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The U11/U12 snRNP 65K protein acts as a molecular bridge, binding the U12 snRNA and U11-59K protein

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U11 and U12 interact cooperatively with the 5′ splice site and branch site of pre-mRNA as a stable preformed di-snRNP complex, thereby bridging the 5′ and 3′ ends of the intron within the U12-dependent prespliceosome. To identify proteins contributing to di-snRNP formation and intron bridging, we investigated protein–protein and protein–RNA interactions between components of the U11/U12 snRNP. We demonstrate that the U11/U12-65K protein possesses dual binding activity, interacting directly with U12 snRNA via its C-terminal RRM and the U11-associated 59K protein via its N-terminal half. We provide evidence that, in contrast to the previously published U12 snRNA secondary structure model, the 3′ half of U12 forms an extended stem-loop with a highly conserved seven-nucleotide loop and that the latter serves as the 65K binding site. Addition of an oligonucleotide comprising the 65K binding site to an *in vitro* splicing reaction inhibited U12-dependent, but not U2-dependent, pre-mRNA splicing. Taken together, these data suggest that U11/U12-65K and U11-59K contribute to di-snRNP formation and intron bridging in the minor prespliceosome.

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Introduction

Two spliceosomes, the multi-subunit complexes that catalyze pre-mRNA splicing, have been identified in higher eukaryotes (Burge *et al*, 1999). The U2-dependent (major) spliceosome is found in all eukaryotes and excises U2-type introns, which represent the vast majority of pre-mRNA introns. The U12-dependent (minor) spliceosome is found in only a subset of organisms and removes U12-type introns, which constitute less than 1% of introns in humans (Burge *et al*, 1998; Levine and Durbin, 2001). In contrast to U2-type introns, they contain highly conserved sequences at their 5′ splice site and branch site, but lack a polypyrimidine tract upstream of the 3′ splice site (Burge *et al*, 1998). U12-type introns coexist with U2-type introns within the same gene, but are spliced more slowly, indicating that U12-type splicing is a rate-determining step in gene expression (Patel *et al*, 2002). The U12-dependent spliceosome contains the U11, U12, U5 and U4atac/U6atac snRNPs, whereas the U2-dependent spliceosome consists of the U1, U2, U5 and U4/U6 snRNPs (reviewed by Patel and Steitz, 2003). U11, U12 and U4atac/U6atac have been shown to be functional analogs of U1, U2 and U4/U6, respectively (Hall and Padgett, 1996; Tarn and Steitz, 1996a, b; Kolossova and Padgett, 1997; Yu and Steitz, 1997).

The assembly pathways of both spliceosomes are very similar, with major differences occurring only during the earliest stages (Patel and Steitz, 2003). Major spliceosome assembly is initiated by interaction of U1 with the 5′ splice site, followed by the stable interaction of U2 with the branch site, forming the so-called prespliceosome. Subsequent entry of the U4/U6.U5 tri-snRNP triggers structural rearrangements that lead to catalytic activation of the spliceosome. Formation of the U12-type prespliceosome is initiated by association of the U11 and U12 snRNPs, which, in contrast to U1 and U2, bind as a highly stable, preformed di-snRNP. U11 and U12 recognize the 5′ splice site and branch site, respectively, in a cooperative manner (Frilander and Steitz, 1999). A catalytically active U12-type spliceosome is formed after integration of the U4atac/U6atac.U5 tri-snRNP and subsequent conformational changes (Tarn and Steitz, 1996b; Yu and Steitz, 1997; Frilander and Steitz, 2001). The excision of both types of introns occurs via two successive transesterification reactions, and an analogous, dynamic network of snRNA–snRNA and snRNA–pre-mRNA interactions is formed in both splicing machineries (Patel and Steitz, 2003).

Spliceosome assembly and splicing catalysis require the activity of numerous proteins, as well as the formation of an extensive network of RNA/protein and protein/protein interactions. The major spliceosome is well characterized in terms of its protein composition, with over 150 proteins identified by mass spectrometry (reviewed by Jurica and Moore, 2003). Insight into the protein composition of the minor spliceosome is now slowly emerging. Many proteins appear to be shared by both spliceosomes, including most U4/U6.U5 tri-snRNP proteins (Luo *et al*, 1999; Nottrott *et al*, 2002; Schneider *et al*, 2002), the U2-associated complex SF3b (Will *et al*, 1999, 2004) and SR proteins (Hastings and Krainer, 2001). Recently, the human U11/U12 di-snRNP particle was shown to contain not only SF3b and Sm proteins, but also seven novel proteins (denoted 65K, 59K, 48K, 35K, 31K, 25K and 20K) not found in U2-type spliceosomes (Will *et al*, 2004). Four of these proteins (59K, 48K, 35K and 25K) are U11 associated and thus potentially facilitate the U11/5′ splice site interaction.

The existence of unique U11/U12 proteins indicates that many interactions contributing to minor prespliceosome formation, in particular those mediating 5′ splice site

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recognition and intron bridging, differ from those in the major prespliceosome. To date, little is known about protein–protein interactions juxtaposing the ends of U12-type introns. As U11 and U12 bind in the form of a stable di-snRNP to the 5′ and 3′ ends of U12-type introns, U11/U12-specific proteins have been proposed to be involved in intron bridging interactions in the minor prespliceosome. During the earliest step of major spliceosome assembly, a molecular bridge is formed between U1 at the 5′ splice site and SF1/mBBP and U2AF, which bind to the branch site and the polypyrimidine tract, respectively (reviewed by Reed, 1996). SR proteins play a major role in intron bridging at this stage, binding the U1-70K protein and U2AF. Upon association of U2 with the branch site, SF1/mBBP is displaced and a new set of interactions is thought to mediate intron bridging in the major prespliceosome. The DEAD-box protein Prp5 appears to play a role in bridging the U1 and U2 snRNPs (Xu *et al*, 2004). However, the precise nature of the molecular bridge juxtaposing the reactive groups of U2-type pre-mRNAs at this stage is not clear.

At present, little is known about the molecular architecture of the U11/U12 di-snRNP, including which of the U11/U12 proteins contact the U11 and U12 snRNAs. Candidate snRNA binding proteins include the U11/U12-65K, -35K and -31K proteins, which contain one or more RNA recognition motifs (RRMs), a structurally well-characterized domain found in a large family of RNA binding proteins (Birney *et al*, 1993). The RRM contains two highly conserved submotifs, the RNP-1 octamer and RNP-2 hexamer, and has a βαββαβ structure that folds into a four-stranded antiparallel β-sheet that packs against the two α-helices (reviewed by Varani and Nagai, 1998). Protein–protein interactions within the U11/U12 snRNP, which potentially mediate di-snRNP formation and/or intron bridging, also remain to be elucidated. Here we have investigated the protein and RNA binding properties of polypeptides of the U11/U12 di-snRNP. These studies provide the first information regarding intermolecular interactions within the U11/U12 snRNP and reveal potential players in di-snRNP formation.

Results

The 65K protein interacts with U12 snRNA via its C-terminal RRM

The human U11/U12-65K protein contains an N- and C-terminal RRM and a centrally located proline-rich region (Figures 1 and 2B). Database searches revealed likely 65K

