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Secondary structure of U6 small nuclear RNA: implications for spliceosome assembly

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Abstract

U6 snRNA (small nuclear RNA), one of five RNA molecules that are required for the essential process of pre-mRNA splicing, is notable for its high level of sequence conservation and the important role it is thought to play in the splicing reaction. Nevertheless, the secondary structure of U6 in the free snRNP (small nuclear ribonucleoprotein) form has remained elusive, with predictions changing substantially over the years. In the present review we discuss the evidence for existing models and critically evaluate a fundamental assumption of these models, namely whether the important 3′ ISL (3′ internal stem–loop) is present in the free U6 particle, as well as in the active splicing complex. We compare existing models of free U6 with a newly proposed model lacking the 3′ ISL and evaluate the implications of the new model for the structure and function of U6's base-pairing partner U4 snRNA. Intriguingly, the new model predicts a role for U4 that was unanticipated previously, namely as an activator of U6 for assembly into the splicing machinery.

U6 snRNA (small nuclear RNA) and pre-mRNA splicing

Nuclear pre-mRNA splicing involves the removal of pre-mRNA regions (introns) that do not code for functional molecules and the subsequent joining of the coding regions (exons) to produce a mature mRNA transcript. Splicing is complex, requiring dynamic interactions between five snRNAs, U1, U2, U4, U5 and U6, and over 100 proteins. A number of these proteins associate with a specific snRNA in an snRNP (small nuclear ribonucleoprotein) particle. These particles are thought to assemble on each new pre-mRNA transcript in an ordered and step-wise fashion to generate a large RNA–protein complex, known as the spliceosome, which is responsible for catalysing splicing reactions.

Mechanistic and structural similarities to group II self-splicing introns have led to the proposal that, like self-splicing introns, pre-mRNA splicing may be an RNA-catalysed event [1,2]. Of the five snRNAs, U6 is the most likely to play a direct role in catalysis since it associates with the 5′ splice site of the pre-mRNA in the active core of functional spliceosomes [3] (Figure 1). Furthermore, the highly conserved AGC triad has been shown to base-pair to U2 in a structure that has been implicated in exon ligation [4], and the adjacent U6 3′ ISL (3′ internal stem–loop), which bears structural resemblance to domain V of group II introns [1,2,5], co-ordinates a catalytically essential Mg^{2+} during the splicing reactions [6]. Lastly, U6 is unusually highly conserved, both in size and sequence, with approx. 60% sequence identity between yeast and human U6 snRNAs, increasing to 80% identity across the middle third of these sequences [7,8].

Despite the high level of sequence conservation, many mutations in U6 snRNA result in only weak conditional growth phenotypes, or show no observable growth phenotype at all. One stretch of nucleotides, however, known as the ACAGAGA box, is unexpectedly intolerant of mutation [9–11]. Point mutations within this stretch of seven nucleotides result in lethality *in vivo* and severe inhibition of splicing *in vitro* with various levels of first- and/or second-step blocks [9,10]. The ACAGAGA sequence has been shown to genetically interact with, as well as to cross-link to, the 5′ splice site region of the pre-mRNA and has also been proposed to play an as-yet-undefined role throughout the splicing reaction [9,12–15].

In order for U6 snRNA to enter into functional spliceosomes, it must first interact with U4 snRNA through an extensive base-pair interaction in a di-snRNP (Figure 1). The functional relevance of this particle, or the large conformational rearrangements required in order to accommodate its formation, has yet to be revealed. The role of U4 in splicing is even less clear as U4 snRNA is thought to leave the spliceosome following formation of the di-snRNP, but prior to any catalytic event [16,17]. It is clear, however, that the U4/U6 complex can form either via a biogenesis pathway, from newly assembled U4 and U6 snRNPs, or via a 'recycling' pathway, using free U4 and U6 snRNPs that have been disassembled from previous rounds of splicing [18,19]. Both of these pathways are thought to require the splicing factor Prp24 to catalyse U4/U6 formation [20].

