change the sequence but do not disrupt the structure of stem II, thus retaining 15.5K binding, do not effect hU3-55K association (lanes 12 and 18). Therefore, the structure but not the sequence of stem II is important for both 15.5K and hU3-55K association. This means that the conserved sequence of stem II is not essential for 15.5K and hU3-55K association and may therefore function in the recruitment of other U3-associated proteins (see “Discussion”).

**hU3-55K Directly Binds the U3BC RNA**—The association of hU3-55K with the box B/C motif is dependent on the binding of 15.5K as well as the conserved structure of the flanking RNA. This implies that hU3-55K, or an additional protein other than 15.5K, directly contacts the U3BC RNA. We therefore performed UV cross-linking experiments in order to determine whether hU3-55K indeed directly contacts the U3 snoRNA. To achieve this we used U3BC wild-type and U3BC mutB tran-

scripts (Fig. 1C) synthesized in the presence of 4-thio-UTP. Following incubation of the RNA in nuclear extract, the reaction was UV-irradiated and subsequently digested with RNase T1 (see “Experimental Procedures”). Cross-linked proteins were then analyzed by SDS-PAGE.

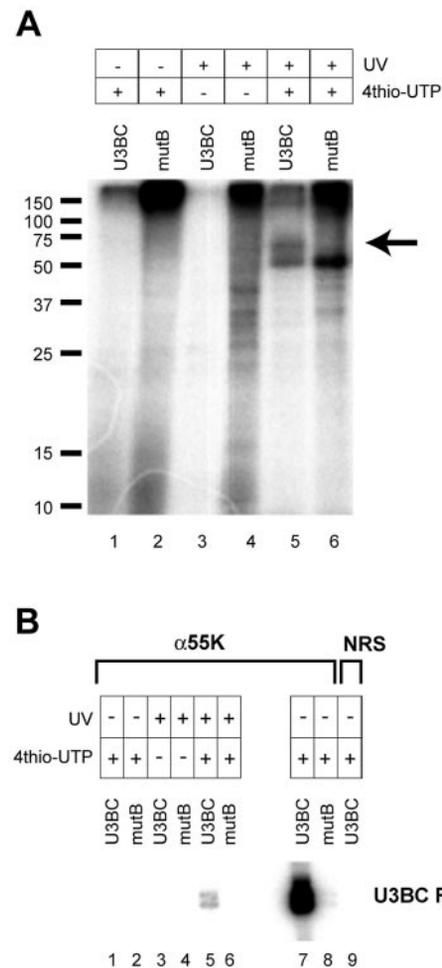
Analysis of the cross-linked products revealed that one specific product of about 65 kDa was observed when U3BC RNA was used as a substrate (Fig. 6A). This product was dependent on both UV irradiation and the presence of 4-thio-UTP in the transcript. Importantly, this cross-link was not observed when the mutB RNA (Fig. 1C) was used, suggesting that the interaction of this protein with the U3BC RNA is specific and dependent on 15.5K binding to the box B/C motif. We next analyzed the cross-link products by immunoprecipitation in order to determine whether hU3-55K is specifically cross-linked to the U3BC RNA. To achieve this, the complexes were immunoprecipitated under semi-denaturing conditions with anti-hU3-55K antibodies (see “Experimental Procedures”). Under these conditions, complexes present within the binding reaction are disrupted, and therefore only RNA covalently attached to the hU3-55K protein, by an UV-induced cross-link, will be co-precipitated by the anti-hU3-55K antibodies. Precipitated complexes were subsequently treated with proteinase K, and the extracted RNAs were resolved on an 8% polyacryl-



