Leading Edge
Review

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The Spliceosome: Design Principles of a Dynamic RNP Machine

Markus C. Wahl,[2,3,*] Cindy L. Will,[1,*] and Reinhard Lührmann[1,*]
[1]Zelluläre Biochemie
[2]Makromolekulare Röntgenkristallographie
Max-Planck-Institut für biophysikalische Chemie, Am Faßberg 11, D-37077 Göttingen, Germany
[3]Fachbereich Biologie, Chemie, Pharmazie, Institut für Chemie und Biochemie, Freie Universität Berlin, AG Strukturbiochemie, Takustr. 6, D-14195 Berlin, Germany
*Correspondence: mwahl@gwdg.de (M.C.W.), cwill1@gwdg.de (C.L.W.), reinhard.luehrmann@mpi-bpc.mpg.de (R.L.)
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Ribonucleoproteins (RNPs) mediate key cellular functions such as gene expression and its regulation. Whereas most RNP enzymes are stable in composition and harbor preformed active sites, the spliceosome, which removes noncoding introns from precursor messenger RNAs (pre-mRNAs), follows fundamentally different strategies. In order to provide both accuracy to the recognition of reactive splice sites in the pre-mRNA and flexibility to the choice of splice sites during alternative splicing, the spliceosome exhibits exceptional compositional and structural dynamics that are exploited during substrate-dependent complex assembly, catalytic activation, and active site remodeling.

At the Interface of RNA and Protein Worlds

RNAs and proteins cooperate extensively in ribonucleoproteins (RNPs) to bring about the biological functions of these molecular machines. Despite their vastly different chemical properties, these two classes of macromolecules exhibit overlapping functional repertoires. For example, both can act as biological catalysts and both can function as versatile binding platforms. RNA-protein collaborations form the basis of numerous enzymatic machineries that mediate key cellular functions.

RNPs are particularly prevalent in the processes of gene expression and its regulation. Consider for example the life cycle of a typical eukaryotic mRNA. The mRNA is synthesized as a precursor (pre-mRNA) during transcription in the nucleus. There, it undergoes a series of processing steps before being transported to the cytoplasm where it serves as a template for protein biosynthesis and where it is eventually degraded (Figure 1). An mRNA invariably exists as an RNP whose protein inventory changes during each phase of its existence (see SnapShot by M. Bergkessel, G.M. Wilmes, and C. Guthrie on page 794 of this issue). For example, different heterogeneous nuclear RNP (hnRNP) proteins associate with the pre-mRNA during transcription. Some of these proteins are then removed and others are added as the RNP is remodeled during mRNA processing, nuclear export, and translation. The particular repertoire of proteins present at each stage of the mRNA life cycle and their locations on a pre-mRNA or mRNA determine the fate of the RNA molecule.

RNPs direct many of the cellular processes involving pre-mRNAs or mRNAs (Figure 1). Uridine-rich (U-rich) small nuclear RNPs (snRNPs) form the major building blocks of the spliceosome, the large RNP that carries out pre-mRNA splicing, whereas the specialized U7 snRNP directs the 3′-end processing of intronless histone pre-mRNAs. Protein biosynthesis is mediated by another large RNP, the ribosome, which directs mRNA translation through the combined actions of its small and large RNP subunits. The ribosome further cooperates with the signal recognition particle (SRP) RNP to translocate some newly synthesized proteins into the endoplasmic reticulum. Protein biosynthesis can also be regulated by microRNPs containing small noncoding RNAs, as was discovered relatively recently (Figure 1). microRNPs typically recognize 3′ untranslated regions of target mRNAs and, depending on the degree of base-pairing complementarity between their guide RNAs and the target mRNA, induce transient suppression of translation or degradation of their target mRNAs (see Review by R.W. Carthew and E.J. Sontheimer on page 642 and Review by O. Voinnet on page 669 of this issue).

RNA-protein complexes are also involved in the biogenesis of RNAs, including the RNA components of RNPs (Figure 1). For example, box C/D and box H/ACA small nucleolar RNPs (snoRNPs) methylate 2′-hydroxyl groups and convert uridines into pseudouridines, respectively, in ribosomal RNAs. A related group of RNPs, the small Cajal body RNPs (scaRNPs), introduces similar modifications in snRNAs. The maturation of transfer-RNAs (tRNAs) essential to protein translation also requires the actions of the RNP RNase P, which generates the mature tRNA 5′ end. Indeed, RNPs play a broad role in cellular processes as even the maintenance of genome stability and regulation of chromatin states require the action of RNA-protein complexes such as telomerase and the RNA-induced transcriptional silencing (RITS) complex (Figure 1).

Job Sharing and Cooperation between RNAs and Proteins

RNPs are involved in a large spectrum of molecular activities. Whereas assemblies such as the signal recognition particle or the U7 snRNP serve as adaptors or transport devices, other