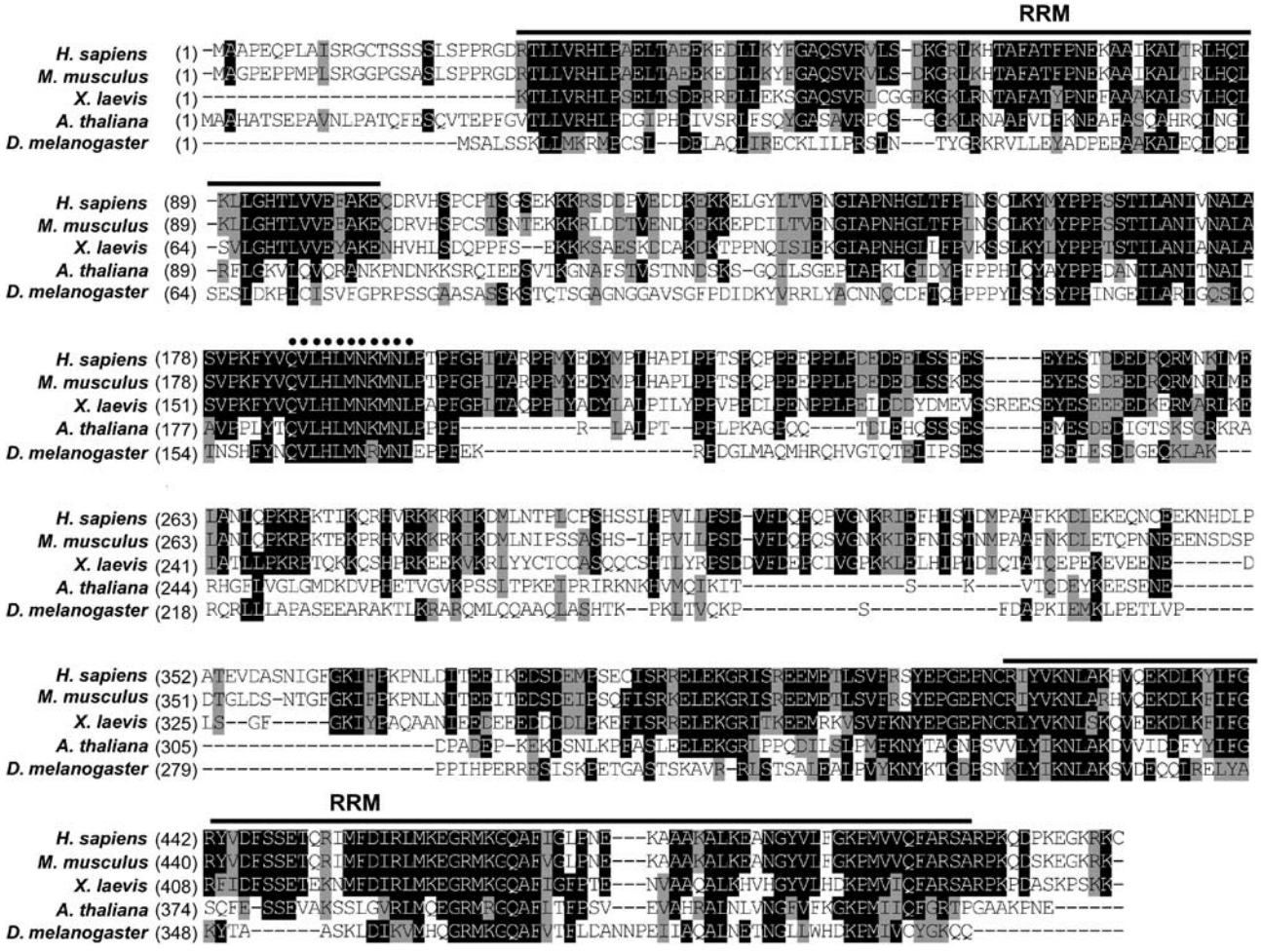


Figure 1 The U11/U12-65K protein is evolutionarily conserved. Sequence alignment of the *H. sapiens* U11/U12-65K protein (gi|48427628) with putative orthologs from *M. musculus* (gi|31981077), *X. laevis* (deduced from ESTs gi|26035864 and gi|17496030; note that the 5′ end appears to be incomplete), *A. thaliana* (gi|15217461) and *D. melanogaster* (gi|21626517). Residues identical in at least three proteins are highlighted in black and conserved residues (gray) are grouped as follows: (D, E), (K, R), (A, S, T, G, C), (N, Q), (Y, F, M, H), (I, L, M, V) and (P). RRMs are indicated by a bar. The conserved sequence QVLHLMN(K/R)MNL is marked by dots.

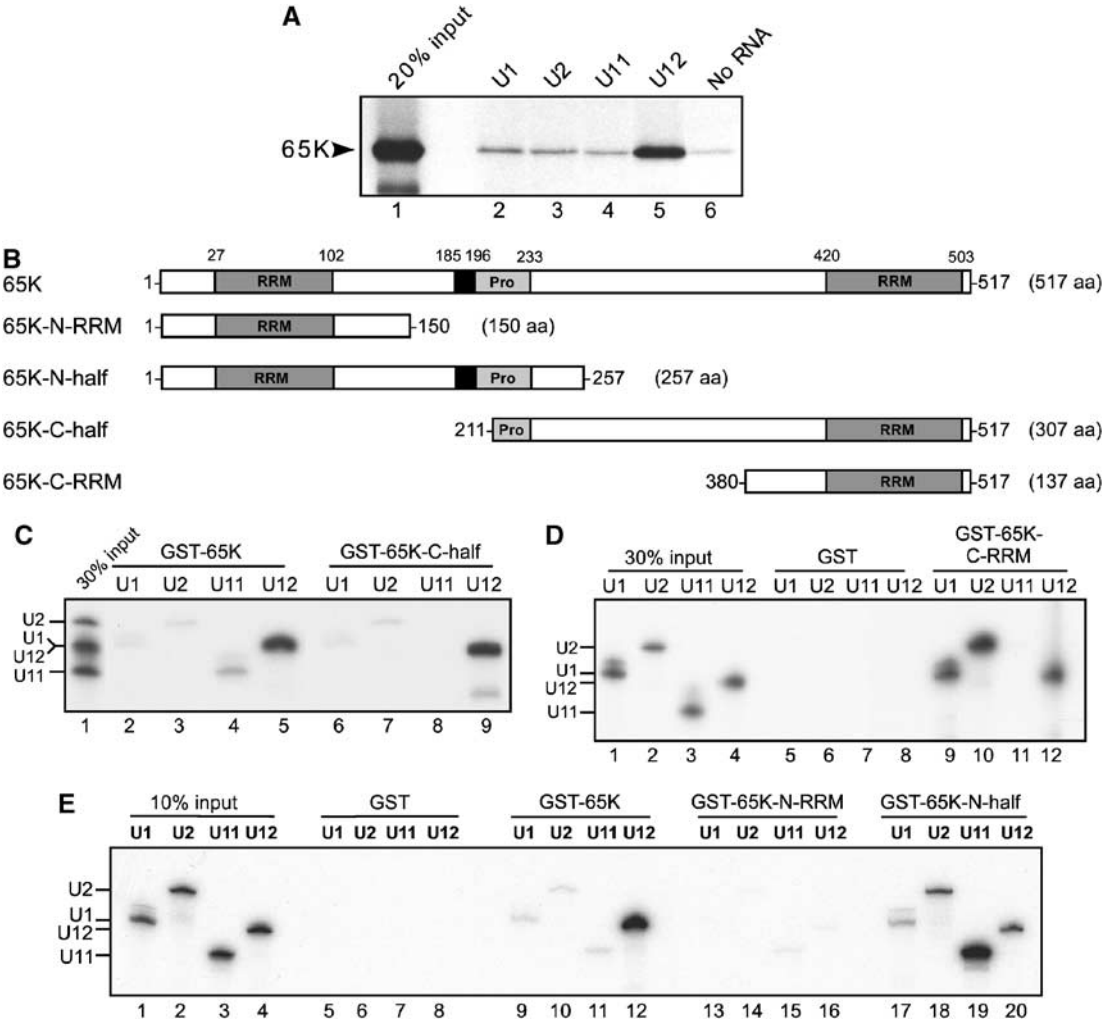


Figure 2 65K binds U12 snRNA via its C-terminal RRM. (**A**) ^{35}S-labeled, *in vitro*-translated 65K was incubated with *in vitro*-transcribed, m^{7}G-capped U1, U2, U11 or U12 snRNA (lanes 2–5, as indicated) or no RNA (lane 6) and co-IPs with anti-cap antibodies were performed as described in Materials and methods. Lane 1: 20% of input 65K. (**B**) Schematic of 65K deletion mutants used in GST pulldowns. RRMs and the proline-rich region (Pro) are shaded in gray and the QVLHLMN(K/R)MNL conserved motif in black. (**C–E**) GST pulldowns of *in vitro*-transcribed, ^{32}P-labeled UsnRNAs (indicated above each lane) by GST-65K wild-type or deletion mutants (as indicated). (C) Lane 1: 30% of a mixture of input snRNAs. (D, E) Lanes 1–4: 30 or 10% of each input snRNA. RNAs were separated by denaturing PAGE and visualized by autoradiography.

orthologs in a variety of organisms known to contain U12-type introns. An alignment of a subset of these (Figure 1) revealed that the majority of sequence conservation lies within the RRMs, with the C-terminal RRM exhibiting higher homology than its N-terminal counterpart—that is, 64% (amino acids (aa) 27–102) versus 49% (aa 420–503) when comparing human with *Arabidopsis thaliana*. In *Drosophila melanogaster*, the N-terminal region has diverged so significantly that it no longer is recognized as an RRM by motif search programs. A 100% conserved sequence, QVLHLMN(K/R)MNL (aa 185–195 in humans), is found downstream of the N-terminal RRM and is detected only in putative 65K orthologs in the nonredundant (nr) database of the National Center for Biotechnology Information (NCBI).

To test whether 65K directly binds U11 and/or U12 snRNA, we translated ^{35}S-labeled 65K protein *in vitro* and assayed its interaction with *in vitro*-transcribed, m^{7}G-capped U1, U2, U11 and U12 snRNA. RNA was precipitated with anti-cap antibodies and co-immunoprecipitation (co-IP) of 65K was analyzed by SDS–PAGE. Whereas the level of 65K co-precipitated with U1, U2 and U11 did not exceed background (Figure 2A, cf. lanes 2–4 and 6), 65K was clearly co-precipitated with U12 snRNA (lane 5). We next overexpressed 65K as a GST fusion and performed GST pulldowns with ^{32}P-labeled U1, U2, U11 or U12 snRNA. No co-precipitation was observed with GST alone (Figure 2D and E, lanes 5–8). In contrast, a significant amount of U12 (~49%) and only a low level of U1, U2 and U11 (~1–4%) co-precipitated with GST-65K (Figure 2C, lanes 2–5; Figure 2E, lanes 9–12). Thus, 65K specifically binds the U12 snRNA.

To determine which RRM of 65K binds U12, several deletion mutants were created (Figure 2B) and expressed as GST fusion proteins. These include mutants containing the N-terminal RRM plus C-terminal extensions of 48 aa (65-N-RRM) or 155 aa (65K-N-half), and those possessing the C-terminal RRM plus N-terminal extensions of 40 aa (65K-C-RRM) or 209 aa (65K-C-half). Pulldown of U12 snRNA was observed with GST-65K-C-half and GST-65K-C-RRM (Figure 2C, lane 9; Figure 2D, lane 12), but not with GST alone or GST-65K-N-RRM (Figure 2E, lanes 8 and 16), or only to a low level with GST-65K-N-half (Figure 2E, lane 20). Enhanced precipitation of U11 snRNA was observed with

GST-65K-N-half (Figure 2E, lane 19) but no other deletion mutant (Figure 2C, lane 8; Figure 2D, lane 11; Figure 2E, lane 15). However, as precipitation of U11 was not observed with full-length 65K, and 65K is not a component of U11 monoparticles (Will *et al*, 2004), U11 binding does not appear to be a property of the wild-type protein. Upon truncation of 65K to within 40 aa of the C-terminal RRM (65K-C-RRM), binding specificity was lost to some extent; both U1 and U2, but not U11, were also efficiently precipitated (Figure 2D, lanes 9–12). Likewise, in electrophoretic mobility shift assays (EMSA), GST-65K-C-RRM interacted with U12, U1 and U2, but not U11, U4 or U5 (not shown). These results indicate that residues 211–380 contribute to 65K binding specificity. Indeed, the N- and C-terminal extensions of RRMs often play important roles in the specificity of RNA recognition (reviewed by Varani and Nagai, 1998). Taken together, we conclude that 65K directly binds the U12 snRNA via its C-terminal RRM.

