Role of the snRNAs in spliceosomal active site

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The spliceosome, the ribonucleoprotein assembly that removes the intervening sequences from pre-mRNAs through splicing, is one of the most complex cellular machines. In humans it is composed of ~150 proteins and five RNAs (snRNAs). One of the snRNAs, U6, contains sequences analogous to all the RNA elements that form the active site of the group II introns, ribozymes that perform a splicing reaction mechanistically identical to spliceosomal splicing. Interestingly, U6 is the only snRNA that is indispensable for splicing and in vitro, in complex with another snRNA, it can catalyze a primordial splicing reaction in the absence of all other spliceosomal factors. On the other hand, discovery of an RNase H-like domain in a spliceosomal protein that is closely associated with splice sites suggests that proteins may be involved in formation of the active site. Thus, whether the spliceosome is an RNA or RNA-protein catalyst remains uncertain.

Introduction

The continuity of the information encoded within modern eukaryotic genes is frequently interrupted by intervening sequences, or introns, which need to be accurately removed or "spliced" out from primary transcripts before they can be used by the cell. Furthermore, introns themselves often harbor regulatory or otherwise functional sequences and their timely removal is essential for their cellular function. Intronic sequences are in most cases much longer than the non-intronic sequences—called the exons—especially in higher eukaryotes, and the sequence elements that specify the intron-exon boundaries are highly complex. Thus, accurate separation of these two sets of functional sequences contained in eukaryotic primary transcripts poses a significant challenge to all eukaryotic cells. While the modern mammalians have one of the most complex splicing patterns among extant eukaryotes, it is likely that in primordial eukaryotes, splicing was already a highly challenging task.

Current research suggests that introns likely originated from self-splicing ribozymes dating from pre-cellular life and constituted the majority of the ancient eukaryotic genomes.[1] Since removal of this large fraction of intervening sequences was

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essential to the survival of the ancient eukaryotes, a highly elaborate cellular machine dedicated to removal of the introns, called the spliceosome, was already present in the last common ancestor of all eukaryotes.[2] Indeed, current data suggest that the ancient spliceosomes were very similar to their modern counterparts, with the majority of the spliceosomal components already in place.[2,3] Spliceosomes are, as expected, very complex: they consist of five RNA molecules (U1, U2, U4, U5 and U6 snRNAs) and ~60 to 150 different proteins depending on the species.[4,5] The origin and evolution of the spliceosome is still largely mysterious, however, several lines of evidence suggest that the eukaryotic spliceosomes might have evolved from self-splicing introns.[6-11] The mechanism of intron removal by the spliceosome, performed through two consecutive transesterification reactions resulting in removal of a branched lariat intron, is identical to the splicing reaction performed by a class of self-splicing introns called the group II introns.[12-15] Intriguingly, the RNA components of the spliceosome, the snRNAs, unmistakably resemble fragments of the catalytically essential domains of group II introns in sequence, structure and function. Although the possibility of convergent evolution cannot be formally ruled out, the above similarities suggest that a detailed knowledge of the function of the snRNAs and their relationship to group II introns may be the key to understanding the spliceosomal function. This review focuses on several recent and exciting discoveries in both the spliceosome and group II intron fields which have greatly increased our knowledge of spliceosomal function, splicing mechanism and evolution of the spliceosomes.