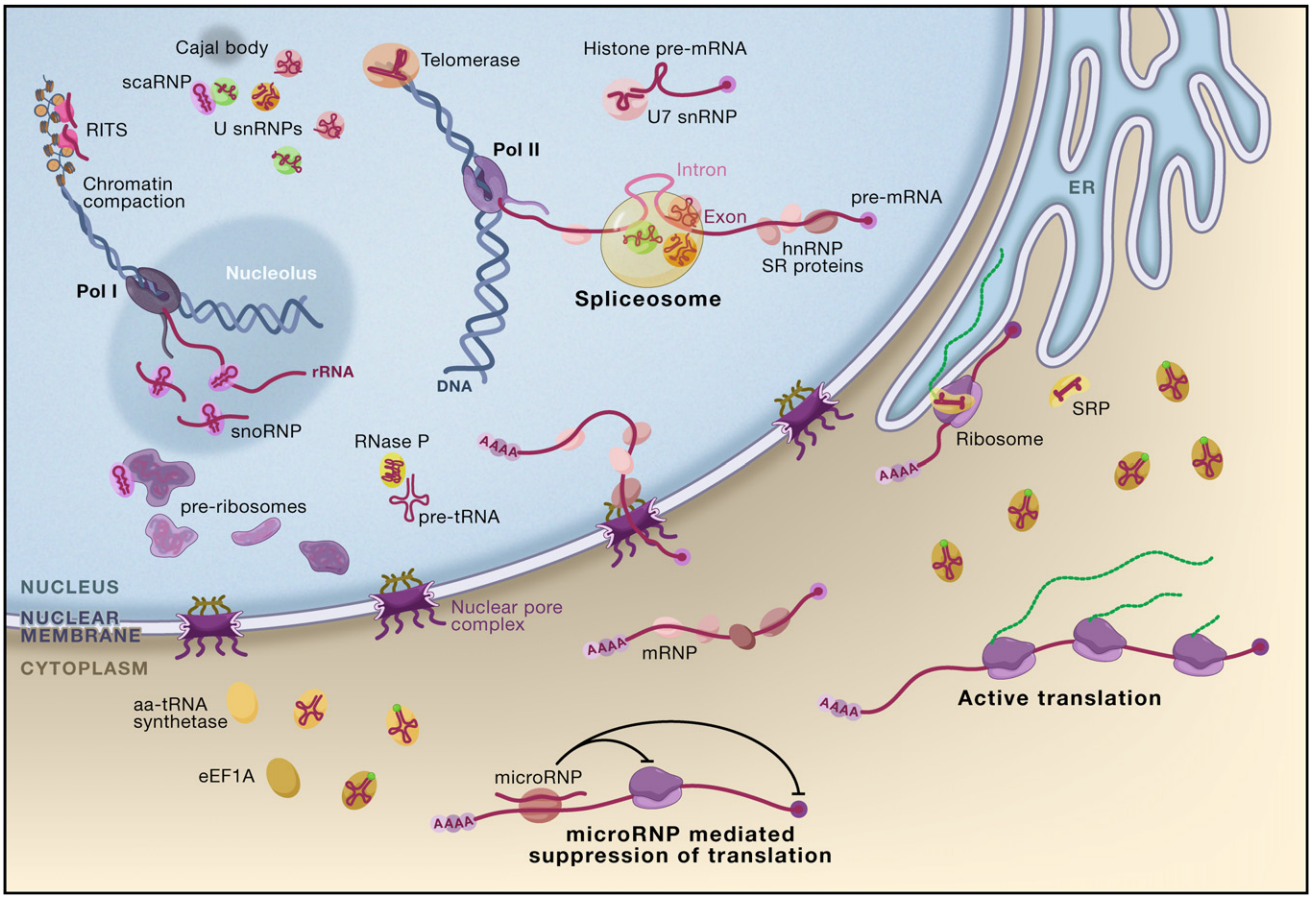

Figure 1. RNPs in Gene Expression and Its Regulation

RNPs play extensive roles in gene expression and its regulation. Here, the major activities of RNPs during gene expression in a eukaryotic cell are depicted. Following transcription by RNA polymerases II (RNA Pol II), pre-mRNAs are bound by diverse proteins, such as hnRNP and SR (serine-arginine-rich) proteins. Pre-mRNAs, containing exons (red) and introns (pink), are subjected to processing by a range of RNPs that include uridine-rich (U-rich) small nuclear RNPs (U snRNPs) that make up the spliceosome. Certain RNAs such as pre-transfer RNAs and mRNA transcripts encoding histones also undergo processing by specific RNPs (RNase P and U7 snRNP, respectively). Small nucleolar RNPs (snoRNPs) and small Cajal body RNPs (scaRNPs) mediate maturation of RNA components of RNPs such as ribosomal RNAs (transcribed by RNA polymerase I, RNA Pol I) and snRNAs, respectively. Small RNAs can form microRNPs that function to regulate translation. In certain organisms, RNA-induced transcriptional silencing (RITS) complexes, which contain small-interfering RNAs, mediate heterochromatin formation and maintenance. Telomerase, a box H/ACA snoRNP, replenishes the terminal telomeric repeats of chromosomes to maintain genomic stability. In the cytoplasm, the ribosome is the key RNP that directs the translation of mRNA into protein. It also functions with the signal recognition particle (SRP) RNP to direct protein translocation into the endoplasmic reticulum (ER). tRNAs also form complexes in the cytoplasm with aminoacyl-tRNA (aa-tRNA) synthetases, which charge tRNAs with the corresponding amino acid, and with translation elongation factor eEF1A.

complexes such as the sno/scaRNPs, the spliceosome, and the ribosome act as enzymes (Figure 1). As RNAs and proteins can in principle functionally substitute for each other (even in their catalytic activity), RNPs span a compositional continuum (Hoogstraten and Sumita, 2007). At one end of this continuum are true ribozymes in which RNA is the catalytic entity. These include self-splicing group II introns, which are found in coding and noncoding RNAs in all kingdoms of life. Although these ribozymes function within RNPs in vivo, the proteins in these complexes play only supporting roles. At the other end of the continuum are RNPs in which proteins carry out the catalytic function with RNA playing solely a scaffolding or templating role. This group includes the sno/scaRNPs, in which the sno/scaRNAs serve both as scaffolds to assemble the catalytic protein subunits and as guides to identify substrate molecules by Watson/Crick base pairing. It also includes microRNPs in which guide RNAs mediate target selection and Argonaute proteins provide the enzymatic target-slicing activity.

Many RNP machines have acquired larger numbers of proteins during the course of evolution, as illustrated by RNase P enzymes or the ribosome. It seems that functions encoded by RNAs alone in primordial systems have been transferred to proteins during evolution. These functions include promoting RNA folding and stability, supporting substrate binding, increasing the range of substrates, enhancing product release, and substituting for metal ions that are required for catalysis or for RNA folding (Hoogstraten and Sumita, 2007). Through these acquired functions, proteins support catalytic functions even in

RNPs where the RNA originally appeared to exclusively mediate chemical catalysis.

This interdependence of RNA and protein for catalytic function is particularly evident in the most elaborate and fascinating of extant RNP machines: the ribosome and the spliceosome. Both of these assemblies are composed of several RNAs and numerous proteins that constitute RNP enzymes in which neither proteins nor RNAs can function without one another. Some similarities between these two systems have been noted in the past (reviewed in Konarska and Query, 2005). However, the fundamentally different nature of the processes mediated by these machines also calls for different design principles.

Stable versus Dynamic RNP Machines

In many respects, the ribosome is representative of the vast majority of RNPs, which are compositionally stable and harbor preformed active sites. The concept of the ribosome as a molecular machine becomes most obvious in the elongation phase of protein biosynthesis. During elongation, amino acids are added to a growing peptide chain in a repeating polymerization process reminiscent of a robotic assembly line. It is also during elongation that the ribosome carries out its primary functions: the decoding of mRNA codons in the decoding center of the small subunit and the formation of new peptide bonds in the peptidyl transferase center of the large subunit (see Review by H.S. Zaher and R. Green on page 746 of this issue). The ribosome undergoes a number of local and global conformational transitions during peptide chain elongation (Korostelev et al., 2008; Steitz, 2008). For example, correct tRNA-mRNA pairing is verified by coupling of a conformational change in the 16S rRNA to new favorable interactions with the minor groove of a tRNA-mRNA mini-helix (Ogle et al., 2001). This conformational rearrangement is accompanied by a global transition of the small subunit from an open to a closed state. The allosteric cascade is transmitted to the GTPase-associated region on the large subunit, which stimulates GTP hydrolysis by elongation factor 1A (eEF1A; EF-Tu in bacteria). GTP hydrolysis triggers the release of the aminoacyl-tRNA for accommodation within the peptidyl transferase center. After peptide bond formation, the ribosome must translocate the message with the associated tRNAs by one codon. This translocation is driven by eEF2 (EF-G in bacteria) GTP hydrolysis (Rodnina et al., 1997) and involves a ratcheting motion of the small subunit with respect to the large subunit (Frank and Agrawal, 2000).