The C-terminal RRM of 65K and N-terminal RRM of U1-A /U2-B″ are highly homologous

The ability of 65K-C-RRM to additionally bind both U1 and U2 (but not other snRNAs) suggests that 65K may be evolutionarily related to the U1 snRNP A protein (U1-A) and the U2 snRNP B″ protein (U2-B″). These proteins are highly related at the amino-acid sequence level and, similar to 65K, both contain N- and C-terminal RRMs (Figure 3A). However, in contrast to 65K, both U1-A and U2-B″ bind their cognate snRNAs (i.e., U1 stem-loop (SL) II and U2 SLIV, respectively) via their N-terminal RRMs (Varani and Nagai, 1998 and

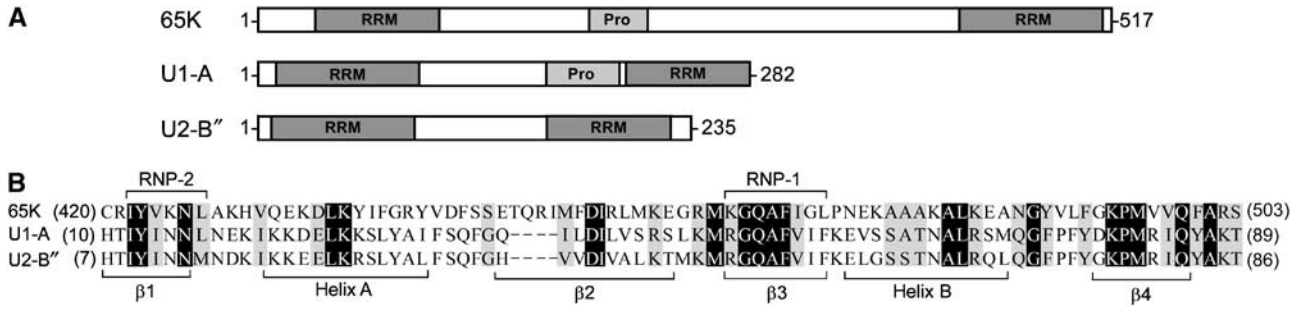


Figure 3 The C-terminal RRM of 65K is homologous to the N-terminal RRM of U1-A and U2-B″. (**A**) Domain structure of the human 65K, U1-A and U2-B″ proteins. (**B**) Sequence alignment of the C-terminal RRM of 65K (nt 420–503) and the N-terminal RRMs of U1-A (nt 10–89) and U2-B″ (nt 7–86). Identical amino acids are highlighted in black and conserved residues are grouped as in Figure 1. Secondary structure elements according to the atomic structures of U1-A and U2-B″ are indicated below.

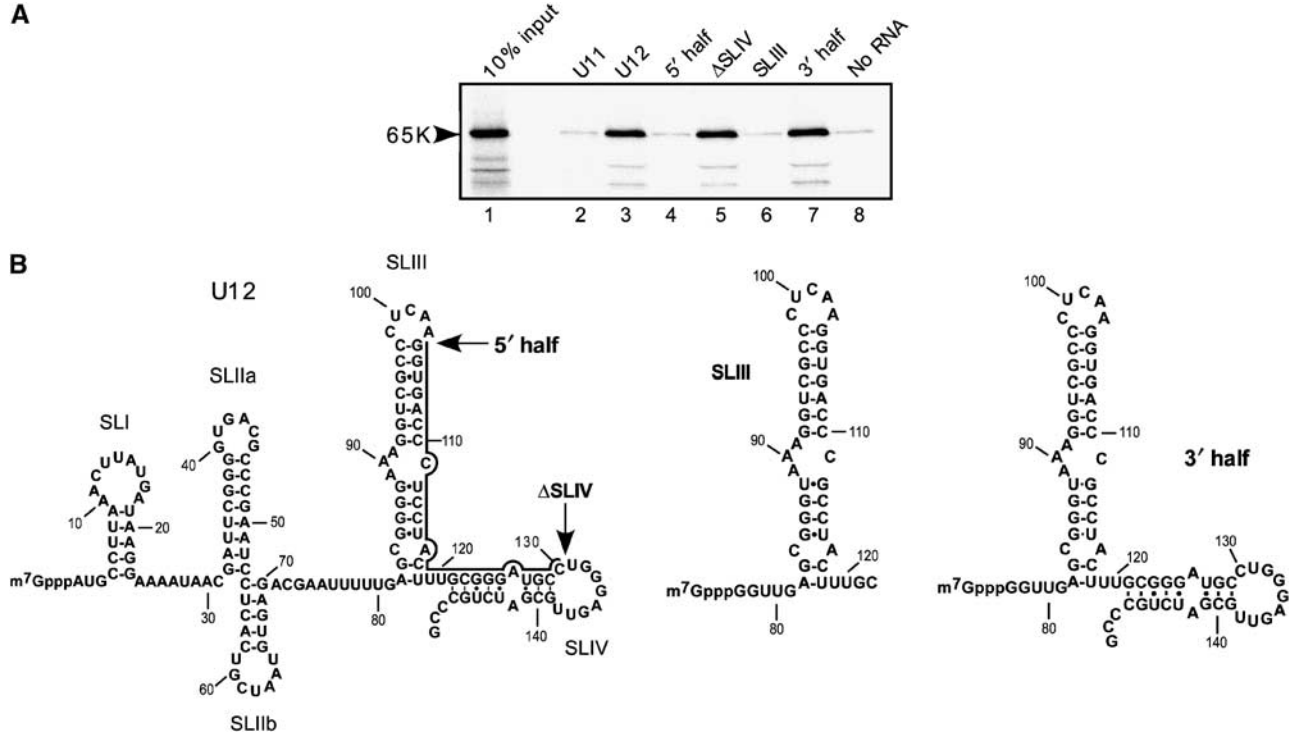


Figure 4 The 65K protein binds the 3′ half of the U12 snRNA. (**A**) Co-IPs were performed as described in the legend to Figure 2 with ^{35}S-labeled 65K and m^{7}G-capped U11 (lane 2), U12 (lane 3) or U12 deletion mutants 5′ half, ΔSLIV, SLIII or 3′ half (lanes 4–7) or no RNA (lane 8). (**B**) Structure of wild-type and truncated U12 snRNAs (according to Wassarman and Steitz, 1992). 5′ half: nt 1–104; ΔSLIV: nt 1–130; SLIII: nt 79–122; 3′ half: nt 79–150. Digestion sites generating 5′ half and ΔSLIV are indicated by an arrow and nucleotides (104–130) required for 65K binding are indicated by a line.

references therein). Intriguingly, aside from 65K orthologs, the C-terminal RRM of human 65K shares one of the highest degrees of homology (i.e., 50%) with the N-terminal RRM of U1-A and U2-B″ from various organisms, when BLAST searches are performed with the NCBI nr database. The RNP-1 and RNP-2 submotifs of those RRMs of 65K and U1-A/U2-B″ known to bind RNA are highly conserved (Figure 3B); only the last two residues of RNP-1 and the fourth residue of RNP-2 are not conserved, and all three proteins contain a glutamine (Q) at the third position of their RNP-1 motif as opposed to an aromatic residue typically found in the vast majority of RNP-1 motifs (Birney *et al*, 1993 and references therein). Thus, it is conceivable that the C-terminal RRM of 65K may have diverged from the N-terminal RRM of U1-A/U2-B″ or *vice versa*.

65K binds the 3′ half of the U12 snRNA

The proposed structure of human U12 (Figure 4B) resembles that of U2 snRNA. Three SLs in its 5′ half (designated I, IIa and IIb) are separated from SLIII and IV by the single-stranded Sm site. To map the binding site of 65K, we performed co-IPs with ^{35}S-labeled 65K and *in vitro*-transcribed m^{7}G-capped deletion mutants of human U12 (Figure 4). Deletion of 19 nucleotides (nt) from the 3′ end (mutant ΔSLIV), which disrupts SLIV, had no effect on 65K binding (Figure 4A, cf. lanes 3 and 5). However, truncation to nt 103 (mutant 5′ half), which leads to the loss of both SLIII and IV, reduced 65K binding to background levels (cf. lanes 4 and 8), suggesting that SLIII is required for 65K interaction. Surprisingly, a U12 snRNA consisting solely of SLIII did not support 65K binding (lane 6). In contrast, when both SLIII and IV were present (3′ half), wild-type binding of 65K was observed (lane 7). Thus, the 3′ half of U12 is sufficient for 65K interaction, and nucleotides within positions 104–130 are required for binding. The latter is unexpected as RRM-containing proteins typically bind single-stranded nucleotides (Varani and Nagai, 1998) and this region contains predominantly base-paired nucleotides in the current U12 model.