snRNAs and the Group II Self-Splicing Introns

Discovery of group II introns, self-splicing ribozymes found in all three kingdoms of life, coincided with intense experimental efforts to elucidate the mechanism of pre-mRNA splicing in eukaryotes.[9-11] Soon it was revealed that both the spliceosome and the group II introns perform splicing through an identical, two step catalytic strategy. In both systems, the first step involves a nucleophilic attack by the 2' hydroxyl group of a certain adenosine in the intron, the branch site adenosine (**Fig. 1**), on the 5' splice site. This results in a transesterification reaction in which the 2' oxygen of the branch site adenosine replaces the 3' oxygen of the last nucleotide of the upstream exon, which in turn leads to the release of the first exon and the formation of an unusual 2'–5' linkage between the branch site adenosine and the first nucleotide of the intron. During the second step, the free 3'
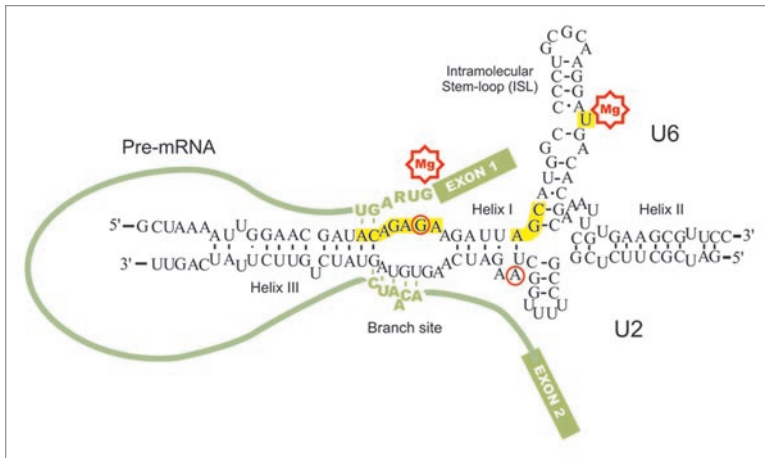
Figure 1. The basepaired complex of the human U2 and U6 snRNAs and the pre-mRNA substrate. The basepairing interactions between the pre-mRNA and the branch-binding site of U2 and the ACAGAGA box of U6 are shown. The position of the basepaired U2/U6 helices I, II and III are indicated. Circles mark the nucleotides involved in a long range tertiary interaction both in vivo and in vitro. Highlights mark the invariant, catalytically essential nucleotides of U6. The location of proven magnesium binding sites in the invariant U6 sequences and at the 5' splice site is shown.
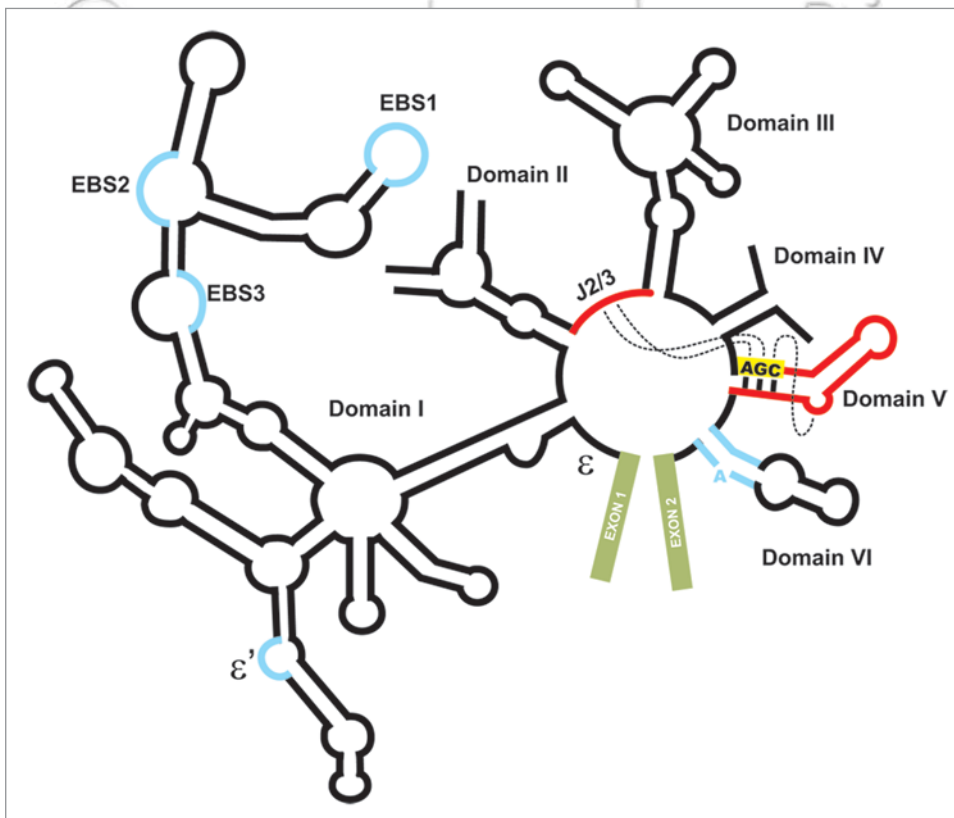

Figure 2. The secondary structure of a self-splicing group II intron. The arrangement of the basepaired domains I through VI is shown. Regions in group II intron domains which have a functional equivalent in the spliceosome are shown. The locations of the EBS1, 2 and 3 (functionally corresponding to U5 snRNA), branch site-containing duplex of domain VI (corresponding to U2 snRNA-branch site interaction) and the ε-ε' interaction (the functional homolog of U1 snRNA-5' splice site interaction) are shown. The location of J2/3 and the AGC triad in domain V which participate in formation of the active site is shown. The dotted lines connect the nucleotides involved in base triple formation with the AGC triad in the active site.

hydroxyl moiety of the newly released exon is activated for a similar nucleophilic attack on the 3' splice site, resulting in ligation of the two exons and release of the intron as a branched lariat.[5,16] Both group II intron and spliceosomal splicing reactions require divalent cations and phosphorothioate substitution analyses at the splice sites have indicated an almost identical interference pattern, which could result from a similar arrangement of catalytically required metal ions in the active site of the two systems.[17-22]

In addition to these intriguing parallels, the significant similarities in the sequence and secondary structure of the snRNAs and several domains of group II introns led to the hypothesis that the snRNAs might be evolutionary descendants of group II-like introns and thus, may have a catalytic role. Group II introns have a conserved secondary structure that consists of six domains, named domains I through VI, which fold into a complex tertiary structure[13,23,24] (**Fig. 2**). The structure of the catalytically critical domain V, the most conserved of the group II intron domains, closely resembles an intramolecular stemloop of U6 snRNA in both overall structure and primary sequence (**Fig. 3**). Both structures contain two short helices separated by an asymmetric bulge known to form a metal-binding pocket[25-30] and are capped by a GNRA-type tetraloop. In addition, they both contain a catalytically critical AGC triad at their 5' ends which interacts with functionally essential divalent cations in group II introns and likely also in the spliceosome.[24,29,31-33] Indeed, domain V could replace the intramolecular stemloop of U6 in an in vivo splicing assay,[34] further demonstrating the functional equivalence of the two structures and strengthening the possibility of an evolutionary relationship between them.