Notably, except for the repetitive coming and going of elongation factors, the composition of the ribosome remains stable during elongation. Furthermore, the decoding center and the peptidyl transferase center are fully preformed in the small and large subunits, respectively (Steitz, 2008). Structural (Ogle et al., 2001; Selmer et al., 2006; Simonovic and Steitz, 2008) and kinetic studies (Wohlgemuth et al., 2006) revealed that the ribosomal subunits function identically either in isolation or within the framework of the entire ribosome. Elongation factors act to increase the fidelity and directionality of the translation process but do not seem to be essential for peptidyl transfer or decoding per se. Evidently, subunits with relatively stable compositions and preformed active sites are well-suited to support the highly processive polymerase activity of the ribosome.

The vectorial process of pre-mRNA splicing facilitated by the spliceosome calls for different design principles. One of the most distinguishing features of the spliceosome is that it forms stepwise on the pre-mRNA (Burge et al., 1999). The substrate pre-mRNA plays an integral role in the formation of an active site immediately after the initial assembly steps of the complex. The intricate involvement of the substrate in building the active sites of the spliceosome goes far beyond the substrate-assisted catalysis recently revealed as a chemical catalytic principle of the ribosomal peptidyl transferase center (reviewed in Beringer and Rodnina, 2007). Another unique principle of the spliceosome is the initial sequestration of active site RNA components into inactive conformations for release at the appropriate times to engage in new interactions (catalytic activation). Indeed, the extensive structural and compositional dynamics of the spliceosome, unprecedented among RNP machines, give it the remarkable plasticity that also renders it particularly susceptible to regulation. Regulation is frequently implemented by two combinatorial control principles. First, functional sites on the pre-mRNA are recognized several times by different factors to ensure splicing fidelity, and second, multiple weak binary interactions act synergistically to consolidate or repress splice-site choices. A final important aspect of the spliceosome is that its protein components not only play key roles in the formation of the RNA/RNP structures required for splicing catalysis but are also intimate parts of the active sites and may even directly participate in splicing catalysis. This unparalleled degree of cooperation between RNA and proteins makes the spliceosome a paradigm for a mixed RNA/protein enzyme (Abelson, 2008). In the following, we will describe the molecular mechanisms underlying the exceptional dynamics of the spliceosome.

The Spliceosome, a Highly Dynamic RNP Machine

Nuclear pre-mRNA splicing entails two S_N2-type transesterification reactions, relatively simple chemical reactions involving functional groups from three reactive regions in the pre-mRNA. First, the phosphodiester bond at the 5′ splice site (SS) is attacked by the 2′-hydroxyl of an adenosine of the branch point sequence (BPS) in the intron, which generates a free 5′ exon and an intron lariat-3′ exon. Subsequently, the 3′-hydroxyl of the 5′ exon attacks the phosphodiester bond at the 3′SS, leading to exon ligation and excision of the lariat intron (Figure 2). The 5′SS, BPS, and 3′SS of nuclear pre-mRNA introns are defined by very short consensus elements that, in metazoans, are also very poorly conserved (Figure 2A). As a consequence, these introns contain relatively little consistent secondary or tertiary structural information. Therefore, unlike self-splicing group II introns that, on their own, can adopt a three-dimensional fold with an active site in which the reactive groups of the intron are juxtaposed (Toor et al., 2008), the folding of nuclear pre-mRNA introns in a manner conducive to splicing is dependent on a multitude of *trans*-acting factors that comprise the spliceosome. During splicing, the spliceosome must overcome a number of challenges. These include the correct recognition and pairing of the splice sites within a multitude of similar sequences, as well as the positioning of these splice sites (which may lie tens

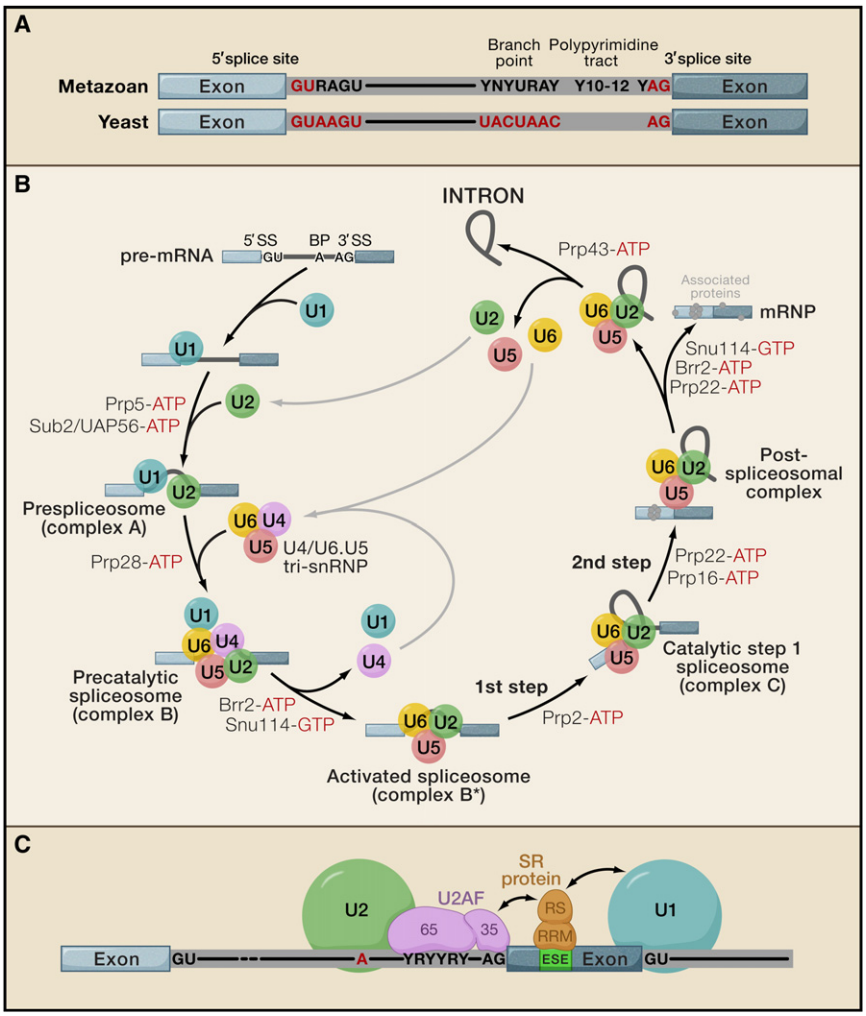

Figure 2. Pre-mRNA Splicing by the Major Spliceosome

(A) Conserved sequence elements of metazoan and yeast pre-mRNAs. Here, two exons (blue) are separated by an intron (gray). The consensus sequences in metazoans and yeast at the 5′ splice site (SS), branch point sequence (BPS), and 3′ splice site (SS) are as indicated, where N is any nucleotide, R is a purine, and Y is a pyrimidine. The polypyrimidine tract is a pyrimidine-rich stretch located between the BPS and 3′SS.