The 3′ half of U12 snRNA can adopt an alternative structure

To determine whether alternative structures of the human U12 snRNA might exist, putative secondary structures were generated using the MFOLD prediction program (Zuker, 2003). Indeed, the most energetically favorable U12 structure generated differed considerably in its 3′ half (but not 5′ half) from that proposed by Wassarman and Steitz (1992). That is, SLIII and IV were replaced by a single extended SL structure containing a 7-nt loop (Figure 5A). Strikingly, the terminal hairpin contains nucleotides required for 65K binding. In contrast, the structure of the human U2 snRNA predicted by the MFOLD program did not differ significantly from the published one (not shown).

Previous chemical/enzymatic structure probing of human U12 snRNA provide limited information about the structure of its 3′ half (Wassarman and Steitz, 1992), but the results obtained are consistent with both the previously proposed U12 structure and that generated by MFOLD. To obtain additional structural information, *in vitro*-transcribed U12 snRNA was incubated with increasing concentrations of lead acetate and cleavage sites were identified by primer extension analyses (Figure 5B). At higher lead concentrations, nt 114–120 and A102 were cleaved, as evidenced by an increase in reverse transcriptase stops at these positions (lanes 8–10). Similar results were obtained with endogenous U12 snRNA purified from HeLa U12 snRNPs (not shown). As lead(II) ions specifically cleave phosphodiester bonds of single-stranded nucleotides (Brunel and Romby, 2000), these results support the existence of the 7-nt loop at positions 114–120 (Figure 5C). In contrast, in the published U12 structure, four of these nucleotides are base-paired and thus should not be cleaved. Additional primer extension analyses also revealed cleavage sites at A89 and A90 (not shown). Significantly, only one of 5 nt comprising loop III in the published structure (i.e., A102) (Figure 5D) was cleaved (Figure 5B, lanes 7–10). Owing to their proximity to the 3′ end of U12, only a subset of nucleotides proposed to comprise loop IV (i.e., nt 130–133) could be analyzed. Significantly, no cleavage was observed at these positions (Figure 5B, lanes 7–10). These data provide additional evidence that the 3′ half of human U12 folds into a single stem with a 7-nt terminal loop.

A single, extended 3′ SL is an evolutionarily conserved feature of U12 snRNA

In addition to humans, U12 snRNAs have been identified in a number of organisms including *Mus musculus*, *Gallus gallus*, *Xenopus laevis*, *A. thaliana* and *D. melanogaster* (Shukla and Padgett, 1999; Otake *et al*, 2002). We have identified putative U12 snRNAs from *Pan troglodytes* (chimp), *Oryza sativa* (rice), *Apis mellifera* (honeybee), *Anopheles gambiae* (mosquito), *Drosophila pseudoobscura* (fruit fly), *Fugu rubripes* (pufferfish) and *Ciona intestinalis* (sea squirt) by performing BLAST searches with the human U12 snRNA. Putative structures of the 3′ half of U12 from various organisms were generated by the MFOLD program; a subset of these is shown in Figure 6A. Significantly, the 3′ halves of all U12 snRNAs identified to date theoretically adopt a similar conformation, namely a single SL containing a 7-nt (or 8-nt in case of *A. thaliana* and *D. pseudoobscura*) loop. The length and sequence of the 3′ stem, as well as the nature and number of internal loops, varies. However, 4 nt of the loop (i.e., UACYU) and the loop-closing C-G base pair are 100% conserved (Figure 6B). An alignment of the loop and adjacent stem nucleotides yields the loop consensus sequence YUACYUY, where Y is a pyrimidine. Thus, an extended 3′ SL with a conserved loop appears to be a common feature of all U12 snRNAs.

U11/U12-65K binds the 7-nt loop of the extended 3′ SL of U12

To determine whether 65K binds the terminal loop of the 3′ SL of U12, we chemically synthesized nt 109–125 of human U12, which comprise the 7-nt loop plus a five base pair stem (Figure 7A; designated U12wt oligo). Indeed, ^{32}P-labeled U12wt oligo was precipitated more efficiently than a control oligonucleotide (the complementary sequence of U12wt) by both GST-65K and GST-65K-C-RRM (but not GST) in GST pulldowns, indicating that this region of U12 serves as the 65K binding site (Figure 7B). EMSAs with GST-65K-C-RRM or GST-65K-C-half and ^{32}P-labeled U12wt oligo or full-length U12 snRNA were subsequently performed. Owing to its inefficient expression and instability in *Escherichia coli*, full-length recombinant 65K protein could not be used in these studies. The apparent K_D of both GST fusion proteins with

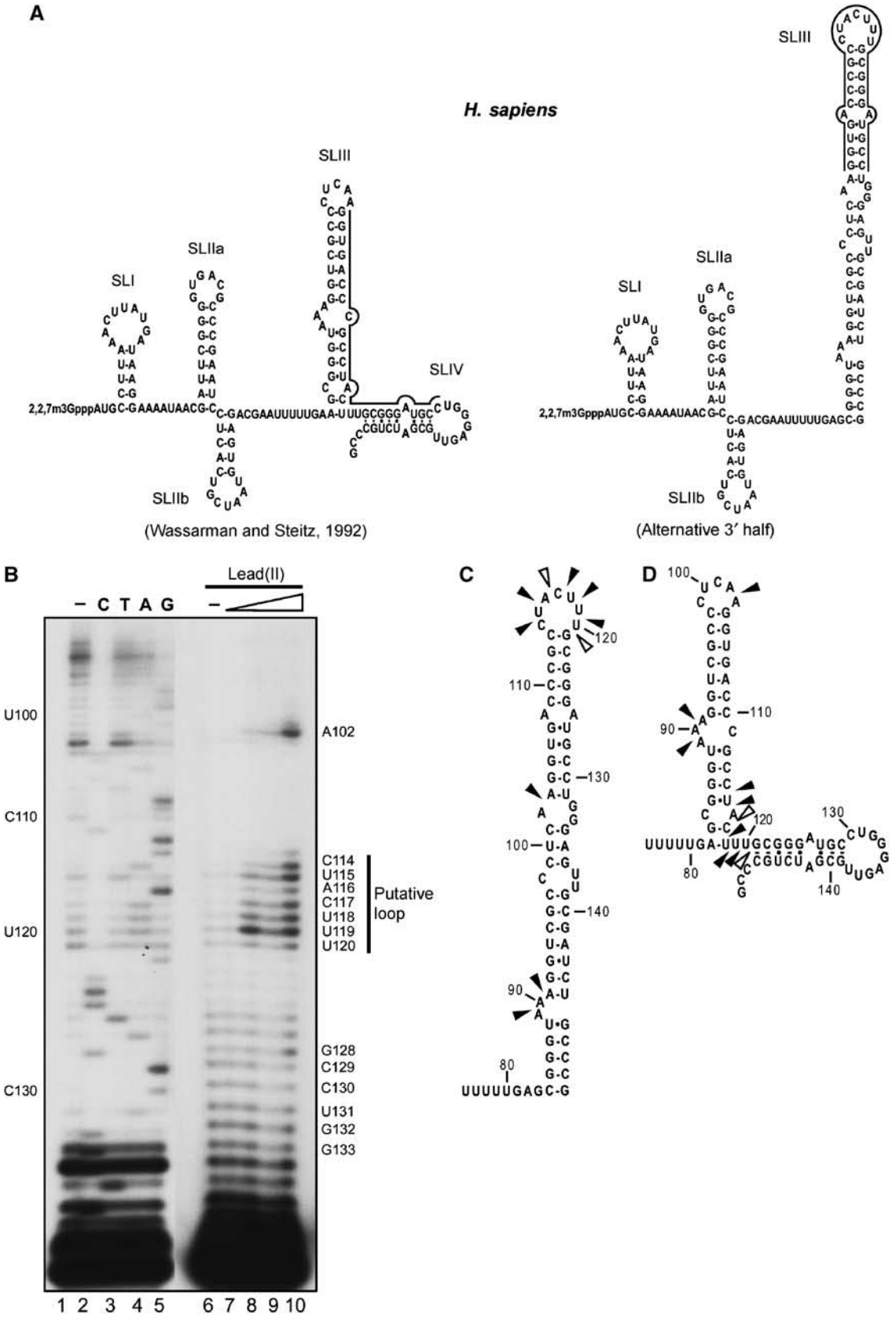
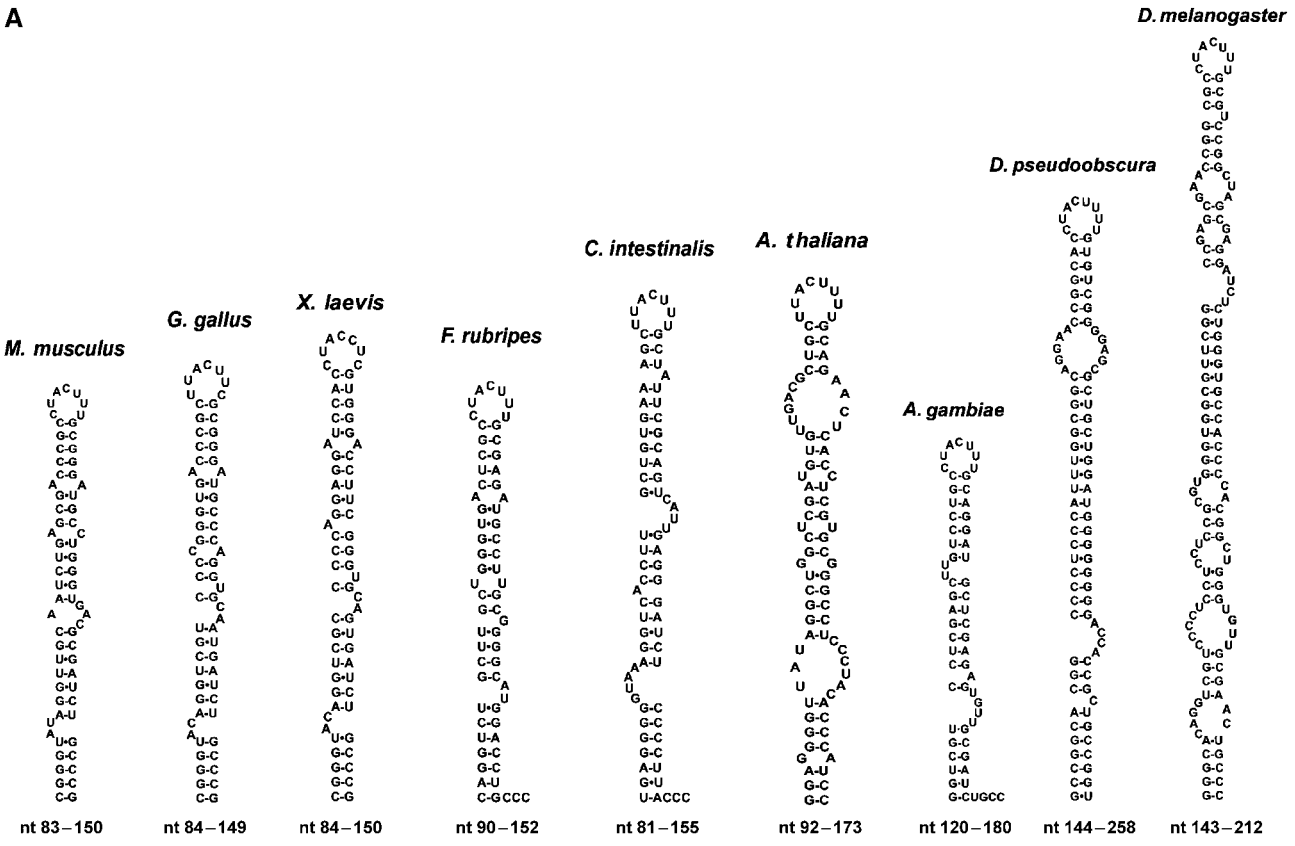