Another domain of group II introns, Domain VI, contains the equivalent of the basepaired structure formed between U2 snRNA and the branch site of introns (**Figs. 1 and 2**). In both cases, a particular adenosine residue which carries the nucleophile of the first step of splicing is kept in a constrained, bulged conformation required for optimal activation.[16,23,35] Further, at least three sequences in Domain I (EBS1,2 &3; **Fig. 2**) bind the exonic sequences in a manner similar to U5 snRNA, which binds to and aligns the two exons for the
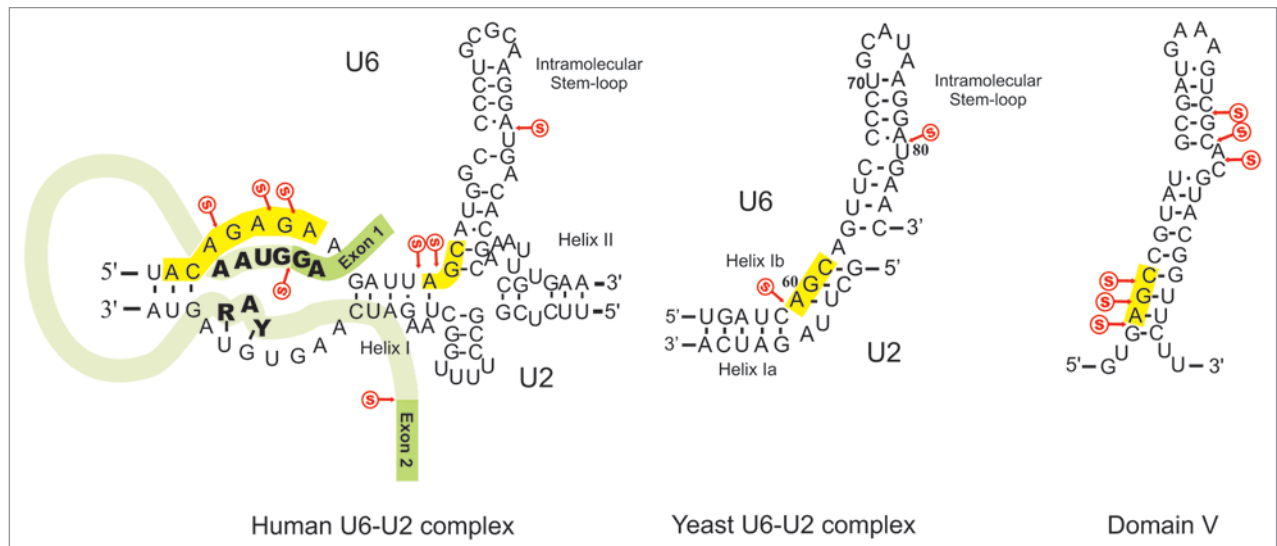
Figure 3. Phosphorothioate interference sites in the human and yeast U6/U2 complex and the group II intron domain V. The partial structure of the human and yeast U6/U2 complex and domain V of group II introns is shown. The highlights mark the location of the ACAGAGA sequence in the human U6/U2 complex and the AGC triad in the three structures. Sequences within the pre-mRNA substrate are shown with bold letters. Location of the phosphorothioate interference sites are shown with circled s signs. The position of helices Ia and Ib in the yeast U2/U6 complex is shown.

second step of splicing. Domain swapping experiments in which a fragment of Domain I was replaced by U5 further support their functional equivalence.[23,36] Another subdomain of Domain I (ε', **Fig. 2**) is involved in recognition of the 5' splice site and can be considered the functional counterpart of the U1 snRNA.[23] There are no known equivalents for the rest of domain I and for domains II and III of the group II introns among the spliceosomal RNAs and it is likely that in the spliceosome, these RNA domains are replaced by proteins. Similarly, there are no clear equivalents for the U4 snRNA in group II introns and its evolutionary origin is completely unknown. Nonetheless, the above structural and mechanistic similarities suggest an evolutionary relationship between the two systems and a prominent role for the snRNAs in the active site of the spliceosome.

snRNAs in the Spliceosomal Active Site

Purification of spliceosomes at various stages of the spliceosomal assembly and catalysis cycle indicated that of the five spliceosomal snRNAs, U1 and U4 leave the spliceosome during the spliceosomal assembly and thus, at the time of catalytic activation of the spliceosome, only U2, U6 and U5 snRNAs were present.[5,16] Later, it was shown that at least in vitro, a conserved loop in U5, which was previously shown to be the functionally important domain of the molecule, was dispensable for the first step of splicing.[37,38] These results suggested that U2 and U6 (**Fig. 1**) are the only snRNAs required for both steps of splicing, further narrowing down the number of spliceosomal RNAs that may be involved in splicing catalysis.

More recently, it has been shown that in spliceosomes assembled on pre-mRNAs harboring a mutant 5' splice site, several positions within the branch binding sequence of the U2 snRNA can be erroneously recognized as the 5' splice site. This results

in an aberrant splicing reaction in trans in which the nucleotides 5' to the branch binding sequence of the U2 snRNA are treated as an exon and undergo both steps of splicing during which this region of U2 is ligated to the downstream exon of the pre-mRNA substrate.[39] These results are highly intriguing, since they imply that the branch binding site of U2 snRNA, which is treated as a substrate and is consumed in this aberrant splicing reaction, is dispensable for spliceosomal catalysis at least under certain conditions. The branch binding sequence of U2 is functionally the most critical region of this snRNA and the only part thought to be present in the active site, as the rest of the RNA fulfills structural roles by forming basepairing interactions with other U2 sequences or with U6 snRNA[15,16] (**Fig. 1**). Thus, these results suggest that U6 snRNA may be the only RNA that is absolutely crucial for splicing catalysis, at least under the conditions studied so far.