(B) Cross-intron assembly and disassembly cycle of the major spliceosome. The stepwise interaction of the spliceosomal snRNPs (colored circles), but not non-snRNP proteins, in the removal of an intron from a pre-mRNA containing two exons (blue) is depicted. Only the spliceosomal complexes that can be resolved biochemically in mammalian splicing extracts are shown. Eight evolutionarily conserved DExD/H-type RNA-dependent ATPases/helicases act at specific steps of the splicing cycle to catalyze RNA-RNA rearrangements and RNP remodeling events. These enzymes include Sub2 (UAP56 in humans), Prp5, Prp28, Brr2, Prp2, Prp16, Prp22, and Prp43 (with Brr2 and Prp22 acting at more than one step in the cycle). The GTPase Snu114 also functions at several steps during the cycle. In yeast, Prp28 acts at a later stage during spliceosome activation (the B complex to B* complex transition) (Staley and Guthrie, 1998). Several of these proteins, such as Prp5, Prp16, and Prp22, also carry out proofreading functions at the stages where they are shown.

(C) Cross-exon splicing complexes form on long introns during the earliest stage of spliceosome assembly. An SR protein containing an arginine-serine-rich (RS) domain and RRM (RNA recognition motif) is depicted as interacting with an exonic splicing enhancer (ESE). The U1 (blue) and U2 (green) spliceosomal snRNPs and the two subunits of the U2 auxilliary factor (U2AF), U2AF65 and U2AF35, are also shown interacting with the splice sites flanking the exon.

of thousands of nucleotides apart) within the atomic distance that allows the transesterification reactions to proceed. Solutions to these problems come from the large number of subunits in the spliceosome and the principles by which the various protein and RNA players are brought together on the substrate pre-mRNA.

Whereas some exons are constitutively spliced, that is, they are present in every mRNA produced from a given pre-mRNA, many are alternatively spliced (especially in higher eukaryotes) to generate variable forms of mRNA from a single pre-mRNA species (reviewed in Black, 2003; Blencowe, 2006; Graveley, 2001; Smith and Valcarcel, 2000). Alternative splicing enhances the complexity of the proteomes of higher organisms. Understanding regulated splicing is of prime medical relevance, as many human genetic diseases are associated with aberrant pre-mRNA splicing (Nissim-Rafinia and Kerem, 2005; Wang and Cooper, 2007) (see Review by T.A. Cooper, L. Wan, and G. Dreyfuss on page 777 of this issue). To accommodate the highly regulated nature of the splicing process in higher eukaryotes, the spliceosome must not only catalyze splicing with great precision but also exhibit a high degree of flexibility that allows a rapid response to regulatory signals.

Stepwise Assembly of the Splicing Machinery

The U1, U2, U4/U6, and U5 snRNPs are the main building blocks of the major spliceosome, which is responsible for removing the vast majority of pre-mRNA introns. Some metazoan species and plants contain a second, minor spliceosome that is composed of the compositionally distinct but functionally analogous U11/U12 and U4atac/U6atac snRNPs, with the U5 snRNP shared between the machineries (reviewed in Patel and Steitz, 2003). Each snRNP consists of an snRNA (or two in the case of U4/U6) and a variable number of complex-specific proteins. In addition, the U1, U2, U4, and U5 snRNPs all contain seven Sm proteins. In contrast to ribosomal subunits, none of these particles possess a preformed active center and several of the snRNPs are substantially remodeled in the course of the splicing reaction.

In the consensus view of spliceosome assembly (Figure 2B) (based on in vitro studies using native gel electrophoresis, affinity

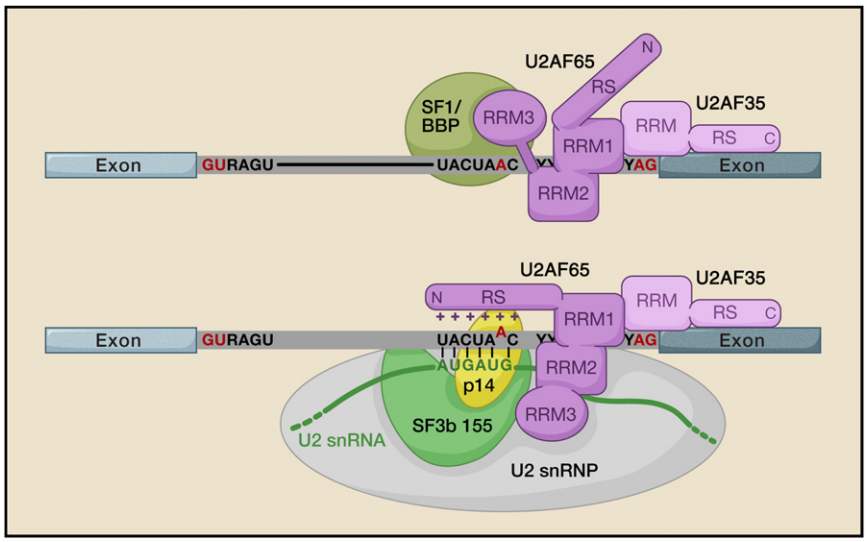

Figure 3. Molecular Interactions at the Branch Site and 3′SS within the Spliceosomal E and A Complexes

(Top) In the spliceosomal E complex, the pre-mRNA (exons, blue; introns, gray) branch site is bound by SF1/BBP, whereas the polypyrimidine tract and 3′ splice site (SS) are bound by the U2 auxilliary factor (U2AF) subunits U2AF65 and U2AF35, respectively. U2AF65 binds both SF1/BBP and U2AF35.