Figure 5 The 3′ half of U12 snRNA can adopt an alternative structure. (**A**) Secondary structure of the human U12 snRNA as proposed by Wassarman and Steitz (1992) (left) or generated by the MFOLD secondary structure prediction program (right). Nucleotides required for 65K binding are indicated by a line. (**B**) Lead(II)-induced cleavage of human U12 snRNA. Primer extension was performed after treatment of *in vitro*-transcribed U12 snRNA with 0, 1, 5, 10 and 50 mM lead acetate (lanes 6–10) using a primer complementary to nt 139–150. A sequencing ladder (lanes 2–5) was generated by performing primer extension of U12 snRNA in the presence of dideoxynucleotides. Lane 1: reaction without ddNTPs. Nucleotide positions are indicated on the right. Summary of cleaved sites in the alternative (**C**) or previously published (**D**) 3′ half structure of human U12 snRNA. Weak (open arrowheads) and strong (closed arrowheads) cleavage sites are indicated.



B

	Stem	Loop	Stem
H. sapiens	CCCGC	CUACUUU	GCGGG
M. musculus	CCCGC	CUACUUU	GCGGG
G. gallus	CCCGC	UUACUUC	GCGGG
X. laevis	UCCAC	CUACCUC	GUGGG
F. rubripes	CUCGC	CUACUUU	GCGAG
C. intestinalis	AGC	UUACUUU	GCU
A. thaliana	CUGC	UUACUUUU	GCAG
A. gambiae	CCUGC	CUACUUU	GCAGG
D. pseudoobscura	GGCAC	CUACUUUU	GUGUC
D. melanogaster	GGCGC	CUACUUU	GCGUC
Consensus	C	YUACYUY	G

Figure 6 An extended 3′ SL in U12 snRNA is evolutionarily conserved. (**A**) Predicted secondary structures of the 3′ half of U12 from various organisms (as indicated) were generated by the MFOLD program. (**B**) Phylogenetic comparison of nucleotides comprising the terminal loop and adjacent stem of the 3′ SL of U12.

full-length U12 (i.e., protein concentration required for 50% complex formation) was 0.5 μM (Figure 7C, lanes 5–11; not shown), which is within the range typically observed for RRM-containing proteins (i.e., 10^{-11}–10^{-6} M; Varani and Nagai, 1998). In contrast, the apparent K_D observed with the U12wt oligo was 10 μM for GST-65K-C-RRM (Figure 7C, lanes 1–4) and greater than 30 μM for GST-65K-C-half (not shown), indicating an ~20-fold higher affinity of 65K for full-length U12. Consistent with these results, in competition studies, a much higher excess of the U12wt oligo (~10-fold), as compared to the U12 snRNA, was required to compete out complex formation between 65K-C-RRM and U12 snRNA (not shown). Removal of GST or extending the stem of U12wt oligo by two base pairs (to stabilize the hairpin) had little or no effect on 65K binding affinity (not shown). These results indicate that nucleotides comprising the loop and adjacent stem of the extended 3′ SL of U12 are recognized by the 65K protein, but that additional U12 nucleotides contribute to 65K binding affinity.

To determine which nucleotides of the 3′ loop and adjacent stem are required for 65K binding, we mutated one or more nucleotides of the U12wt oligo (Figure 7A) and performed EMSAs with GST-65K-C-RRM. Mutation of conserved loop nucleotides U115, A116 and C117 (i.e., exchanging a pyrimidine with a purine and *vice versa*; mutants L1, L2 and L3) abolished 65K–RNA complex formation (Figure 7D, lanes 9–20). Conversion of the conserved C-G loop-closing base pair to U-A (mutant S1) severely reduced complex formation (lanes 21–24). Disruption of the stem (mutant S2) nearly abolished complex formation (lanes 25–28) indicating that, like U1-A and U2-B″, an SL structure, and not single-stranded RNA alone, is required for binding. Changing the orientation of the loop-closing C-G base pair to a G-C base pair (mutant S4) had only a moderate effect, whereas altering the entire

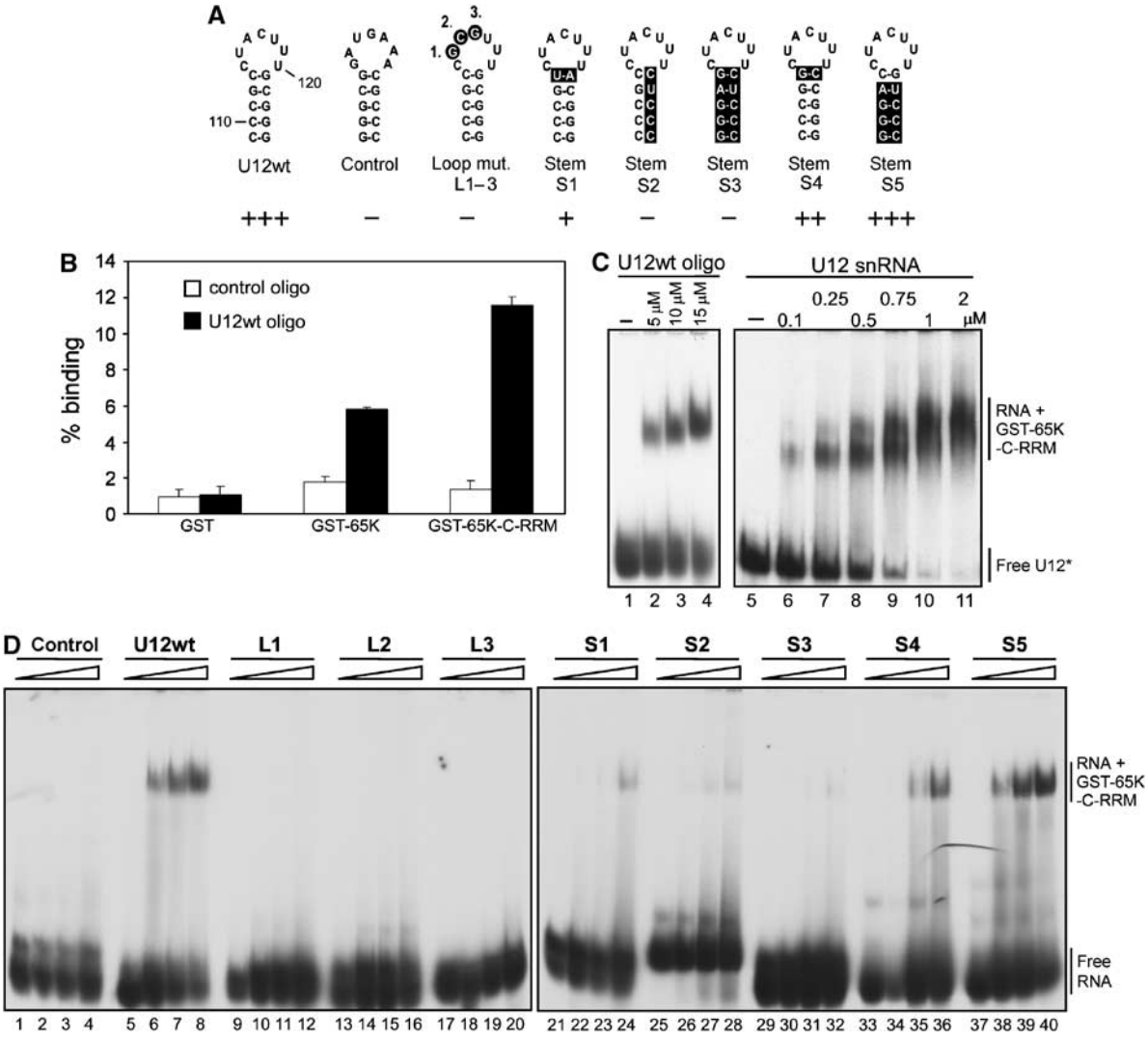


Figure 7 The 65K protein binds the terminal hairpin of the extended 3′ SL of U12. (**A**) Sequence of the wild-type U12 SL oligonucleotide (U12wt) and mutants (L1–L3 and S1–S5). Altered nucleotides are highlighted in black. The relative affinity of 65K-C-RRM for each mutant is indicated by plusses or a minus. (**B**) 65K and 65K-C-RRM interact with U12wt in GST pulldowns. (**C**) EMSA of 65K-C-RRM with U12wt oligo versus full-length U12 snRNA. Increasing concentrations of GST-65K-C-RRM (as indicated above each lane) were incubated with 0.01 or 0.25 pmol of the indicated oligonucleotide (lanes 1–4) or RNA (lanes 5–11), respectively. RNP complex formation was analyzed by native gel electrophoresis and visualized by autoradiography. Note that similar results were obtained when equimolar amounts (10 μM each) of U12wt oligo and U12 snRNA were used. (**D**) Effect of U12wt oligo mutation on 65K-C-RRM interaction. ^{32}P-labeled oligos (as indicated above) were incubated with increasing concentrations of GST-65K-C-RRM (0, 5, 10 or 25 μM) and analyzed as in panel C.