Is U6, similar to its counterpart in the group II introns, domain V, the catalytic domain of the spliceosome? Both U6 and domain V are highly conserved, and the presence of two evolutionarily invariant sequences in U6, the ACAGAGA and the AGC boxes, suggests a critical role in splicing for this snRNA.[15,16,23,40] Several mutations in the nucleobases or the backbone of these two regions lead to either a complete or partial block to splicing, while mutations elsewhere in U6 or in other snRNAs often have a mild phenotype.[41-46] Further, crosslinking and mutational complementation analyses have indicated that the first step of splicing occurs in close proximity of the ACAGAGA box, suggesting that this sequence is in close vicinity of or even forms part of the spliceosomal active site.[47,48] In the recently published high resolution structure of a self-splicing group II intron,[13,24] the active site is formed by the AGC triad and the asymmetric internal bulge of domain V along with J2/3, a short purine-rich sequence which joins domains II and III and is thought to be

functionally equivalent to the ACAGAGA box in U6 (**Fig. 2**). Through a complex series of tertiary interactions, an extrahelical nucleotide in the asymmetric internal loop of domain V and two residues in J2/3 form base triples with the portion of the duplex at the base of domain V which contains the AGC triad (**Fig. 2**). This surprising arrangement, which brings together all the known active site elements in group II introns, also helps form inner sphere binding sites for two metal ions which likely participate in catalysis. Interestingly, U6 contains the equivalent of all these sequences which form the active site in group II introns. If the spliceosomal active site indeed resembles the one in group II introns, it is likely that U6 constitutes a major part, if not all, of the spliceosomal active site.[12,14]

In addition to the above mentioned mechanistic and structural similarities, recent data have uncovered a number of additional parallels between the two systems. For example, similar to the recently proven dispensability of U2 snRNA in the spliceosome, the U2-like Domain VI of group II introns is also not essential for catalytic activity; however, in its absence the first step of splicing is performed through hydrolytic cleavage of the 5' splice site rather than branching.[23,35] In a further parallel between the spliceosome and group II introns, it has recently been shown that the spliceosomal active site can also catalyze hydrolytic cleavage reactions. In spliceosomes stalled after the second step of splicing, addition of manganese to the buffer results in release of cleaved 5' and 3' exons, likely via hydrolytic cleavage reactions on fully spliced mRNAs in a spliced exon reopening reaction.[23,49] While the RNA requirements for catalysis of this reaction and its relationship, if any, to the spliced exon reopening reactions observed in self-splicing ribozymes remains to be established, the existing data draw strong parallels between the spliceosomal active site and that of group II self-splicing introns.[12-14,23] This, in turn, further strengthens the likelihood that the U6 sequence elements equivalent to those forming the active site of the group II introns may be involved in forming the spliceosomal active site.

Another interesting conclusion from the aberrant reactions described above, in which U2 is used as the 5' splice site, is the flexibility of the spliceosomal active site with respect to the choice of the splice sites. This is consistent with previous results, in which mutations in the U6 sequences upstream of the ACAGAGA box in nematodes led to the aberrant use of these mutant sequences as the 5' splice site.[50] In a recent analysis, Smith and colleagues[51] systematically probed the positioning of the branch site adenosine within the active site. Using a mutant U2 snRNA which could only participate in the splicing of a pre-mRNA bearing complementary mutations in its branch site, the authors could show that while there is considerable flexibility in the sequence of the branch site-U2 basepairing interactions which flank the branch site adenosine, the location of the bulged adenosine within this duplex could be moved only one nucleotide upstream or downstream of its original position. Further analyses indicated that this narrow window is at least partially defined by the distance between the branch site adenosine and the U2/U6 helix I (**Fig. 3**, see below), which likely reflects its positioning in relationship to the rest of the catalytically critical elements in the active site. Thus, despite a significant degree of sequence flexibility in the choice of splice sites, maintaining the spatial positioning of the RNA elements of the active site in relationship to the reacting groups seems to be critical for splicing.

The Three Dimensional Positioning of snRNAs in the Active Site

While under certain conditions U2 snRNA may be dispensable for splicing, the very low efficiency of these aberrant reactions indicate that U2 is essential for physiological splicing. In the activated spliceosome, in addition to forming basepairing interactions with the branch site of introns, U2 forms a number of basepaired helices with U6 which are thought to act as scaffolds for juxtaposing the reactive groups and the rest of the active site components[15,16,40] (**Figs. 1 and 3**). While the details of the basepairing interactions may be different in yeast and human spliceosomes[52-54] (**Fig. 3**), the general architecture of the U6/U2 basepaired complex is conserved. The invariant ACAGAGA box of U6 and the branch binding sequence of U2 are juxtaposed via the basepairing interaction which forms the U6/U2 helix I. This basepaired structure also connects these two functionally critical regions with the other invariant sequence of U6, the AGC triad, and the intramolecular stemloop (ISL) of U6, which contains a conserved, functionally required metal binding pocket[26,30] (**Fig. 3**).