(Bottom) Upon stable U2 snRNP binding during A complex formation, SF1/BBP is displaced, allowing the U2-associated protein p14 to contact the BPS and U2AF65 to interact with SF3b155. The U2/BPS base-pairing interaction is stabilized by components of the U2 snRNP and by the arginine-serine-rich (RS) domain of U2AF65. Adapted in part from Mackereth et al. (2005).

selection, and glycerol gradient centrifugation), landmark assembly intermediates are operationally defined by the sequential association and release of the spliceosomal snRNPs (reviewed in Brow, 2002; Will and Lührmann, 2006). Assembly begins with the ATP-independent binding of the U1 snRNP through base-pairing interactions of the 5′ end of the U1 snRNA to the 5′SS of the intron. This interaction in higher eukaryotes is stabilized by members of the serine-arginine-rich (SR) protein family and proteins of the U1 snRNP. Indeed, most of the functionally important RNA-RNA interactions formed within the spliceosome are weak and generally require the assistance of proteins to enhance their stability. In addition to the U1-5′SS interaction, the earliest assembly phase of the spliceosome also involves the binding of the SF1/BBP protein and the U2 auxiliary factor (U2AF) to the BPS and the polypyrimidine tract just downstream of the BPS, respectively (Figure 3). These proteins bind cooperatively, with SF1/BBP interacting with the 65 kDa subunit of U2AF (U2AF65) through its C-terminal RNA recognition motif (RRM). In addition, the 35 kDa subunit of U2AF, which is tightly bound to U2AF65 in the U2AF heterodimer, binds the AG dinucleotide of the 3′SS. Together, these molecular interactions yield the spliceosomal E complex and play crucial roles in the initial recognition of the 5′SS and 3′SS of an intron.

After the formation of the spliceosomal E complex, the U2 snRNA engages in an ATP-dependent manner in a base-pairing interaction with the pre-mRNA's BPS, leading to the formation of the A complex. This base-pairing interaction is stabilized by heteromeric protein complexes of the U2 snRNP, namely SF3a and SF3b (Gozani et al., 1996), and also by the arginine-serine-rich domain of the U2AF65 protein (Valcarcel et al., 1996). Association of U2 leads to the displacement of SF1/BBP from the BPS (Figure 3). The latter interaction is replaced (at least in higher eukaryotes) by association of SF3b14a/p14 with the BPS adenosine (Will et al., 2001). Moreover, SF3b155 now interacts with the C-terminal RNA recognition motif of U2AF65 (Gozani et al., 1998). These steps illustrate three reoccurring principles of the splicing process. First, the reactive groups of the pre-mRNA are recognized multiple times by RNA or protein to ensure the precision of the splicing reaction. Second, many functionally important binary interactions in the spliceosome are often weak but are enhanced by a combination of multiple interactions. This is a design principle that is crucial for the flexibility of the spliceosome, in particular during regulated splicing events. Third, RNP rearrangements during spliceosome assembly (and later during catalytic activation) generally involve the handing over of one or more binding partners to new interaction partners. These RNP rearrangements are relatively well understood for this early stage of the splicing process but are more poorly characterized for the subsequent steps of spliceosome assembly and catalytic activation. It is known that U2AF dissociates at later stages of splicing and also that the U2AF35-3′SS interaction is replaced by a different set of factors after the first transesterification reaction (reviewed in Umen and Guthrie, 1995).

Subsequent to A complex formation, the U4/U6 and U5 snRNPs are recruited as a preassembled U4/U6.U5 tri-snRNP, forming the B complex (Figure 2B). Although all snRNPs are present in the B complex, it is still catalytically inactive and requires major conformational and compositional rearrangements (catalytic activation) in order to become competent to facilitate the first transesterification step of splicing. During spliceosome activation, U1 and U4 are destabilized or released, giving rise to the activated spliceosome (the B* complex). The activated spliceosome then undergoes the first catalytic step of splicing, generating the C complex. Prior to the second catalytic step, additional rearrangements occur in the spliceosomal RNP network (Konarska et al., 2006). After the second catalytic step, the spliceosome dissociates, releasing the mRNA in the form of an mRNP. It also releases the U2, U5, and U6 snRNPs to be recycled for additional rounds of splicing.

A number of recent observations, foremost the isolation under low-salt conditions (50 mM NaCl) of a yeast complex consisting of all five spliceosomal snRNPs (termed the penta-snRNP; Stevens et al., 2002), have led to the hypothesis that the spliceosome can also exist in a more extensively preassembled form

such that all of the snRNPs could interact concomitantly with the pre-mRNA. However, all of the major RNA-RNA and RNP remodeling events described below, as well as changes in the composition of the spliceosome, would still be required to generate its catalytically active RNP structure (Brow, 2002).

Alternative Spliceosome Assembly Pathways

The initial assembly of the spliceosome across an intron appears to be limited to pre-mRNAs containing single or very short introns. In the case of long introns (which are the rule rather than the exception in metazoans), spliceosomal components first assemble across an exon (so-called exon definition; Berget, 1995), with U1 recognizing the downstream 5′SS, U2AF, and U2 snRNP binding the upstream polypyrimidine tract and BPS, respectively, and SR proteins mediating cross-exon interactions (Figure 2C). In a subsequent step, these cross-exon interactions must be replaced by cross-intron interactions, a process that at present is very poorly understood. The ability of the spliceosome to initially form a cross-exon or cross-intron complex with a pre-mRNA substrate illustrates that alternative spliceosome assembly pathways exist, at least during the earliest stages of assembly.

Cis-Acting Regulatory Elements in Splice-Site Selection

Which 5′SS and 3′SS are recognized and subsequently paired by the spliceosome clearly influences the sequence of the mRNA that is ultimately produced. Splice site selection in higher eukaryotes is determined by multiple factors. First, the relative strength of a given splice site can play an important role in this process, with sites that have greater affinity for U1 or U2AF, in general, being more efficiently recognized. However, as most splice site consensus sequences are relatively degenerate, at least in higher eukaryotes where alternative splicing is predominant, splice sites alone are not capable of efficiently directing spliceosome assembly. Recognition and selection of splice sites is in most cases influenced by flanking pre-mRNA regulatory sequences—so-called intronic and exonic splicing enhancers or silencers—that can have positive or negative effects on splice-site usage (Cartegni et al., 2002; Singh and Valcarcel, 2005). These *cis*-acting elements mediate their effects primarily by functioning as binding sites for *trans*-acting regulatory factors that in turn recruit the snRNP subunits of the splicing machinery to the adjacent splice site or, in the case of negative regulators, prevent their association. Exonic splicing enhancers (ESEs) are often bound by SR proteins whereas exonic splicing silencers (ESSs) are typically bound by hnRNP proteins. Indeed, these two classes of proteins often have antagonistic effects on splice-site usage. The SR protein ASF/SF2 and hnRNP A1 are a well-characterized example of factors that exhibit antagonistic effects (Black, 2003; Caceres et al., 1994; Zhu et al., 2001). Ultimately, it is the sum of multiple factors, some exerting positive effects and others exerting negative effects, that decides whether a particular site is recognized by the spliceosome for inclusion of the adjacent exon in the mRNA product.