**FIG. 5. The structure but not the sequence of stem II in the box B/C motif is essential for both 15.5K and hU3-55K binding to the U3 snoRNA.** *A*, schematic representation of the U3BC RNA stem II mutants. Conserved nucleotides of the box B/C motif are represented as in Fig. 1*A*. *B*, binding of GST15.5K to U3BC stem II mutants. GST pull-down experiments were performed with either recombinant GST or GST15.5K using the RNAs outlined in *A*. The bound RNAs were then resolved on an 8% polyacrylamide, 7 M urea gel (indicated by *Pellet*). The use of GST or GST15.5K is indicated. The RNA used is indicated above each lane. *Input* represents 10% of the starting material. *C*, *in vitro* reconstitution experiments performed in HeLa nuclear extract with the U3BC stem II mutants. The U3BC as well as the mutant transcripts outlined in *A* were incubated in HeLa nuclear extract, and the binding of hU3-55K was assayed by immunoprecipitation. The bound RNAs were then separated on an 8% polyacrylamide, 7 M urea gel. The RNA mutants used are indicated above the respective lanes. *NRS*, normal rabbit serum;  *$\alpha$ 55K*, anti-hU3-55K antibodies; 5% *I*, 5% of the input material after incubation in nuclear extract.

amide, 7 M urea gel. As seen in Fig. 6*B*, hU3-55K specifically cross-links to the U3BC RNA. The co-precipitation of the U3BC RNA was only observed with the wild-type RNA and not with the box B mutant (Fig. 6*B*, lanes 5 and 6). In addition, this signal was dependent on UV irradiation and the presence of 4-thio-UTP in the transcript (Fig. 6*B*, lanes 1–4). This therefore clearly demonstrates that the WD40 protein, hU3-55K, specifically binds the U3BC RNA.

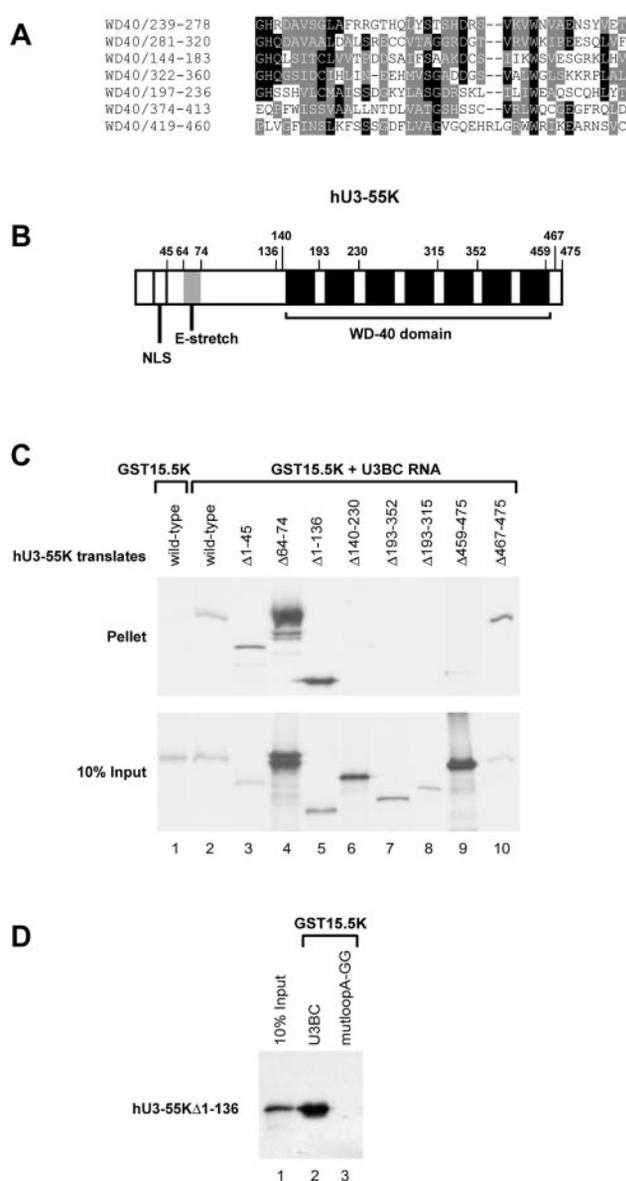
*The WD40 Repeats Alone Are Sufficient for the 15.5K-dependent Binding of hU3-55K to the U3BC RNA*—It was initially reported that hU3-55K contained six WD40 repeats, located between amino acids 144 and 405 (31). In re-examining its amino acid sequence, we have recently observed a seventh WD40 repeat located between amino acids 419 and 461 in the C terminus of the protein. An alignment of the seven WD40 repeats is shown in Fig. 7*A*. It was demonstrated previously