As mentioned above, the sequences in group II introns that are equivalent to the U6 ACAGAGA and AGC boxes and the ISL metal binding pocket converge to form the active site and thus, it is conceivable that in the three dimensional structure of the U6/U2 complex in activated spliceosomes, these regions may be juxtaposed to create a similar active site. Experimental evidence for such a spatial arrangement was provided by hydroxyl radical footprinting experiments in assembled spliceosomes both before and after the first step of splicing.[55] In these studies, hydroxyl radicals generated by a tethered Fe-BABE probe attached to the 10th nucleotide of the intron resulted in cleavage of the nucleotides upstream of the ACAGAGA box in U6 and the residues close to the metal binding pocket in the U6 ISL, indicating the proximity of these two structures in the folded U6/U2 complex both before and after the first step of splicing. Further, in vivo mutagenesis studies had shown a long-range interaction between U2 residues that are located across the helix from the AGC triad and the second G in the ACAGAGA box[56] (**Fig. 1**), which suggested the proximity of these two invariant domains of U6 in the spliceosomes. Taken together, the existing data suggest that the catalytically critical regions in U6 are positioned close to each other in the folded, active structure of the U6/U2 complex found in the activated spliceosomes, perhaps in an arrangement similar to the one found in group II introns.[12,14]

While several studies indicate that the transition from the first step of splicing to the second involves a conformational change,[54,57-59] it is likely that the same three domains of U6 are involved in catalysis of both steps of splicing. Mutagenesis studies have suggested a critical role for all three regions in both the first and second steps of splicing.[15,16] Further, as mentioned above, the hydroxyl radical footprinting experiments performed on

activated spliceosomes before and after the first step of splicing[55] suggest that the remodeling of the snRNA structures is rather minor. Thus, it is likely that the main conformational changes involve the removal of the first step products and positioning of the second step substrates in the active site. Consistent with this possibility, mutational complementation analyses in yeast have indicated that while strengthening the 5' SS-ACAGAGA interaction by increasing the basepairing potential between the two allowed the first step of splicing to go to completion, it significantly reduced the efficiency of the second step.[48] This result not only confirms the previous data indicating that the first step of splicing occurs in close proximity of the ACAGAGA box, it also indicates that removal of the first step products from the vicinity of this critical region of U6 is required for the second step of splicing to happen. In summary, current data suggest that similar to what has been observed in group II introns,[60,61] the same RNA elements are likely to be involved in catalysis of both steps of splicing. While the active site elements undergo a minor remodeling between the two steps of splicing, several other RNA-RNA interactions including snRNA-substrate interactions seem to be significantly rearranged during the catalytic cycle of the spliceosome.[57-61]

Spliceosomal snRNAs Sans Proteins: An RNA-Centric View of the Active Site

The identity of the catalytic domains and the extent of involvement of the RNA and protein components of the spliceosome in catalysis has been a central and long-standing question in the splicing field. As mentioned above, a significant body of data point to the formation of a functionally critical, complex folded structure for the snRNAs in the activated spliceosomes. This folded structure not only helps to form binding pockets for coordinating catalytically essential metal ions, but also juxtaposes RNA sequence elements that are thought to participate in the formation of the spliceosomal active site. In a long-held RNA-centric view of the spliceosomal active site, the snRNAs are thought to be fully competent to form the majority, if not all, of the active site and to perform catalysis, much like the case with the self-splicing group II introns. However, in the activated spliceosomes several protein factors interact with the snRNAs and the pre-mRNA throughout the spliceosomal cycle (see below). While the exact role played by the proteins in the active site is mostly unknown, their possible roles could range from assisting the snRNAs in assuming their functional structure, assisting in or independently coordinating critical metal ions and participating in the positioning of the substrates, to independently forming part of the active site and direct involvement in catalysis.[62] As a first step toward defining the share of the spliceosomal RNA and protein components in the formation of the active site and catalysis, the most direct and fruitful approach is likely the analysis of the function of snRNAs and proteins in isolation using simplified, in vitro models.

The folding and structure of protein-free snRNAs have now been studied by several groups using a variety of biochemical and structural biology techniques. Initially, it was shown that in vitro-transcribed, protein-free RNAs corresponding to the functionally required domains of human U2 and U6 snRNAs can efficiently form a basepaired complex in vitro in the presence of magnesium.[63] Psoralen-mediated crosslinking analyses indicated the formation of U6/U2 basepaired helices I, II and III in this basepaired complex (**Fig. 1**, Valadkhan S and Manley, unpublished data). In addition, crosslinking studies indicated that a previously described, functionally required interaction between the second G residue in the ACAGAGA box and the U2 sequences across the helix from the AGC triad[56] is also present in this protein-free, in vitro-assembled complex.[63] This result suggested that the spontaneous three-dimensional folding of the in vitro-assembled U6/U2 complex is close to the functionally required structure that these snRNAs assume in the spliceosome. Recently, Fluorescence Resonance Energy Transfer (FRET) experiments have shown the presence of at least three different folded structures for the U6/U2 basepaired complex in solution.[64] Interestingly, in one of these structures the ACAGAGA-containing stem and the U6 ISL are positioned in close proximity, similar to the arrangement previously shown to exist in activated spliceosomes,[55] and the conserved, bulged U residue in the U6 ISL is required for the formation of this folding arrangement.[64]

In addition to stabilizing the proximity of the ACAGAGA and the ISL in the folded structure of protein-free U6/U2 complexes, the bulged U residue in the U6 ISL is part of a functionally critical metal-binding pocket. Phosphorothioate interference analyses in nuclear extracts have provided evidence for a functionally important metal coordination by the non-bridging phosphate oxygen located 5' to this bulged U[30-32] (**Fig. 3**). Interestingly, NMR studies have provided evidence for metal binding to this phosphate group in isolated U6 ISLs, indicating that the metal-binding ability was an inherent property of this snRNA structure.[26,28] NMR and biochemical analyses have similarly demonstrated the presence of an analogously positioned metal binding pocket in the counterpart of the ISL in group II introns, domain V[25,27] (**Fig. 3**), which helps coordinate the active site metal ions.[13,24] It is conceivable that the metal binding pocket of the U6 ISL may play a similar role in the spliceosomal active site.