sequence of the stem (S3) nearly abolished binding (lanes 29–36). However, altering all stem nucleotides with the exception of the C-G loop-closing base pair (mutant S5) had no effect (lanes 37–40). Thus, an SL structure, as well as the identity of several of the loop nucleotides and the loop-closing base pair, plays a critical role in the 65K/3′ SL interaction.

An excess of the U12wt oligo inhibits U12-dependent splicing in vitro

To investigate the role of the 65K–U12 snRNA interaction in U12-dependent pre-mRNA splicing, splicing was carried out in HeLa nuclear extract with ^{32}P-labeled U12-type P120 pre-mRNA in the presence of a molar excess (relative to U12) of the U12wt or S5 mutant oligonucleotides, both of which efficiently bind 65K (Figure 7D). Addition of these oligonucleotides is expected to compete for 65K binding and functionally sequester it in the splicing reaction. L3, which contains a single point mutation that abolishes 65K binding, was also used to control for nonspecific effects of oligonucleotide addition. As shown in Figure 8A, addition of 100 or 150 pmol of U12wt and S5 oligonucleotide led to a reduction in splicing as evidenced by a drop in the level of mRNA and spliced intron. In contrast, L3 had little effect on splicing even at the highest concentration tested. Quantitation with a phosphorimager revealed a 50–60% reduction in the level of mRNA relative to pre-mRNA in the presence of U12wt and S5 (Figure 8B). Significantly, addition of the U12wt oligo had no effect on splicing of a U2-type pre-mRNA (not shown). These studies suggest that the 65K protein plays an important role in U12-dependent splicing.

The N-terminal half of the U12-65K protein interacts with the U11-59K protein

To identify potential protein interaction partners of the 65K protein, we first performed two-hybrid interaction assays in

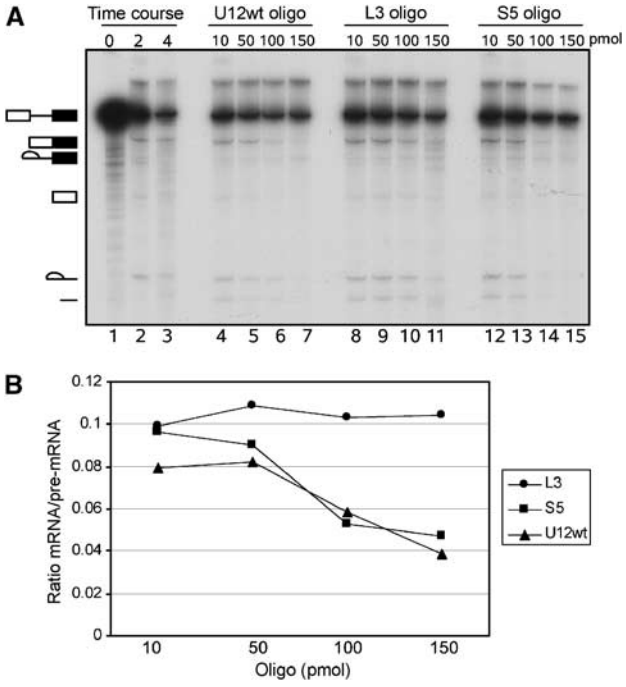


Figure 8 An excess of U12wt and S5 oligo inhibits P120 pre-mRNA splicing *in vitro*. (**A**) *In vitro* splicing was performed for 4 h with ^{32}P-labeled P120 pre-mRNA in the presence of increasing concentrations (as indicated) of U12wt oligo (lanes 4–7), mutant L3 oligo (lanes 8–11) or mutant S5 oligo (lanes 12–15). Lanes 1–3: P120 splicing in the absence of oligo after 0, 2 and 4 h. RNA was separated on an 8% polyacrylamide–8 M urea gel and visualized by autoradiography. The positions of the splicing intermediates and products are indicated schematically on the left. (**B**) Quantitation of splicing efficiency. The amounts of pre-mRNA and mRNA were determined using a PhosphorImager and the ratio of mRNA to pre-mRNA was plotted as a function of oligonucleotide concentration.

yeast. When 65K was used as bait, it interacted strongly with 59K but not with any of the other U11/U12-specific proteins tested (not shown). This interaction was also observed in the reciprocal two-hybrid screen with 59K as bait (Figure 9A), indicating that both proteins tightly interact with one another. To further define the interacting domains of 65K and 59K, deletion mutants were generated. $65K_{1–257}$ and $65K_{257–517}$ consist of 65K's N-terminal RRM plus the proline-rich region (aa 1–257) or its C-terminal RRM plus upstream amino acids (aa 257–517), respectively (Figure 9D). 59K was divided into its N-terminal proline-rich region ($59K_{1–135}$), arginine-rich region ($59K_{130–337}$) and C-terminal region rich in glutamic acid ($59K_{332–481}$). When 59K was used as bait, it interacted with full-length 65K and $65K_{1–257}$, but not with $65K_{257–517}$ (Figure 9A). When the 59K deletion mutants were used as bait, interactions were only observed between $59K_{332–481}$ and either full-length 65K or $65K_{1–257}$. Significantly, no interaction was observed between $59K_{332–481}$ and the U11/U12-specific 35K, 25K or 20K proteins when used as either bait or fused to the activating domain (not shown). These results indicate that the N-terminal half of 65K interacts specifically with the C-terminal 149 aa of the 59K protein.

Next, Far Western overlays were performed with *in vitro*-translated, ^{35}S-labeled U11/U12 proteins and nitrocellulose strips containing U11/U12 proteins. Interestingly, both full-length 65K and $65K_{1–257}$ (Figure 9B, lanes 2 and 3), but not $65K_{257–517}$ (not shown), interacted predominantly with 59K. The 65K–59K interaction appears to be specific, as other equally abundant proteins (as evidenced by Ponceau staining; Figure 9B, lane 1) were not efficiently bound by 65K. Furthermore, *in vitro*-translated 35K or 20K protein did not react with 59K in Far Western overlays (not shown). Owing to the extremely high GC-rich 5′ end of the 59K cDNA, it was not possible to translate full-length 59K *in vitro* or overexpress it, thereby preventing overlays or GST pulldowns. However, *in vitro*-translated $59K_{332–481}$ reacted specifically with 65K (Figure 9B, lane 4), consistent with the $65K_{1–257}$/$59K_{332–481}$ interaction observed by two-hybrid assays.

Finally, pulldowns were performed with GST fusions containing full-length or truncated 65K, and *in vitro*-translated, ^{35}S-labeled $59K_{332–481}$. Co-precipitated protein was subsequently analyzed by SDS–PAGE. Significantly, $59K_{332–481}$ was precipitated efficiently with GST-65K and GST-65K-N-half, but not with GST, GST-65K-N-RRM, GST-65K-C-half or GST-65K-C-RRM (Figure 9C). These results demonstrate that the N-terminal half of 65K interacts with the C-terminal 149 aa of 59K and further suggest that residues between positions 150–257 of 65K are required for this interaction.

Discussion

65K binds the terminal hairpin of the 3′ SL of U12

We have investigated protein–RNA and protein–protein interactions involving proteins specifically associated with the human 18S U11/U12 snRNP. We show that the U11/U12-65K protein directly interacts with the U12 snRNA (Figures 2 and 4). These data provide the first information about proteins that directly contact snRNA within the U11/U12 snRNP. Co-IP studies with truncated U12 snRNA revealed that the 65K binding site is located in the 3′ half of U12 (Figure 4). We provide evidence that, in contrast to the currently accepted structure, the 3′ half of the human U12 snRNA folds into a single, extended SL with a terminal 7-nt loop. Evidence for the 3′ SL structure includes the following: (i) all known U12 snRNAs from diverse organisms, including vertebrates, plants and insects, can theoretically be folded into this structure and it represents the most energetically favorable in each case; (ii) structure probing studies with lead(II) ions (Figure 5) were most consistent with the 3′ SL structure; and (iii) the sequence of the terminal loop of the 3′ SL is evolutionarily highly conserved (Figure 6) and a chemically synthesized RNA oligo comprising the terminal hairpin binds recombinant 65K both in GST pulldowns and EMSAs (Figure 7).