**FIG. 6. A direct contact between hU3-55K and the U3BC RNA.** *A*, single protein specifically cross-links to the U3BC RNA in HeLa nuclear extract. *In vitro* reconstitution experiments were performed in HeLa nuclear extracts using 4-thio-UTP containing  $^{32}$ P-labeled U3BC transcripts. The extracts were then UV-irradiated, RNase T1-digested, and then resolved on an SDS-13% polyacrylamide gel. The RNA used, the inclusion of 4-thio-UTP in the transcript, and whether the sample was UV-irradiated is indicated above each lane. *NRS*, normal rabbit serum;  *$\alpha$ 55K*, anti-hU3-55K antibodies. *B*, hU3-55K specifically cross-links to the U3BC RNA. *In vitro* reconstitution and UV cross-linking experiments were performed as in *A*. After UV irradiation, the samples were immunoprecipitated either under semi-denaturing conditions (lanes 1–6) or normal native conditions (lanes 7–9) with either anti-hU3-55K antibodies ( *$\alpha$ 55K*) or normal rabbit serum (*NRS*). The bound RNA was then separated on an 8% polyacrylamide, 7 M urea gel. The RNA used, the inclusion of 4-thio-UTP in the transcript, and whether the sample was UV-irradiated is indicated above each lane.

(31) that deletions in the region of amino acids 140–460, the seven WD40 repeats, inhibited the association of hU3-55K with the U3 snoRNA. We therefore decided to re-examine the role the WD40 repeats play in 15.5K-dependent binding of hU3-55K to the U3BC RNA.

Radiolabeled hU3-55K or one of the mutant proteins was incubated with U3BC RNA and recombinant GST-tagged 15.5K. The resulting complex was isolated using GST beads, and the association of the *in vitro* translated protein was analyzed by SDS-PAGE. As seen in Fig. 7*C*, the binding of hU3-55K to the GST 15.5K requires the presence of the U3BC RNA (lanes 1 and 2). Analysis of the previously published hU3-55K deletion mutants clarifies that, as with the previous experiments, mutations in the WD40 domain abolish binding to the 15.5K-bound U3BC RNA, whereas mutations in the N-terminal domain have little or no effect (Fig. 7*C*, lanes 3 and 4 and 6–10) (17, 31). In order to refine this analysis further, we



**FIG. 7. The WD40 domain of hU3-55K is necessary and sufficient for the interaction with the 15.5K-U3BC RNA complex.** *A*, alignment of the seven putative WD40 repeats identified in hU3-55K. By using a motif search program ([www.embl-heidelberg.de/~andrado/papers/rep/search.html](http://www.embl-heidelberg.de/~andrado/papers/rep/search.html)), the seven WD40 repeats were identified in the amino acid sequence of hU3-55K. Highly conserved amino acids are shown in white on a black background. Conserved amino acids are represented in white on a gray background and are grouped according to Ref. 44. The WD40 repeat profiles used for these studies have been described (45) ([bmerc-www.bu.edu/bioinformatics/wdrepeat.html](http://bmerc-www.bu.edu/bioinformatics/wdrepeat.html)). *B*, schematic representation of the hU3-55K protein. The putative nuclear localization signal (NLS) and the glutamic acid stretch (E-stretch, gray box) have been described previously (17, 31). The WD40 repeats are represented as black boxes. The amino acid positions relevant to the deletions used in *C* are indicated. *C*, *in vitro* translated wild-type or mutant <sup>35</sup>S labeled hU3-55K protein was incubated with GST 15.5K in the absence or presence of U3BC RNA. Reconstituted complexes were precipitated using glutathione-Sepharose beads and the co-precipitated proteins resolved on an SDS-13% polyacrylamide gel. The identity of the *in vitro* translated protein used is indicated above each lane. The designation of the mutants indicates which regions of hU3-55K were deleted, as described previously (17, 31). Ten percent of the input for each <sup>35</sup>S-labeled protein is shown in the lower panel. *D*, the WD40 domain of hU3-55K requires RNA elements flanking the 15.5K-binding site for its association with the U3BC RNA. The binding of the hU3-55K mutant Δ1-136 to the GST 15.5K-RNA complex was performed as described above using either the U3BC wild-type or mutloopA-GG RNAs (see Fig. 4B). The RNA transcript used is indicated above each lane. Lane 1, 10% of the *in vitro* translated protein used in each reconstitution.