In addition to the ISL, phosphorothioate interference analyses in nuclear extracts have suggested the presence of two additional metal binding sites in U6 in the ACAGAGA and AGC boxes[31,32] (**Fig. 3**). Analogous sequences in group II introns, together with the metal binding pocket in the asymmetric internal loop of domain V (see above), contain the functional groups involved in coordination of the catalytic metal ions in the active site.[13,24,65] Using protein-free snRNAs, Yuan and colleagues[29] used a FRET-based assay that took advantage of the increase in the luminescence of lanthanide ions (e.g., terbium III) upon forming inner sphere coordination with a metal binding site. Based on the changes in the luminescence of the site bound Tb(III) ions and fluorescence resonance energy transfer between a covalently attached fluorescent dye and the site-bound Tb(III), three metal binding sites could be detected in the U6/U2 complex, corresponding to the ACAGAGA sequence, the vicinity of the AGC triad and the asymmetric bulge of the U6 ISL. These results suggest that the reduction in splicing efficiency observed upon phosphorothioate

substitutions in the ACAGAGA and AGC sequences in U6 may result from the loss of functionally required inner sphere coordinated metal ions and perhaps more importantly, that in the absence of all other spliceosomal factors, the complex of U6 and U2 snRNAs has the inherent ability to coordinate metal ions at these functionally critical sequences.

Do these metal binding sites represent the functional groups coordinating active site metal ions? By replacing the 3'-linked oxygen at either the 5' or the 3' splice site with sulfur and demonstrating a metal specificity switch, Piccirilli and colleagues provided evidence for functionally critical inner sphere metal coordination by these two oxygens, which are the leaving groups of the first and second step of splicing, respectively.[20,22] Previous phosphorothioate substitution studies had indicated that replacing the pro-Rp non-bridging phosphate oxygen at the 5' splice site blocks the first step of splicing, a finding which could indicate the involvement of this oxygen in coordinating functionally important metal ions.[66,67] As mentioned above, crosslinking and mutational complementation analyses have indicated that at the time of catalysis of the first step of splicing, the 5' splice site is positioned in close proximity of the ACAGAGA sequence, partially via basepairing to this sequence[16,48] (**Fig. 1**). Interestingly, this interaction positions the 5' splice site directly across the duplex from the phosphorothioate interference sites in the ACAGAGA sequence[16] (**Fig. 3**). Thus, it is conceivable that the metal ion possibly bound by the ACAGAGA sequence may be the same one which is coordinated by the non-bridging oxygen and the leaving group at the 5' splice site, likely facilitating the departure of the leaving oxyanion.

Taken together, significant evidence suggest that the primary sequence of U2 and U6 snRNAs is sufficient to direct their folding into a basepaired structure that not only contains many of the long-range interactions observed in the catalytically active spliceosomes in vivo, but also has the ability to form binding pockets for functionally critical metal ions. Further, basepairing interactions between the two snRNAs, the 5' splice site and the branch site play a significant role in positioning of the substrates in the active site of the spliceosomes[16] (**Fig. 3**), suggesting that a large share of the RNA-based interactions found in the spliceosomal active site can be reconstituted in protein-free systems in vitro.

## Catalytic Activity of the RNA Elements of the Active Site in Isolation

As mentioned above, the in vitro-assembled, basepaired complex of U2 and U6 snRNAs to a large extent recapitulates the structural aspects and metal binding properties of the U2-U6 complex formed in the activated spliceosomes, which in turn has close structural and functional similarities to the catalytically essential domains of group II introns. These similarities pose the tantalizing possibility that the folded structure of the in vitro-assembled U2-U6 complex may contain a sufficiently large share of the spliceosomal active site for being functionally active in isolation.

Several attempts at investigating this possibility has been made using the in vitro-assembled U2-U6 complex and various minimal splicing substrates. In one case, a catalyzed reaction was

observed in which the designated branch site adenosine in a short intron-like substrate was covalently linked to the G residue in the AGC domain of U6. While the reaction chemistry was clearly distinct from splicing and highly inefficient, it depended on the catalytically critical ACAGAGA and AGC sequences in U6 and the ability of the substrate to bind the branch binding site of U2 in a way that would leave the branch site adenosine in an unpaired, bulged conformation.[68,69] In another observed reaction, a covalent linkage was formed between the branch site sequence of an intron-like short oligonucleotide substrate and a short sequence resembling the 5' splice site consensus. This reaction, which was also dependent on the invariant U6 sequences, resulted in release of a fragment of the 5' splice site-like sequence, which suggested similarity to the release of the 5' exon after the first step of splicing.[70] In both these reactions, the low efficiency of product formation prohibited a thorough analysis of the chemistry of the reaction and its requirements; however, the dependence of both reactions on invariant regions in U6 and the presence of magnesium in the buffer suggested that at least in a limited sense they are related to spliceosomal catalysis.