SR proteins bind both the pre-mRNA and other RS domain-containing spliceosomal proteins with low affinity and specificity. Low binding affinity, essential for the dynamic nature of the spliceosome, is a common characteristic not only of regulatory splicing factors but also of core components of the spliceosome. Canonical SR proteins are for the most part absent from yeast, where alternative splicing events are rare. With the exception of the SR-like protein Npl3 (Kress et al., 2008), SR proteins do not appear to function in yeast pre-mRNA splicing. Significantly, exonic splicing enhancers also appear to be extremely rare in yeast pre-mRNAs. Furthermore, the yeast 5′SS and BPS consensus sequences are typically defined by perfect complementarity to regions of the U1 and U2 snRNAs, respectively. Thus, the degeneration of the consensus elements defining the 5′SS, 3′SS, and the BPS in metazoans correlates with the addition of SR proteins to the repertoire of splicing regulatory factors (reviewed in Izquierdo and Valcarcel, 2006).

Dynamics of the Spliceosomal RNA-RNA Interaction Network

As is the case for the ribosome, the splicing process is accompanied by profound conformational rearrangements. However, the dynamic nature of the spliceosome in terms of its compositional changes and RNP rearrangements is unprecedented among RNP machines. Instead of the repetitive, cyclical conformational changes of the ribosome, the spliceosome undergoes a cascade of major structural rearrangements during the evolution of its active sites. This cascade is best illustrated by the dramatic changes in its snRNA-snRNA and pre-mRNA-snRNA interaction networks (Nilsen, 1998; Staley and Guthrie, 1998). Major players in spliceosomal RNA-RNA rearrangements (and also RNP remodeling events) are the spliceosome-associated DExD/H-type RNA-dependent ATPases/helicases (Staley and Guthrie, 1998).

After the initial base pairing of U1 with the 5′SS and U2 with the BPS of the pre-mRNA, snRNA components of the U4/U6.U5 tri-snRNP engage in base-pairing interactions with the pre-mRNA. In these interactions, U5 contacts nucleotides of the 5′ and 3′ exon, and the 3′ end of U6 base pairs with the 5′ end of U2 (Nilsen, 1998) (Figure 4A). Nucleotides of the U6 snRNA comprise essential components of the active site but are initially delivered to the spliceosome in an inactive form where the catalytically important regions of U6 are base paired with U4. During catalytic activation, U1 is displaced from the 5′SS, U4/U6 base-pairing interactions are disrupted, and the conserved ACAGAG box of the U6 snRNA engages intron nucleotides at the 5′SS. Additional, short U2/U6 duplexes are formed (Figure 4A) and U6 is refolded, forming an intramolecular stem loop (U6-ISL) that in yeast is involved in metal binding (Yean et al., 2000). The initial inactivation of U6 and its liberation by conformational rearrangements are a major principle by which the spliceosome ensures that the pre-mRNA is not cleaved prematurely.

Changes in base-pairing interactions that make up the two-dimensional RNA-RNA interaction network are generally well understood. However, as it is difficult to isolate conformationally homogenous spliceosome assembly intermediates, several uncertainties in the current model of RNA rearrangements still remain. For example, the precise nature of the U2/U6 base-pairing interactions immediately prior to catalysis is unclear and it is not known in which order the various U2/U6 helices form. In principle, the nature and dynamics of the spliceosomal RNA network are likely evolutionarily conserved from yeast to man with only minor differences that include the exact nature of the U2/U6 base-pairing interactions (Hilliker and Staley, 2004; Madhani

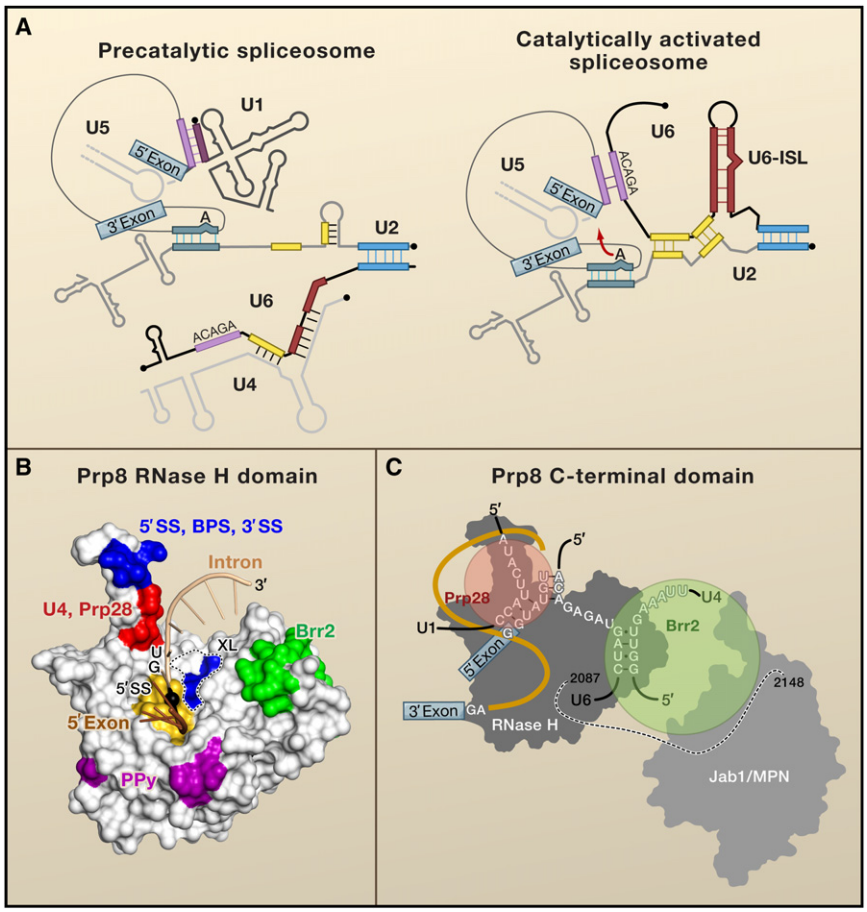

Figure 4. Spliceosomal RNA Network and Coordination of Prp28 and Brr2 Activities by Prp8

(A) The network of RNA interactions in the precatalytic (left) and activated spliceosome (right). During activation of the spliceosome, regions of U6 and U2 (yellow or red) undergo major rearrangements. The 5′ end (black ball) of the U6 snRNA base pairs through its highly conserved ACAGAG motif to the 5′ splice site (SS), displacing U1. U4 and U1 are destabilized or dissociate from the spliceosome at the time of activation and no longer are part of the spliceosome's RNA interaction network. Spliceosomal snRNAs are depicted with secondary structures observed in mammals and are not drawn to scale. Only stem loop I of U5 is shown. Critical base-pairing interactions are highlighted.