Models of the U6 secondary structure in free U6 snRNP

Over the last 15 years, a number of models for the yeast U6 snRNA secondary structure in free U6 snRNP have been proposed, yet they offer little insight into the structural rearrangements leading to the U4/U6 di-snRNP formation,

Key words: pre-mRNA splicing, Prp24, small nuclear ribonucleoprotein biogenesis (snRNP biogenesis), spliceosome assembly, U4 small nuclear RNA, U6 small nuclear ribonucleoprotein.
Abbreviations used: 3′ ISL, 3′ internal stem–loop; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein.
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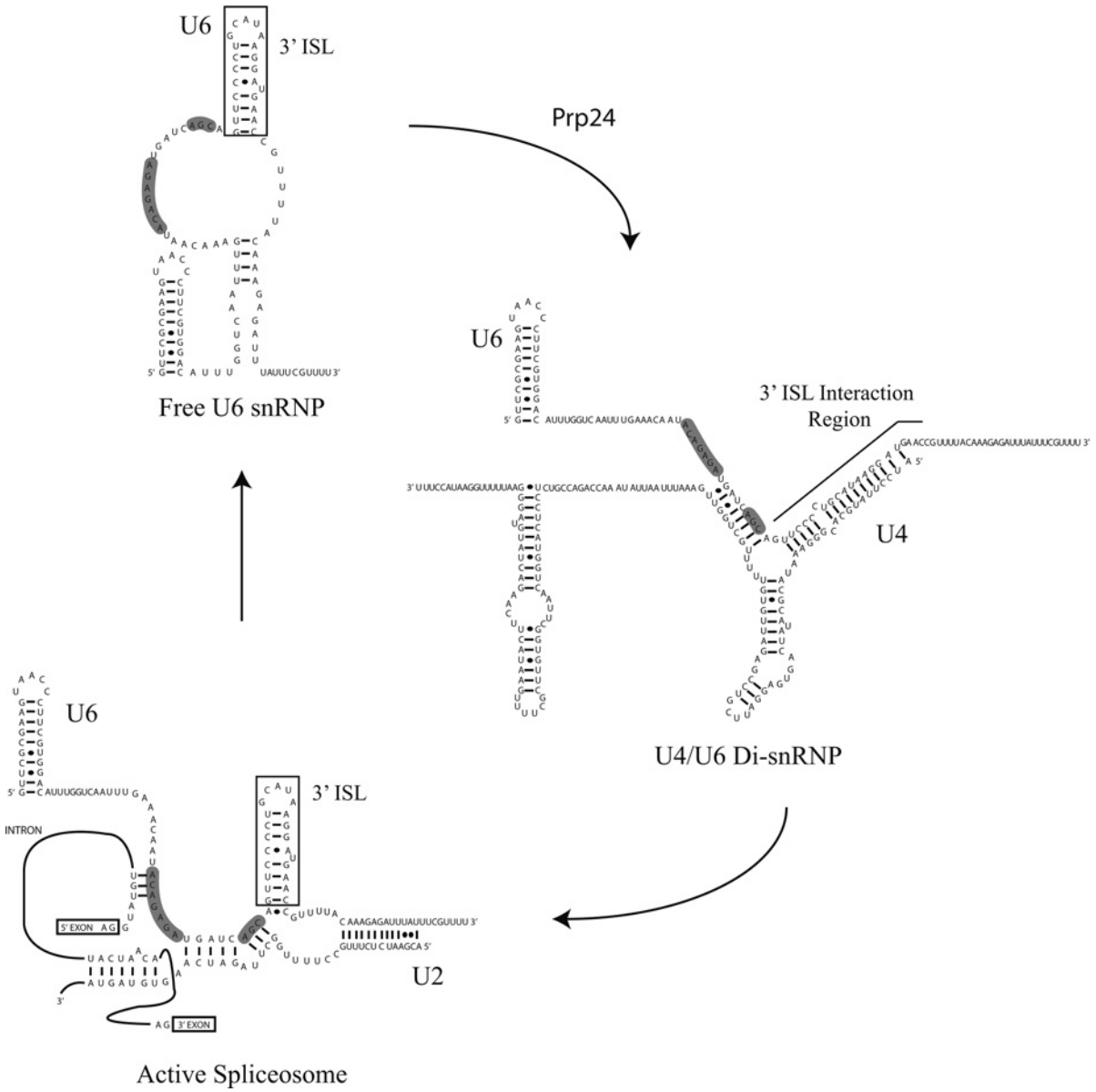
Figure 1 | **U6 snRNA undergoes a number of structural rearrangements during the splicing cycle**