More recently, by redesigning the positioning of the short oligonucleotides used as substrates, it was shown that the in vitro-assembled U2/U6 complex can indeed catalyze a two step splicing reaction similar to the one catalyzed by the self-splicing group II introns and the spliceosome[71] (**Fig. 4**). In the first step of this reaction, the substrate containing the 5' splice site was cleaved via a catalyzed hydrolysis reaction, resulting in the release of an "exon" with a free 3' OH group. The second step involved a transesterification reaction between the newly released 3' OH of the cleaved first exon and an internal phosphate in the 3' splice site substrate, resulting in the release of an "intronic" sequence and formation of a linear product containing the "exonic" fragments of the two substrates joined together via a 3'-5' phosphodiester linkage (**Fig. 4**). Mutations in the ACAGAGA and AGC sequences and the U6 ISL either completely blocked product formation or resulted in significantly reduced efficiency, suggesting that similar to the authentic spliceosomal catalysis, these sequences may play a role in catalysis of this reaction. Further, crosslinking and mutational complementation analyses indicated that the reaction occurred in the vicinity of the ACAGAGA sequence, a further parallel with the spliceosomal catalysis.[71] Finally, the reaction was magnesium dependent, similar to both spliceosomal and group II intron catalysis, both of which require divalent cations.[14,16]

Although there are clear similarities between this reaction and spliceosomal splicing both in the chemistry of the reaction and the RNA sequences involved, the mechanism of the first step of splicing is distinct in the two systems. Unlike the branching reaction observed in the spliceosome, the cleavage of the 5' splice site occurs through hydrolysis in this minimal, RNA-only system. This could be due to the absence of a branch site-like substrate, which precludes a branching reaction. Thus, it is possible that with the careful addition of a third substrate to the reaction, the U6/U2 complex can also catalyze a branching reaction. Alternatively, an additional spliceosomal factor might be necessary for enabling the snRNAs to perform branching. Interestingly, hydrolytic cleavage of the 5' splice site is commonly
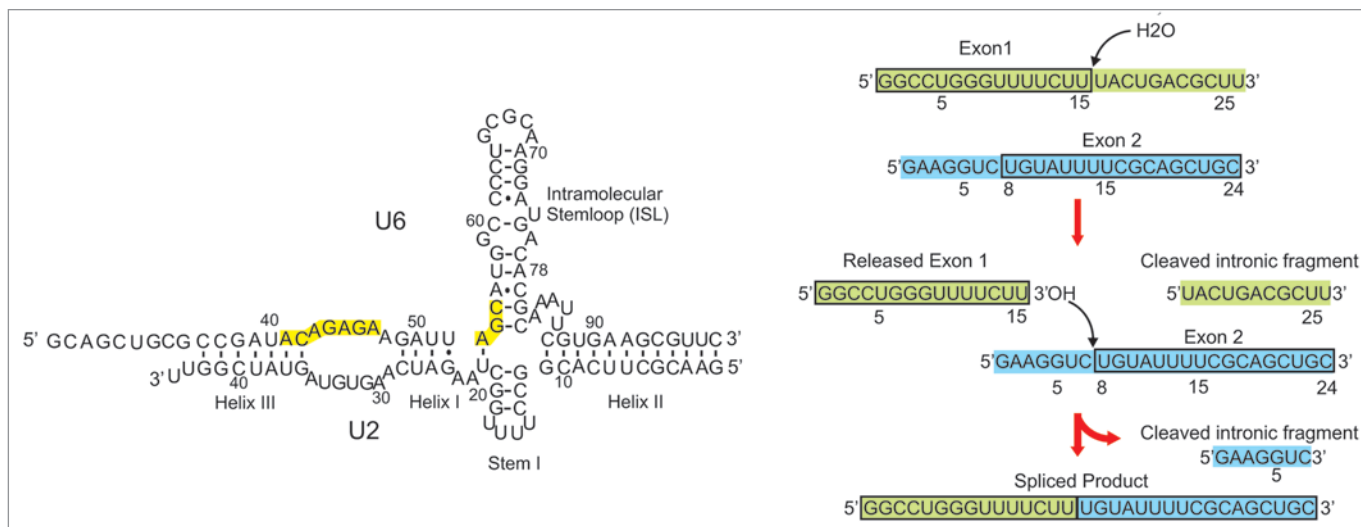
Figure 4. A two-step splicing reaction catalyzed by protein-free snRNAs. The human snRNA constructs which can perform a two-step splicing reaction are shown. The locations of the ISL and helices I, II and III are indicated. Areas highlighted in U6 are the invariant sequences. Numbers correspond to the human numbering system for these two snRNAs. The 5′ and 3′ splice site-containing substrates are shown on the right. The "exonic" sequences are boxed. Numbers indicate position from the 5′ end. In the first step of this splicing reaction, the phosphodiester bond corresponding to the 5′ splice site is cleaved via a catalyzed hydrolysis reaction. Next, the newly released 3′ OH of the released Exon 1 (shown) attacks an internal phosphodiester bond on the second substrate, leading to a transesterification reaction and exon ligation.

observed in group II introns both in vitro and in vivo, and is thought to be a more ancient mechanism for performing the first step of splicing.[23,35,72] Thus, hydrolysis is a physiological alternative to branching at least in group II introns. Further, as mentioned above, it has been recently shown that the spliceosomal active site is also capable of performing hydrolytic cleavage in a reaction similar to the Spliced Exon Reopening (SER) observed in group II introns,[23] providing a precedent for hydrolytic reactions catalyzed by the spliceosomal active site.[49] Although this snRNA-catalyzed two-step splicing reaction is over ten folds more efficient than the previously observed reactions catalyzed by the U6/U2 complex, it is still significantly less efficient than spliceosomal splicing, which suggests that other spliceosomal factors, perhaps some spliceosomal proteins, are necessary for the optimal catalytic function of the snRNAs.