(B) Surface view of the expanded RNase H-like domain of yeast Prp8 with a 5′SS RNA modeled onto the mitten-like structure (reprinted with permission from Pena et al., 2008). Colored regions encompass residues that exhibit genetic interactions to the factors indicated. The numerous genetic interactions are consistent with a role for Prp8 in mediating the kinetic competition between two different spliceosome conformations that support the two catalytic steps of splicing (Query and Konarska, 2004). A surface patch (yellow) is a functional hotspot surrounding a truncated RNase H-like active center. The surface patch of a Prp8 peptide that can be crosslinked to the 5′ splice site (SS) in a *trans*-splicing system after addition of the tri-snRNP and after establishment of a 5′SS-U6 ACAGAG motif contact (Reyes et al., 1996) is circled (XL). During the same assembly stage, the active center of Prp28 can also be crosslinked near the 5′SS (Ismaili et al., 2001), demonstrating its close physical proximity to the Prp8 RNase H domain before spliceosome catalytic activation. PPy—polypyrimidine tract.

(C) Coordination of Prp28 and Brr2 activities by Prp8. A pre-mRNA-snRNA interaction network before catalytic activation (U1 snRNA base paired to the 5′SS, U6 snRNA base paired to U4 snRNA) is shown on an outline of the yeast Prp8 RNase H-like domain (residues 1836–2087; Pena et al., 2008) and the yeast Prp8 Jab1/MPN domain (residues 2147–2391; Pena et al., 2007). A dashed line connects the approximate positions of the C terminus of the RNase H domain and the N terminus of the Jab1/MPN domain in Prp8. Regions of the Prp8 domains interact with Prp28 (red circle) and Brr2 (green circle) to regulate their function. The C-terminal Jab1/MPN domain of Prp8 directly contacts Brr2 and stimulates its helicase activity (Maeder et al., 2009).

and Guthrie, 1992; Sun and Manley, 1995). At present, information about the nature and dynamics of RNA tertiary interactions in the spliceosome is particularly scarce. Thus, conformational rearrangements in the RNA interaction network of the spliceosome are certainly even more complex than current models would suggest.

The Spliceosome Is a Protein-Rich Machine

The spliceosome is a particularly protein-rich RNP where proteins make up more than two-thirds of its mass (in the case of spliceosomes assembled on short pre-mRNA introns). Thus, in addition to protein-RNA interactions, protein-protein interactions are expected to be prevalent and to play functionally important roles. Human spliceosomes contain ~45 distinct snRNP-associated proteins, which contribute ~2.7 MDa of molecular mass. As a consequence of its complexity, assembly of the spliceosome represents a kinetic challenge that is met, in part, by prepackaging many spliceosomal proteins in the form of snRNPs or in stable preformed heteromeric complexes. Human spliceosomes also contain numerous non-snRNP proteins. Initial mass spectrometric analyses of a mixed population of affinity-purified spliceosomal complexes indicated that between 150 (Zhou et al., 2002) and 300 distinct proteins (Rappsilber et al., 2002) copurify with spliceosomes. More recently it has been possible to purify spliceosomes at more defined stages of assembly and function (Behzadnia et al., 2007; Bessonov et al., 2008; Deckert et al., 2006; Hartmuth et al., 2002; Jurica et al., 2002; Makarov et al., 2002, 2004). These studies indicated that the total number of spliceosome-associated factors is approximately 170, with individual assembly intermediates (e.g., A, B, and C complexes) each containing generally about 125 proteins or less (in the case of the A complex) (Figure 5). In addition, they revealed a remarkable exchange of proteins from one stage to the next during the splicing process.

Although the majority of proteins identified in spliceosomes have clear roles in the splicing process per se, the function of many others is unclear. A number of spliceosome-associated proteins are likely involved in coupling the splicing machinery