Proposed secondary structure in: free U6 snRNP [23]; the U4/U6 di-snRNP interaction [7]; and in the active spliceosome [1,3,12–15,41,42]. The nearly invariant ACAGAGA and AGC sequences are shaded grey and the catalytically important 3′ ISL is boxed or overlined.

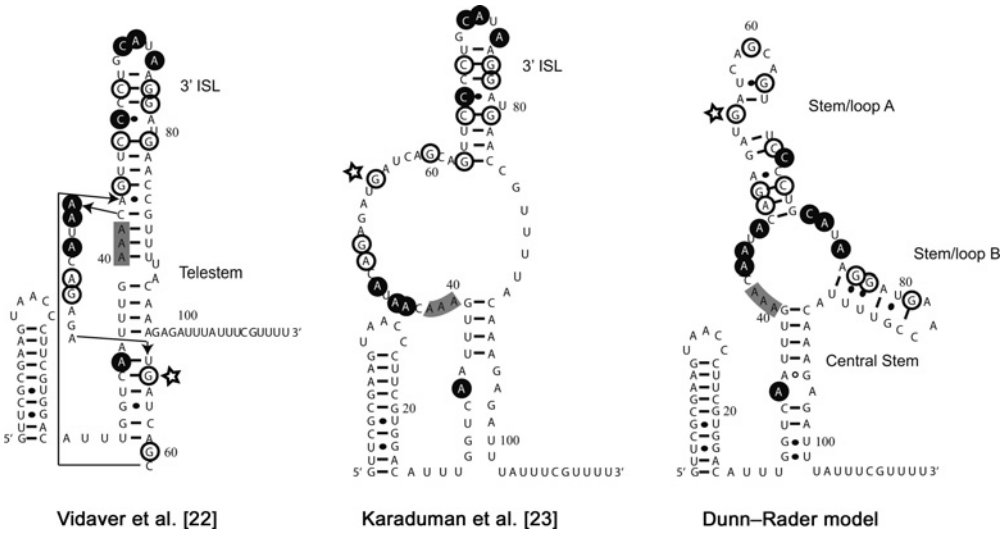


or into the transition of U6 from an inactive species to an active catalytic element at the heart of the functional spliceosome. Common to each of these models is the catalytically important 3′ ISL and an adjacent region of more ambiguous structure. In an early model, the adjacent region was proposed to form a third stem structure that later evolved into the 'telestem' [21,22] (Figure 2). In the most recent model, the upper telestem interaction has been disrupted, generating a large asymmetric bulge consisting of 23 nucleotides on the 5′ side and seven nucleotides on the 3′ side [23] (Figure 2). It is unusual to find such large unstructured segments in RNA molecules; however, Karaduman et al. [23] argue that this large bulge accommodates binding of the U6-snRNP-specific protein Prp24, since, in the absence of protein, chemical modification experiments have suggested a much more structured RNA molecule.

Although the 3′ ISL was first proposed in the mammalian free U6 snRNP in 1980 [24], the yeast U6 3′ ISL was first described in the splicing literature in 1992 in the context of the active spliceosome [1]. Two years later, this structure was proposed for the yeast free U6 snRNP [21] and alternative models for free U6 base-pairing interactions that are also

Figure 2 | **Chemical modification of U6 snRNA in the free U6 snRNP**

Nucleotides that are strongly protected from or strongly modified by chemical modifiers are indicated with white and black circles respectively [23,33]. Nucleotides that are strongly modified in the absence of protein and strongly protected in the presence of protein are shaded grey [23,33]. A UV cross-link to Prp24 is highlighted with a star [23].



consistent with the same experimental results have never been reported.