generated a new mutant, in which the complete N terminus of this protein is deleted (Δ1-136), effectively leaving just the seven WD40 repeats of hU3-55K. This mutation still efficiently binds the 15.5K-U3BC complex (Fig. 7C, lane 5) demonstrating that the WD40 motifs alone are sufficient for this interaction. We next analyzed whether the binding of Δ1-136 is dependent on the flanking RNA sequences. The use of the U3BC RNA mutation, which blocks the binding of hU3-55K in nuclear extract, instead of the wild-type RNA, completely inhibits the binding of the *in vitro* translated WD40 domain of hU3-55K (Fig. 7D) although this mutant still binds the 15.5K protein. Therefore, these data are highly suggestive that the WD40 domain of hU3-55K makes specific contacts to the U3BC RNA.

## DISCUSSION

*The 15.5K Protein Is Essential for the Binding of hU3-55K to the U3 snoRNA*—In this article we have shown that the binding of 15.5K to the box B/C motif is essential for the subsequent association of hU3-55K in either nuclear extract or using *in vitro* translated protein. This demonstrates a hierarchical assembly of the U3 snoRNP in which 15.5K binds first to the box B/C motif in order to recruit hU3-55K. The binding of 15.5K to the box B/C motif functions to direct the specific association of hU3-55K to the U3 snoRNA. The association of hU3-55K is also dependent on the RNA elements surrounding the box B/C motif. This therefore suggests that 15.5K binding to the box B/C motif results in the creation of a highly specific binding site for hU3-55K. The crystal structure of 15.5K bound to the U4 5' stem-loop clearly showed that the binding of this protein has a dramatic effect on the folding of the RNA (37). The data presented in this paper strongly suggest that the box B/C motif probably folds into a stem-internal loop-stem structure very similar to that of the U4 5' stem-loop. It is therefore likely that 15.5K binds the box B/C motif in a similar manner. The binding of this protein to the snoRNA will have a profound effect on the structure of this region of the U3 snoRNA (see below). Thus, we believe that 15.5K binding to the box B/C motif functions to nucleate the assembly of the U3 box B/C motif RNP by the specific creation of the hU3-55K-binding site.

The 15.5K protein has now been shown to function as a nucleation factor in the hierarchical assembly of three distinct RNP complexes, namely the U3-specific box B/C RNP (this work), the box C/D core snoRNP (30), and the spliceosomal U4/U6 snRNP (38). In the box C/D core complex, 15.5K binding is essential for the association of NOP56, NOP58, fibrillarlin, TIP48, and TIP49. In addition, in the U4/U6 snRNP 15.5K binding is essential for the association of 61K (hPrp31) and the heteromeric 20/60/90K complex. Interestingly, hU3-55K and the 60K protein are both WD40 repeat proteins suggesting that there may be some similarity in the way they bind their respective 15.5K-RNA complexes.

*hU3-55K Is a WD40 Protein That Directly Binds RNA*—The results presented in this paper clearly show that there are two equally important elements required for the binding of hU3-55K to the U3 snoRNA. These are the 15.5K protein bound to the box B/C motif as well as the flanking RNA structures, in particular an internal loop structure (nucleotides 115-118/155-157) adjacent to the box B/C motif. Indeed, disruption of the secondary structure of the loop joining stem III and stem IV specifically disrupted the association of hU3-55K. The structure, but not the primary sequence of the U3 snoRNA elements important for hU3-55K association, appears to be conserved among the various U3 snoRNAs. This suggests that the specific structural arrangement of the U3 snoRNA, after the binding of 15.5K, is the essential requirement for hU3-55K binding. It has been shown previously in yeast that the stem-loops surrounding the box B/C motif are not essential for U3 function. Al-

though the data presented in this paper would predict, in the case of the human U3 snoRNP, that hU3-55K binding would be abolished, it is entirely possible that the association of the yeast protein is not affected by these changes or compensated by protein-protein interactions.