The latter result not only supports the existence of the extended 3′ SL, but also precisely maps the binding site of 65K on the U12 snRNA to nucleotides comprising the terminal hairpin. Mutational analyses demonstrated that the nature of the loop nucleotides and the loop-closing base pair, and also the existence of a stem structure are major determinants of 65K binding (Figure 7). The U1-A and U2-B″ proteins also require a hairpin structure for binding and recognize not only loop nucleotides, but also the loop-closing base pair of their respectively bound RNA hairpins in a sequence-specific manner (Oubridge *et al*, 1994; Price *et al*, 1998). In the case of U1-A and U2-B″, the presence of a stem is thought to aid binding, not only through sequence-specific interactions, but also by imposing constraints on the conformation of the loop nucleotides (Varani and Nagai, 1998). Significantly, although nucleotides comprising the terminal loop of the 3′ SL of U12 are also partially single stranded in

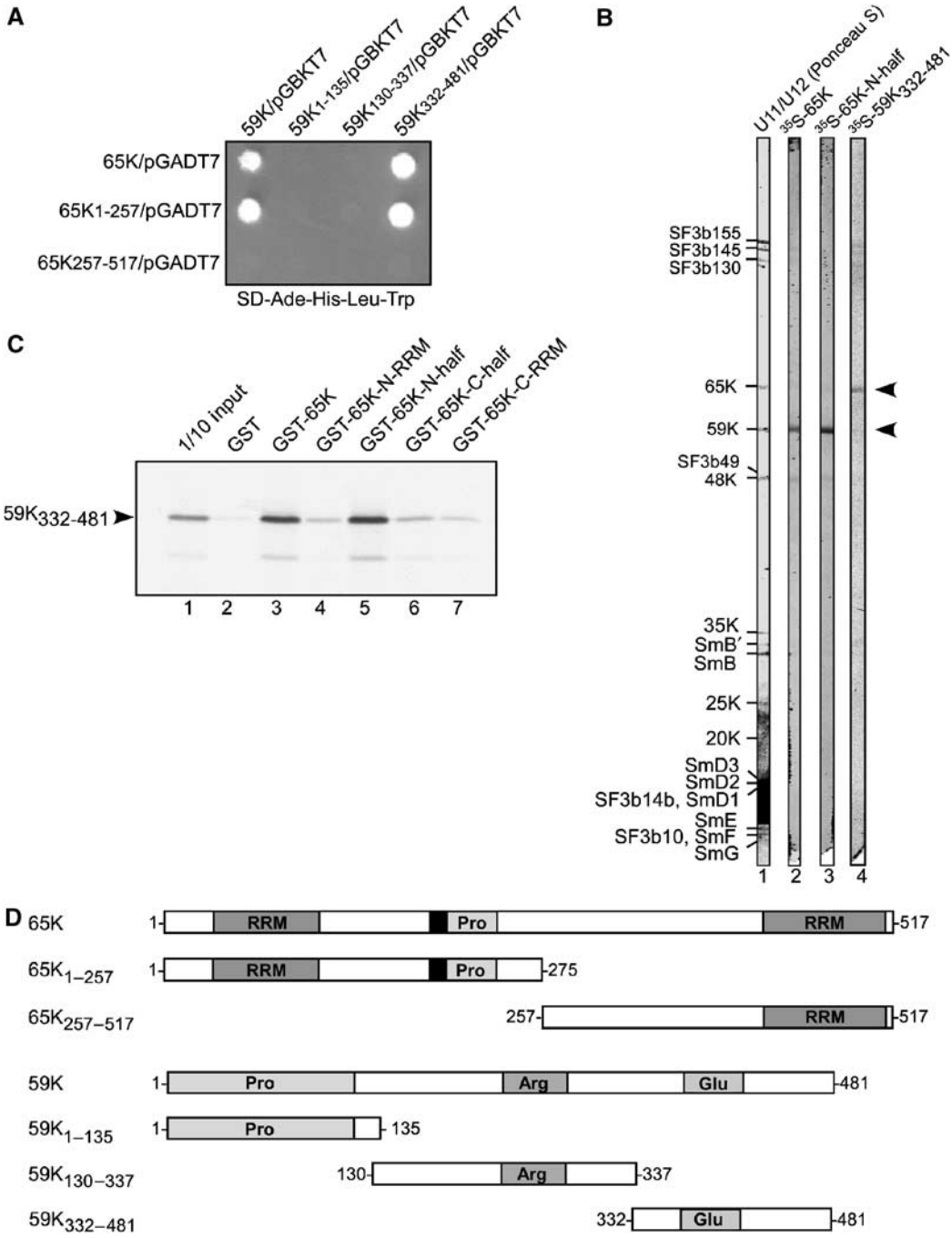


Figure 9 The N-terminal half of 65K interacts with the C-terminal 149 aa of the 59K protein. (**A**) Yeast two-hybrid assays with the 65K and 59K proteins (and deletion mutants thereof). (**B**) 65K and 59K interact in Far Western overlays. U11/U12 proteins on nitrocellulose strips were visualized by staining with Ponceau S (lane 1) or incubated with ^{35}S-labeled, *in vitro*-translated protein (as indicated above each lane) and visualized by fluorography (lanes 2–4). U11/U12 proteins are indicated on the left. (**C**) GST pulldowns with ^{35}S-labeled 59K$_{332–481}$ and GST (lane 2), or GST fusions of 65K and deletion mutants thereof (lanes 3–7; as indicated). Co-precipitated protein or 10% of the input (lane 1) was analyzed by SDS–PAGE and visualized by fluorography. (**D**) Schematic of wild-type and truncated 65K and 59K proteins used in panels A–C. Abbreviations are as in Figure 2. Arginine- (Arg) and glutamic acid-rich regions (Glu) are shaded gray.

the previously published U12 structure (i.e., they are located between stems III and IV), the requirement of a hairpin structure for 65K binding argues against the existence of the latter structure in the U11/U12 snRNP.

The 65K C-terminal RRM deletion mutant binds U1 and U2 snRNA as well as U12

Data presented here demonstrate that the C-terminal RRM of 65K is necessary and sufficient for U12 binding. However, in contrast to full-length 65K or 65K-C-half (aa 211–517), 65K-C-RRM (aa 380–517) also interacted with U1 and U2, but not U11, U4 or U5. Thus, in the absence of residues 211–379, 65K loses its ability to distinguish between the U1, U2 and U12 snRNAs, but retains some binding specificity in that other snRNAs are not bound. By analogy, in the absence of the U2-A′ protein, U2-B″ lacks the ability to distinguish between U1 and U2, binding both with similar affinity (Bentley and Keene, 1991). A comparison of the cocrystals of the

N-terminal RRM of U1-A and U2-B″ bound to U1 SLII and U2 SLIV, respectively, indicates that snRNA discrimination by U1-A and U2-B″ is the result of the formation of a different network of multiple hydrogen-bonding interactions between the RNA and protein (Oubridge *et al*, 1994; Price *et al*, 1998). Presumably, the structure of the C-terminal RRM mutant, in contrast to the full-length protein or the C-terminal half mutant, is altered in some way such that it is capable of engaging in a network of interactions compatible not only with U12 but also U1 and U2 binding.

The U11/U12-65K and U1-A/U2-B″ proteins appear to be related

Among human proteins present in the NCBI nr database, the C-terminal RRM of 65K exhibits the second highest degree of homology with the N-terminal RRM of U1-A and U2-B″. This finding, as well as the ability of 65K-C-RRM to recognize U1 and U2, suggests that these proteins may have evolved from a direct common ancestor or potentially from one another. Significant homology between the RRM of the U11-35K protein and that of the U1-70K protein, which also binds U1 snRNA, was noted previously (Will *et al*, 1999), suggesting that several U11/U12-specific proteins may be evolutionarily related to U1 or U2 proteins.

The RRM is an ancient domain and, because of its modular nature, RRM proteins have been proposed to have evolved by gene duplication of an ancestral RNA binding protein and subsequent diversification (Bandziulis *et al*, 1989). The N-terminal RRM of 65K does not exhibit notable homology with its own C-terminal RRM, indicating that the N- and C-terminal RRMs have not arisen by an intragenic domain duplication event. Likewise, the N- and C-terminal RRMs of U1-A or of U2-B″ do not appear to be related by RRM duplication within the same protein (Birney *et al*, 1993). The N-terminal RRM of the 65K protein also does not exhibit notable homology with the C-terminal RRMs of U1-A or U2-B″. This, coupled with the fact that the position of the related RRMs is different in 65K versus U1-A/U2-B″ (i.e., C- versus N-terminal), suggests that if in fact the 65K C-terminal RRM was duplicated giving rise to U1-A/U2-B″ (or *vice versa*) it must have been inserted into an unrelated protein already containing a second RRM. Alternatively, after gene duplication, unrelated RRMs must have been introduced into the N- or C-terminus of the 65K or U1-A/U2-B″ proteins, respectively. Although less likely, it cannot be excluded that the observed homology between the C-terminal RRM of 65K and the N-terminal RRM of U1-A/U2-B″ is fortuitous in nature.

Three models describing potential evolutionary relationships between the U2- and U12-type spliceosomes have been proposed (Burge *et al*, 1998). These include the so-called fission–fusion and codivergence models, in which both spliceosomes have a homologous origin (i.e., they are derived from a common ancestor). The apparent relatedness of 65K and U1-A/U2-B″ is consistent with a homologous origin of the major and minor spliceosomes. The latter is also supported by the fact that many spliceosomal proteins and the U5 snRNA are shared by both spliceosomes. However, the limited sequence similarities between the U11, U12, U4atac and U6atac snRNAs and their major snRNA counterparts suggest that they have a nonhomologous origin and thus support the so-called parasitic invasion model. In this model, several genes of a common ancestor of animals and plants, which had a pre-existing spliceosome, were invaded by a parasitic group II intron. Fragmentation of this intron gave rise to new snRNAs that associated with many proteins of the pre-existing spliceosome, resulting in snRNPs with shared or similar proteins. In the case of 65K, a pre-existing protein that recognized the U2 snRNA may have evolved such that it also recognized the U12 snRNA (or *vice versa*); at some point in time, the full-length protein would have lost its ability to recognize U2 (or U12), thereby preventing the formation of hybrid U2 and U12 snRNPs. Thus, data presented here also support the idea that a subset of snRNP proteins from one of the spliceosomes directly diverged from proteins of the other pre-existing spliceosome.