The 3′ ISL is a relatively strong intramolecular structure with an estimated melting temperature of 60°C in the absence of proteins [25]. The current model of spliceosome assembly and activation involves the disruption of this structure in free U6 snRNP to accommodate U4/U6 di-snRNP formation, followed by reformation of the 3′ ISL upon release of U4 during spliceosome activation [21] (Figure 1). Such large structural rearrangements are energetically expensive; e.g. unwinding of the U4/U6 duplex requires ATP hydrolysis and the DEXD-box helicase Brr2 [26]. In contrast, rearrangement of free U6 snRNP to allow interaction with U4 during di-snRNP formation does not require ATP [20,27], and, furthermore, free U4 and U6 have been reported to anneal in the absence of protein factors, albeit inefficiently [28,29]. Thus the mechanism and functional significance of unwinding such a stable intramolecular structure in the free snRNP during spliceosome assembly, without input of energy, remains a mystery.

Although NMR structures for the short 3′ ISL, both alone and as part of the U2/U6 active-site conformation, support the formation of the 3′ ISL during splicing [5,30,31], genetic analyses have failed to provide strong support for this structure in free U6 snRNP, mainly because growth phenotypes reveal little about the detailed biochemical milieu in which a mutation exerts its effect(s). For example, a mutation predicted to hyperstabilize the 3′ ISL, U6-A62G, did result in a cold-sensitive growth phenotype and a U4/U6 assembly defect consistent with stem stabilization [21]. However, the U4/U6 assembly defect was also observed at higher temperatures in the absence of a growth defect, demonstrating that the U4/U6 assembly defect did not cause the growth defect [21]. In addition, overexpression of U4 snRNA corrected the U4/U6 assembly defect, whereas the cold-sensitive phenotype was only partially alleviated [21]. Thus the cold-sensitive growth phenotype probably occurs following U4/U6 assembly and may reflect inhibition of 3′ ISL unwinding during spliceosome disassembly, stalling the spliceosome and preventing recycling of U6 snRNA. We therefore conclude that neither U6-A62G nor other genetic analyses provide unambiguous support for the existence of the 3′ ISL in the free U6 snRNP.

A new model of U6 secondary structure in the free U6 snRNP

In the light of the considerations above, we propose a new model for the U6 snRNA secondary structure in free U6 snRNP (henceforth the 'Dunn–Rader model') that provides some insight into the mechanism of activating U6 for splicing through U4/U6 di-snRNP formation. Our model predicts three helical segments that meet at a three-way junction: the central stem, stem–loop A, and stem–loop B (Figure 2). Although the base-pair composition of the central stem is essentially identical with that proposed by Karaduman et al. [23], the presence of two additional stems is unique to our model and replaces the 3′ ISL proposed previously (Figure 2). Consistent with the dynamic nature of U6 snRNA, these intramolecular structures are expected to be much weaker than the 3′ ISL, with an experimentally determined melting temperature of approx. 35°C for stem–loop A (E.A Dunn and S.D. Rader, unpublished work) and an estimated melting temperature of 42°C for stem–loop B [25]. Thus structural rearrangement of free U6 snRNP to allow interaction with U4 during di-snRNP formation is not expected to require a large input of energy and may occur through a concerted

mechanism where bond breaking in the free U4 and U6 intramolecular stems provides the energy required for formation of the intermolecular interactions.

Chemical structure probing of the free U6 snRNP

Investigations of the secondary structure of U6 snRNA by analysis of purified free U6 snRNP particles is limited to two chemical modification studies [23,33]. Notably, neither study demonstrated that their snRNPs were functional. These experiments were carried out on U6 snRNP species isolated and probed under very different conditions: Jandrositz and Guthrie [33] used native free U6 snRNPs that were purified from other U6-containing species on a glycerol gradient, whereas Karaduman et al. [23] introduced a TAP (tandem affinity purification)-tag at the C-terminal end of Prp24 to allow further purification of U6. The highly conserved C-terminal domain of Prp24 has been shown to interact with the Lsm complex [34], and it is not clear whether introducing a large tag at this location has altered the snRNP structure. Consequently, in comparing the two data sets to assess the validity of the models in Figure 2, we only consider those strong protections and strong modifications that were consistent between the two studies, i.e. we explicitly ignore any modifications or protections that were not seen in both studies. This allows us to be confident that the protection pattern observed was not an artefact of the experimental conditions used. We evaluate our new model alongside the two most recently proposed models from the literature, Vidaver et al. [22] and Karaduman et al. [23].