The Role of Proteins in the Spliceosomal Active Site

In vivo many known, well-characterized ribozymes are associated with proteins which improve their catalytic activity via several known mechanisms. These include stabilizing the functional structure of the RNAs, assisting in binding and positioning of the substrates and assisting in binding of functionally important metal ions; however, direct participation in catalysis has not been observed for any of the ribozyme-associated proteins studied so far.[62,73] Assuming that the spliceosome is an RNA enzyme, the spliceosomal snRNAs are unusual ribozymes in many respects, perhaps most importantly, they are unusually small for a ribozyme catalyzing phosphodiester bond cleavage via the activation of a non-adjacent nucleophile. The other known natural ribozymes catalyzing such reactions, namely, group I and II introns and RNase P, are at least two and in many cases several folds longer

than the combined length of human U6 and U2 snRNAs. These ribozymes are also much longer than the rest of the nucleolytic ribozymes which activate an adjacent 2′ hydroxyl nucleophile for phosphodiester bond cleavage.[23,74,75] This longer length is thought to allow them to fold into complex structures stabilized by multiple tertiary interactions, which in turn enable them to create sophisticated active sites capable of accurately positioning the cleavage site, the active site metal ions and the remote nucleophile for in-line nucleophilic attacks. It is conceivable that due to their short length, the snRNAs at best form an inefficient splicing ribozyme, which requires other spliceosomal factors for stable positioning of the active site elements and the reacting groups.

The most conserved spliceosomal protein, Prp8, is known to play such a role in the spliceosomal active site. Crosslinking experiments have indicated the proximity of Prp8 to both the 5′ and 3′ splice sites and the branch site, in addition to U6 and U5 snRNAs.[76,77] Thus, it is clear that Prp8 is closely associated with reacting groups in assembled spliceosomes. Further, mutations in Prp8 are known to affect the efficiency of either the first or second step of splicing, especially for suboptimal substrates.[57,76,78-80] This observation is best explained by a hypothesis stating that Prp8 is involved in stabilization of alternative, mutually-exclusive active site conformations that are either poised for catalysis of the first or the second step of splicing and that certain Prp8 mutants might lead to hyperstabilization of one of these states.[57,79,81] While all evidence suggest that Prp8 is likely involved in positioning of the substrates and stabilization of the active site, evidence for involvement in metal ion coordination or direct participation in catalysis has been lacking.

Intriguingly, recent high resolution structural studies of a fragment of Prp8 close to its C-terminal domain has indicated the presence of a degenerate RNase H-like motif in this protein.[82-85]

While the general topology of the domain is conserved, only one of the three catalytic residues at the active site is present. This residue, an aspartate, is involved in coordinating both catalytic metals in the active site of canonical RNase H domains. Mutation of this residue in Prp8 in yeast in one study resulted in no detectable growth defect, while in another it caused protein misfolding and led to a lethal phenotype.[82,83] No metal binding was observed by the region corresponding to the active site of the RNase H domain when the crystals were grown in up to 200 mM $MgCl_2$, and it bound RNA with a Kd of 20 to over 200 μM, depending on the RNA species tested.[82-84] What made this RNase H-like domain particularly interesting was that the amino acids corresponding to its active site are positioned adjacent to residues in Prp8 that were previously shown to crosslink to the 5' splice site in precatalytic spliceosomes.[86] However, the efficiency of the formation of this crosslink was decreased as the precatalytic spliceosomes progressed to become catalytically active spliceosomal complexes.[86] The observed decrease in crosslinking efficiency could be interpreted to indicate that this interaction may be disrupted in activated spliceosomes or alternatively that the interaction persists, however the formation of the crosslink itself is abolished due to a minor change in the environment of the crosslinked residues. If this degenerate RNase-H-like domain is indeed in proximity of 5' splice site in catalytically active spliceosomes, it is possible that it may participate in positioning of the substrates in the active site and/or coordinating catalytic metals. While the domain by itself cannot bind RNA or metals, it is possible that in the presence of the rest of the active site elements it may form part of the substrate or metal binding pockets. Proving or refuting these possibilities await future biochemical and structural biology experiments.

Insights into Spliceosomal Catalysis in the Years to Come

As discussed above, many fundamental questions in spliceosomal catalysis have remained unanswered, however perhaps the most pivotal question remaining is the extent by which proteins are involved in splicing catalysis. While a high resolution structure of the spliceosome would provide invaluable clues into this and many other remaining questions in the splicing field, it is likely that such structural information unfortunately will not be available in the near future. Further, although the existence of high resolution structural data for many other enzymatic systems has helped guide the direction of scientific inquiry in the field, in many cases it has not been sufficient by itself to determine the mechanism of function of the enzyme and detailed biochemical analysis has been necessary for providing definitive answers. It is very likely that understanding spliceosomal catalysis, likewise, will require in-depth biochemical characterization, which in turn necessitates the development of simplified models for the spliceosomal active site which are amenable to such detailed analyses. On the other hand, the availability of a high resolution structure for a group II intron ribozyme has made it possible to formulate testable hypotheses on the similarity of the active site organization between these introns and the spliceosome and will likely lead to a wave of new insights. Taken together, progress in the last two years has significantly added to our understanding of spliceosomal function. Whether the spliceosome proves to be an RNA enzyme heavily buttressed by proteins, or contain an active site made of both RNAs and proteins, it remains a fascinating example of the transition of the RNA world into the present, protein-dominated one.

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