U12-65K interacts with the U11-59K protein

Data presented here also provide the first insights into protein–protein interactions that likely contribute to U11/U12 snRNP formation. Yeast two-hybrid, Far Western and co-IP assays demonstrated a direct interaction between the 65K and 59K proteins. The latter, in contrast to 65K, which binds the U12 snRNA, is associated with 12S U11 snRNPs. Thus, the 65K–59K interaction appears to bridge 12S U11 and 15S U12 snRNPs, and may play a key role in the biogenesis of the 18S U11/U12 di-snRNP. Importantly, the region of 65K that interacts with 59K was mapped to aa 150–257 in its N-terminal half, which are not involved in U12 binding. Interestingly, this region contains the sequence QVLHLMN(K/R)MNL, which is 100% conserved and uniquely present in all putative 65K orthologs identified to date. Truncation of 59K revealed that aa 332–481 at its C-terminus are involved in 65K binding. That this region of 59K is located at the U11/U12 interface was suggested previously by IP studies in which antibodies against the C-terminus of the protein precipitated 12S U11 but not U11/U12 snRNPs (Will *et al*, 2004). The U11 snRNP does not appear to remain associated with U12 throughout the course of splicing, but rather is likely destabilized during catalytic activation of the spliceosome. Thus, U11 and U12 snRNPs appear to be reassembled after each round of splicing, and interactions mediating di-snRNP formation may repeatedly play a crucial role in splicing. In this respect, it is also interesting to note that 59K (also denoted ES18) has been implicated in apoptosis (Park *et al*, 1999), suggesting that the disruption of U11/U12 di-snRNP formation is potentially linked to programmed cell death.

The concomitant interaction of the U11/U12 di-snRNP with the 5′ splice site and branch site of the pre-mRNA during U12-dependent prespliceosome assembly indicates that U11/U12 proteins contribute to intron bridging at this stage. Although direct evidence is currently lacking, the U11/U12-specific 65K and 59K proteins likely play an important role in this process. In the major spliceosome, SR proteins have been implicated in intron bridging interactions and recent data suggest that the DEAD-box protein Prp5 bridges the U1 and U2 snRNPs (Xu *et al*, 2004). SR proteins are required for U12-type splicing and likely facilitate interactions of the U11/U12 snRNP with the pre-mRNA (Hastings and Krainer, 2001), but they do not appear to function in di-snRNP formation/stability (i.e., they are not found in purified U11/U12 snRNPs). Likewise, hPrp5 is not part of the human 18S U11/U12 snRNP and thus does not appear to contribute to intron bridging interactions in the minor prespliceosome. Thus, unique protein–protein interactions, including

65K-59K, likely contribute to the molecular bridge connecting the ends of an U12-type versus U2-type intron.

A functional role for 65K in U12-dependent splicing

A vital cellular function of the 59K protein was previously demonstrated by RNAi-mediated knockdowns, which led to cell death (Will *et al*, 2004). However, the significant amount of 59K still present after knockdown (~30%) and the inability to efficiently immunodeplete 59K from HeLa nuclear extract have hindered studies of its role in di-snRNP formation. Likewise, owing to inefficient RNAi-mediated knockdown and immunodepletion of 65K, its role in splicing and/or di-snRNP formation could not be assayed by these methods. As an alternative approach, we attempted to functionally sequester the 65K protein by adding a large molar excess of the U12wt oligonucleotide comprising the 65K binding site; similar studies revealed a role for the U4-15.5K RNA binding protein in U2-type splicing (Nottrott *et al*, 1999). Significantly, addition of the U12wt oligo led to a reduction in the efficiency of U12-type (but not U2-type) splicing *in vitro* (Figure 8; not shown). The effect of the U12wt oligo appeared to be specific, as no reduction in splicing was observed with a mutant U12 oligonucleotide (L3) that no longer binds 65K. In the presence of the U12wt oligo, a significant reduction in U11/U12 di-snRNP formation could not be detected by IP studies (not shown), suggesting that the observed reduction in splicing is not due to disruption of the di-snRNP complex. However, more subtle alterations in the molecular architecture of the U11/U12 snRNP upon U12wt oligo addition or a more direct role of the 65K protein in U12-dependent splicing cannot, at present, be ruled out.

Materials and methods

Database searches

Homologs of the human 65K protein and U12 snRNAs were identified by BLAST searches of the nr or EST databases at the NCBI (http://www.ncbi.nlm.nih.gov/entrez/). Proteins were aligned using the Clustal method.

In vitro transcription and 5′-end labeling of RNA

^{32}P-labeled U1, U2, U11 or U12 snRNA was transcribed *in vitro* with T7 or SP6 polymerase from plasmids containing the respective snRNA gene in the presence of ^{32}P-UTP (3000 Ci/mmol). Preparative amounts of m^{7}G-capped, nonlabeled UsnRNA (for co-IPs) were prepared under similar conditions, except that m^{7}GpppG was included in the reaction. RNA oligonucleotides were 5′-end labeled with polynucleotide kinase and [γ-^{32}P]ATP (5000 Ci/mmol).

Co-IP studies and GST pulldowns

Co-IPs with anti-cap antibodies were performed essentially as described by Nottrott *et al* (1999). GST fusion proteins or GST alone were expressed in *E. coli* (BL21 plysS) cells and purified as described previously (Will *et al*, 2001). For mobility shift assays, GST-65K-C-half was subsequently concentrated using a Vivaspin concentrator (Vivascience) according to the manufacturer's protocol. GST pulldowns were performed with the indicated *in vitro*–transcribed, ^{32}P-labeled UsnRNA, ^{32}P-end-labeled RNA oligo or 6 µl of *in vitro*-translated, ^{35}S-labeled 59K$_{332–481}$ protein essentially as described (Will *et al*, 2001). To isolate co-precipitated UsnRNAs, 100 µl of 1 × PK buffer (100 mM Tris–HCl (pH 7.5), 12.5 mM EDTA (pH 8.0), 150 mM NaCl, 1% (w/v) sodium dodecyl sulfate) were added, followed by phenol extraction and ethanol precipitation. RNA was separated on a 10% polyacrylamide–7 M urea gel. Co-precipitated protein was analyzed as described above. Pulldown of oligonucleotides was quantitated by Cherenkov counting in a scintillation counter.

Lead(II)-induced cleavage

U12 snRNA was isolated by phenol extraction from purified UsnRNPs or generated by *in vitro* transcription. 50 ng *in vitro*-transcribed U12 and 2 µg tRNA were incubated for 5 min at 20°C in 20 µl of buffer N1 (25 mM HEPES–NaOH (pH 7.5), 5 mM magnesium acetate, 100 mM potassium acetate) in the presence of 0, 1, 5, 10 or 50 mM lead(II) acetate according to Brunel and Romby (2000). Reactions were stopped by adding 5 µl of 250 mM EDTA and RNAs were recovered by ethanol precipitation. Cleavage sites were detected by primer extension with primers complementary to nt 139–150 of U12 as described by Hartmuth *et al* (1999).

Electrophoretic mobility shift assays

Recombinant proteins (0–100 pmol, as indicated) were incubated with 0.25 pmol of ^{32}P-end-labeled RNA oligonucleotide or 0.01 pmol of ^{32}P-labeled, *in vitro*-transcribed UsnRNA for 45 min at 4°C in the presence of 10 µg of *E. coli* tRNA in a final volume of 10 µl of buffer A (20 mM HEPES–KOH (pH 7.9), 100 mM KCl, 1.5 mM $MgCl_2$, 20 µg/ml of *E. coli* tRNA, 0.25 mM DTE, 5% glycerol, 10 µg/µl BSA). RNA–protein complexes were separated on a native 6% (80:1) polyacrylamide gel.

Far Western overlays and yeast two-hybrid assays

Coupled transcription and translation of the indicated protein were performed using the TNT system (Promega) in the presence of [^{35}S]methionine (1000 Ci/mmol). For Far Westerns, 18S U11/U12 snRNP proteins were isolated by affinity selection with a biotinylated oligonucleotide complementary to U12 and streptavidin agarose beads (Will *et al*, 1999). Proteins were separated on a 13% polyacrylamide–SDS gel, transferred to nitrocellulose and visualized by staining with Ponceau S. Far Western overlays were performed as described by Achsel *et al* (1998). Yeast two-hybrid analyses were performed with the MATCHMAKER Two-Hybrid System 3 as described by the manufacturer (Clontech) (see also Supplementary data).

In vitro splicing

Splicing was performed with *in vitro*-transcribed, ^{32}P-labeled P120 pre-mRNA essentially as described previously (Will *et al*, 2001), except that U2-type splicing was inhibited by adding a 2′Ome RNA oligo complementary to the U6 snRNA (AUGCUAAUCUUCUCU GUA; end concentration 4.8 µM). For splicing inhibition studies, increasing concentrations (10–150 pmol) of U12wt, L3 and S5 oligo were added to a standard 12.5 µl splicing reaction. RNA was recovered by phenol–chloroform extraction and ethanol precipitation, fractionated on an 8% polyacrylamide–8 M urea gel and visualized by autoradiography. Quantitation of splicing efficiency was performed with a PhosphorImager (Molecular Dynamics).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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