When only the consistent protections and modifications were considered, there were 21 data points that map to the region of U6 modelled with different secondary structures (nucleotides 29–102) (Figure 2). Three of these positions, Ala^{40}–Ala^{42}, are strongly modified in the naked RNA controls in both studies, and are strongly protected in the presence of protein, consistent with them serving as a single-stranded protein-binding site, as proposed on the basis of genetic observations [22,35]. This is consistent with the Karaduman et al. [23] and Dunn–Rader models, but not the Vidaver et al. [22] model. Of the remaining 18 data points, 14 fit the Vidaver et al. [22] and Karaduman et al. [23] models, whereas 16 fit the Dunn–Rader model. All eight strongly modified positions are predicted not to base-pair in both the Karaduman et al. [23] and Dunn-Rader models, whereas one of these nucleotides is predicted to be base-paired in the Vidaver et al. [22] model. Of the ten strongly protected positions, seven, six and eight are predicted to be base-paired in the Vidaver et al. [22], Karaduman et al. [23] and Dunn–Rader models respectively. Crucially, two reproducible protections at positions Ala^{49} and Gly^{50} that are inconsistent with both the Vidaver et al. [22] and Karaduman et al. [23] models are explained by stem–loop A in the Dunn–Rader model (Figure 2). The two positions that are inconsistent with the Dunn–Rader model, Gly^{55} and Gly^{60}, are also inconsistent with the Karaduman et al. [23] model. These nucleotides are predicted to lie in the stem–loop A bulge and loop respectively in the Dunn–Rader model, and in the large bulge in the Karaduman et al. [23] model (Figure 2). UV cross-linking of Gly^{55} to Prp24, and protection of these positions from hydroxyl radical cleavage, suggest that these nucleotides may be protected from modification through RNA–protein or tertiary RNA–RNA interactions rather than through base-pairing [23].

A model for allosteric activation of U6 snRNA through interaction with U4 snRNA

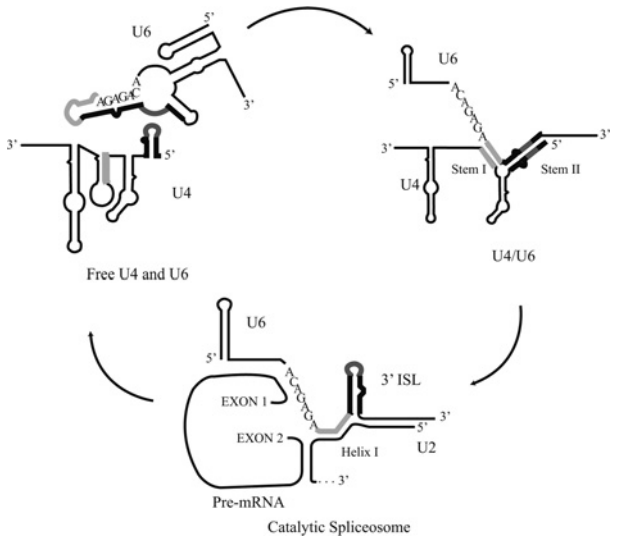
U4 snRNA is an essential gene product; however, its function has remained ambiguous since it is not tightly associated with the active spliceosome and may even dissociate from it following U4/U6 unwinding [16,17]. Brow and Guthrie [36] proposed that U4 might act as an antisense negative regulator of U6 activity, masking catalytically important residues while U6 adopts a conformation that is more favourable for its incorporation into the spliceosome. Alternatively, Stevens et al. [37] suggest that the sub-stoichiometric levels of U4 snRNP, relative to other splicing snRNPs, imply that it is a limiting factor in spliceosome assembly. Thus interaction with U4 might serve as a regulatory point for inclusion of U6 into functional spliceosomes. Although these proposals address the function of U4, they do not explain the large structural rearrangements required in U6 to accommodate the U4/U6 interaction, particularly as the 3′ ISL of U6 has been proposed to be nearly identical in the free snRNP and the active spliceosome.