Due to the solubility problems experienced expressing hU3-55K in *E. coli*, we were not able to study the direct interaction between hU3-55K and the 15.5K-U3BC RNA complex using recombinant proteins. However, several points suggest that the hU3-55K interaction with the 15.5K-bound U3BC RNA complex is direct and not mediated by an additional protein(s). First, the binding of *in vitro* translated hU3-55K to the U3 snoRNA is completely dependent on the presence of recombinant 15.5K (Fig. 2D). Second, the purified yeast U3 snoRNP monoparticle contained just one U3-specific protein, namely Rrp9p (yeast homologue of hU3-55K (11, 19)). This suggests that, at least in yeast, no other U3-specific protein is required for the stable association of Rrp9p with the U3 snoRNA. Third, the hU3-55K protein specifically cross-links to the U3BC RNA. Finally, we have partially purified the U3BC RNP complex assembled in nuclear extract. This complex was shown, by Western blot of the recovered material, not to contain the human homologues of the U3-specific proteins Mpp10, Sof1, and Imp4. In addition, the core box C/D proteins NOP58, NOP56, and fibrillarin were also not associated with this RNA (data not shown). It was shown previously (39) that an intact box C, of the box B/C motif, was important for fibrillarin binding to the U3 snoRNA in HeLa total cell extracts. We did not observe fibrillarin binding to the U3BC RNA in our HeLa nuclear extract; however, under the same conditions we could observe fibrillarin binding to the box C/D motif (30). Furthermore, it has since been shown that the box B/C motif is not required for fibrillarin association with either the yeast or human U3 snoRNAs *in vivo* (40).<sup>2</sup> We therefore believe that the hU3-55K interacts directly with the 15.5K-bound U3BC RNA complex.

The main structural feature of the hU3-55K protein is the presence of seven WD40 repeats (this work and Refs. 17 and 31) that are generally thought to play a role in protein-protein interactions. Cross-link experiments demonstrated that the hU3-55K protein directly contacts the U3 snoRNA in our *in vitro* assembly reaction. Furthermore, we have shown that the WD40 repeats of hU3-55K are sufficient for the RNA-dependent interaction with the 15.5K-bound U3BC RNA. There was no observable interaction between hU3-55K and 15.5K in the absence of RNA suggesting that protein-RNA contacts play a significant role in the binding of the WD40 repeats to the U3BC RNA. To our knowledge, we believe that this is the first example of a WD40 repeat protein directly interacting with RNA, and it is likely that the hU3-55K interaction with the U3 snoRNA is mediated by one or more of the WD40 repeats.

**The U3 snoRNP Contains Two Independent 15.5K Containing RNP Complexes**—The 3' domain of the U3 snoRNA contains two 15.5K-binding sites, namely the C/D and B/C motifs. Several studies have shown that in the U3 snoRNA, the box B/C and box C/D motifs are functionally distinct. Mutations in box B/C motif appear not to affect the stability of the RNA *in vivo*, whereas mutations in the box C/D motif render the RNA unstable<sup>2</sup> (32). The box B/C motif alone was found to be sufficient for targeting the RNA to Cajal bodies, whereas the box C/D motif is essential for nucleolar localization (41). Furthermore, in contrast to the box B/C, the box C/D is essential for 5' cap hypermethylation (42). This functional distinctiveness be-

tween the box B/C and C/D motifs is likely due to the distinct sets of proteins that bind these two RNA elements.