Our model of the U6 snRNA secondary structure is consistent with these proposed roles for U4 snRNA/RNP in splicing; however, we suggest that U4 snRNA performs an additional role as an activator of U6 prior to its role in the di-snRNP (Figure 3). All other proposed yeast and mammalian models of the U6 secondary structure in the free U6 snRNP predict that the U4/U6 interaction domain base-pairs with itself in the 3′ ISL. Our model proposes instead that this region of U6 base-pairs with regions of U6 that flank the U4/U6 interaction domain. Importantly, the ACAGAGA sequence is inaccessible in our model due to base-pairing with the U4/U6 interaction domain (Figure 3). We propose that base-pair formation between U4 and U6 is required to release the ACAGAGA sequence from the intramolecular interaction so that it can subsequently interact with the 5′ splice site of a pre-mRNA transcript. Hence U4 snRNA can be viewed as an allosteric activator of U6.

The mechanism of U4/U6 di-snRNP formation is not yet understood; however, the U6-snRNP-specific protein Prp24 catalyses base-pairing of U4 and U6 snRNAs [20,34,38,39]. Chemical modification/interference experiments on mammalian U6 snRNA suggest that nucleotides 65–70 are important for initiating base-pairing between U4 and U6 [27]. In yeast, the strong chemical modification of the corresponding residues 72–75, located in the single-stranded junction connecting stem-loops A and B in our model, indicate that these residues are indeed accessible in both the naked RNA and in the presence of protein in free U6 snRNP

Figure 3 | A model for allosteric activation of U6 snRNA by U4 snRNA

Mutually exclusive interaction regions in U4 and U6 are shown in light grey, dark grey and black.



[23,33]. Thus Prp24 probably interacts with U6 snRNA in such a way as to present these nucleotides to U4. Base-pairing between U4 and U6 may be initiated by contacts between the loop residues of a short stem–loop, the U4 kissing loop, located at the 5′ end of U4 snRNA (T. Wong and S. Rader, unpublished work) and U6 residues 72–75 (Figure 3).

Following initiation of base-pairing through the loop–bulge interaction, extension of the intermolecular interaction would result in the complete disruption of stem–loops A and B of U6, releasing the ACAGAGA sequence from intramolecular base-pairing. According to our model, the only U6 present in the cell with an exposed ACAGAGA sequence would be found in the di-snRNP. Simultaneously, the U2/U6 helix I and the U6 3′ ISL regions of U6 would be engaged in interactions with U4, preventing premature formation of these functionally important structures (Figure 3). The ACAGAGA sequence is predicted to lie outside of the U4/U6 interaction domain, and strong chemical modification of this sequence in U4/U6 di-snRNPs suggests that the ACAGAGA sequence is in fact accessible in this context [7,33]. Cross-links observed between U4 snRNA and the 5′ splice site in early stages of spliceosome formation imply that a proofreading mechanism may exist to ensure that correct base-pairing between U6 and the 5′ splice site is established prior to U4/U6 disruption [40]. Dissociation of U4 following establishment of the correct U6/pre-mRNA interaction would then allow the formation of U2/U6 helix I and the U6 3′ ISL within the catalytically active spliceosome (Figure 3).

Summary

We have reviewed existing models of U6 snRNA in the free snRNP particle in the light of some of the existing experimental results and functional considerations. Importantly, whereas previous models assumed the existence of the stable 3′ ISL, our model proposes instead two weaker stems, consistent with the low-activation-energy observed for U4/U6 di-snRNP formation. In replacing the 3′ ISL with two smaller stems, our model removes the structurally unprecedented asymmetric bulge in favour of the well-known structural motif of a three-way helical junction, which results in a modestly better fit to the most consistent chemical modification data. Finally, our model sequesters the functionally critical ACAGAGA sequence, suggesting that an important role for the U4 snRNP is to open up U6 to activate it for spliceosome assembly.

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