The B/C motif is associated with hU3-55K and 15.5K, and the core box C/D motif is associated with 15.5K, NOP56, NOP58, and fibrillarin. How do these two RNA motifs, which are very similar in structure and both bound by 15.5K, direct the specific assembly of two distinct RNP complexes? In this paper, we show that a specific structural element, present between boxes B and C in the primary sequence, is essential for the recruitment of hU3-55K to the 15.5K-bound U3BC RNA. In contrast, recent work (30) has shown that the association of the core snoRNP proteins with the box C/D snoRNA-15.5K complex is dependent on the conserved sequence of stem II in the box C/D motif (corresponds to stem II in the U3BC RNA). Interestingly, the sequence of this stem is different in the B/C motif explaining why the core box C/D snoRNP proteins do not associate with the U3BC RNA. In addition, the structure but not the sequence of this stem in the box B/C motif is essential for both 15.5K and hU3-55K protein binding. Likewise, the conserved structural element found between boxes B and C in the U3 snoRNA is not present in the core box C/D motif. Therefore, the distinct flanking sequences/structures, surrounding the highly similar 15.5K-binding sites, provide the specificity for the recruitment of the additional, complex-specific proteins.

**The Structure of the 3' Domain of the U3 snoRNA**—The results presented in this paper provide a significant insight into the structure of the 3' domain of the U3 snoRNA. All the work presented here was performed with a sub-fragment of the U3 snoRNA; however, the U3BC RNA contained all of the sequences necessary for the binding of hU3-55K. Therefore, we believe that this reflects what occurs in the full-length RNA. This approach has been used previously (31, 33, 41, 42) in the systematic analysis of the U3 snoRNA *in vitro* and *in vivo*. Furthermore, since there are two 15.5K-binding sites in the U3 snoRNA, it is essential to physically separate these two motifs in order to enable the clear analysis of the binding requirements of this protein in the BC region of the U3 snoRNA.

The mutational analysis of the U3BC RNA has not only resulted in the definition of the binding sites for the 15.5K and hU3-55K proteins, it has also provided convincing functional evidence for our model of the U3 snoRNA. In this model, we have brought together the boxes B and C to form stems II and III in the 3' domain of the U3 snoRNA. Stem III is only 2 bp in length; however, this interaction is feasible in all U3 snoRNAs examined even though in some cases only one potential base pair is found. Our mutational analysis has shown the importance of base pairing in stem II for 15.5K binding. Furthermore, stem IV appears to be necessary to assist in the formation or stabilization of stem III. This point is emphasized by the dramatic increase in 15.5K binding that was observed upon closing the small internal loop between stems III and IV. These data are therefore highly suggestive that the short stem III, which is probably stable only upon 15.5K binding, is found in the U3 snoRNP. The short nature of this stem is probably necessary to form the correct structure for hU3-55K binding. Base pairing in stem II of the B/C RNA is also required for 15.5K binding. This stem contains a G-C base pair that is found in all of the available U3 snoRNA sequences. Although the structure, but not the sequence, of this base pair is essential for 15.5K binding, it is possible that this conserved element may play a role, perhaps along with the hU3-55K protein, in the sequence-specific recruitment of additional U3-specific proteins.

**Acknowledgments**—We thank Stuart Maxwell for the U14 and U3 plasmids; Hossein Kohansal and Peter Kempkes for excellent technical assistance; Heiko Manninga for providing 4-thio-UTP and help with

<sup>2</sup> S. Granneman, N. J. Watkins, J. Vogelzangs, R. Lührmann, W. J. van Venrooij, and G. J. M. Pruijn, manuscript in preparation.

cross-linking; Claudia Schneider for critically reading the manuscript; and Sylviane Muller for generating the anti-hU3-55K antibodies.

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**RNA: STRUCTURE METABOLISM AND  
CATALYSIS:**

**The hU3-55K Protein Requires 15.5K  
Binding to the Box B/C Motif as Well as  
Flanking RNA Elements for Its Association  
with the U3 Small Nucleolar RNA *in Vitro***

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*J. Biol. Chem.* 2002, 277:48490-48500.

doi: 10.1074/jbc.M206631200 originally published online October 14, 2002

Access the most updated version of this article at doi: [10.1074/jbc.M206631200](https://doi.org/10.1074/jbc.M206631200)

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