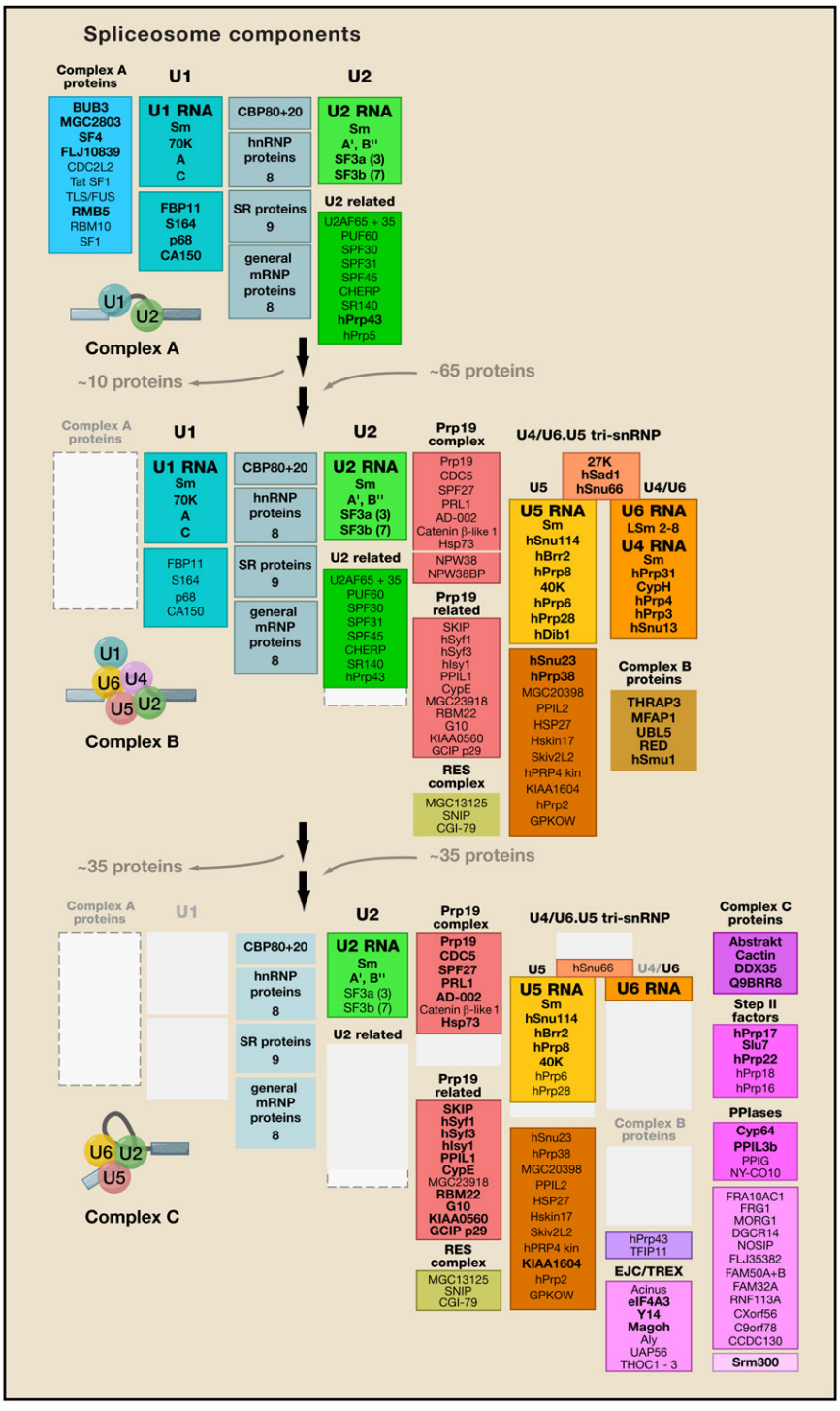

Figure 5. Compositional Dynamics of Human A, B, and C Spliceosomal Complexes

The protein composition of the human A complex (formed on MINX pre-mRNA) (Behzadnia et al., 2007), the human B complex (a consensus of complexes formed on MINX and PM5 pre-mRNA) (Bessonov et al., 2008; Deckert et al., 2006), and the human C complex (formed on PM5 pre-mRNA) (Bessonov et al., 2008) as determined by mass spectrometry. Proteins (human nomenclature) are grouped according to snRNP association, function, presence in a stable heteromeric complex, or association with a particular spliceosomal complex, as indicated. The relative abundance of proteins is indicated by light (substoichiometric amounts) or dark (stoichiometric amounts) lettering and is based on the relative amounts of peptides sequenced or, in some cases, also by immunoblotting experiments. Numbers indicate the total number of individual proteins in a particular group. SR proteins identified include ASF/SF2, 9G8, SRp20 Tra2-α, SRp30c, SRp38, SRp40, Srp55, and SRp75. HnRNP proteins include hnRNPA1, A3, C, G, K, M, U, and PCBP1. mRNP proteins include ASRBP, NFAR, NF45, BCLAF-1, YB-1, HuR, LOC124245, and RNPC2. Some exon junction complex (EJC) proteins (Acinus and eIF4A3) and factors involved in step 2 of the splicing reaction (Prp17) are also observed in B complex preparations, though this is likely due to the presence of low amounts of contaminating B* complexes. A small subset of Prp19 complex proteins are also detected in low amounts in A complex preparations isolated under low-salt conditions.

to other molecular machines in the cell such as those responsible for transcription, 3′-end processing, or quality control of spliced mRNPs (reviewed in Jurica and Moore, 2003; Maniatis and Reed, 2002). Many of the non-snRNP proteins identified appear to be present in substoichiometric amounts in the individual assembly intermediates. Rather than reflecting simply the partial loss of some proteins during the purification procedure, the presence of less abundant proteins, in particular those involved in splicing regulation, hints at another design principle. These proteins could represent examples of a host of loosely associated proteins whose regulated actions provide flexibility and specificity to spliceosome function.

Dramatic Changes in the Spliceosome Protein Inventory

Mass spectrometric analyses of different assembly intermediates indicate that there is a dramatic exchange of proteins during spliceosome assembly and activation. A comparison of the protein compositions of human A, B, and C complexes affinity-purified under identical physiological conditions (Figure 5) reveals three principles regarding the spliceosome's composition during the course of splicing. First, several different groups of spliceosomal proteins are present in the spliceosome throughout the splicing cycle, including U2-associated proteins and members of the SR and hnRNP protein families (which are present in varying abundance). Second, during the A complex

to B complex and B complex to C complex transitions, a large number of additional spliceosomal proteins are recruited. Third, numerous spliceosomal proteins are released or destabilized at each stage during the splicing process. During the A complex to B complex transition, ~25 proteins are recruited as part of the tri-snRNP complex. However, more than 35 non-snRNP proteins also associate at this stage. These include components of the heteromeric Prp19/CDC5 protein complex (composed of at least seven subunits and the functional equivalent of the yeast *n*ine*t*een *c*omplex [NTC]; Makarova et al., 2004) and a group of proteins designated Prp19/CDC5-related (which either physically interact with the Prp19 or CDC5 proteins in humans or yeast or were present with Prp19 in the human 35S U5 snRNP) (Ajuh et al., 2000; Chen et al., 1999, 2002). They also include proteins of the RES (retention and splicing) complex (Dziembowski et al., 2004) and an additional group of 15 proteins, 5 of which are abundant in the B complex but essentially absent from the C complex. During the conversion of the B complex to the C complex (which encompasses catalytic activation), most so-called step 2 factors (proteins known to function just prior to or during the second transesterification reaction) are recruited. In addition, a large number of proteins designated C complex specific associate together with several DExD/H-box helicases and peptidyl-prolyl *cis/trans* isomerases (PPIases). These helicases and isomerases may be responsible for RNP conformational changes at this or later stages of the splicing process. Finally, most members of the exon junction complex (EJC), which is deposited upstream of the exon-exon splice junctions of the mRNA and influences its subsequent metabolism in the cell (reviewed in Tange et al., 2004), are also recruited at this stage of transition from the B complex to the C complex.

In addition to these elaborate recruitment events, spliceosomal proteins are released or destabilized during the A complex to B complex and B complex to C complex transition. Thus, several non-snRNP proteins of the A complex (designated "A complex proteins"; Figure 5) are no longer found in the B complex. Likewise, all U1- and U4/U6-associated proteins dissociate or are destabilized during the B complex to C complex transition, together with most U2-related proteins and several non-snRNP proteins (designated "B complex"; Figure 5). Recent evidence also indicates that the U2-associated heteromeric complexes SF3b and SF3a are less stably bound at this stage (Bessonov et al., 2008), indicating an RNP remodeling event that may be related to the rearrangement of the spliceosome's active site prior to the second transesterification step (Konarska et al., 2006; Smith et al., 2008). Significantly, a large portion of those proteins originally present in the A complex are no longer a part of the catalytically active C complex, attesting to the large turnover of spliceosomal proteins. Most of what is known about the dynamics of the spliceosome's composition is based on experiments performed in vitro. Thus, the loss of some proteins may result from the isolation procedure itself. Some of the changes observed in vitro may also simply reflect binding destabilization rather than bona fide dissociation events. However, recent data support the idea that many of the compositional changes observed in vitro also occur in living cells (see discussion of tri-snRNP remodeling).

To date, human B* complexes have not been isolated under physiological conditions. However, mass spectrometric analyses of B versus B* complexes isolated under nonphysiological, stringent conditions indicate that the loss of U4/U6-associated proteins occurs during the B to B* transition (Makarova et al., 2004). In addition, subunits of the Prp19/CDC5 complex and its related proteins become more stably integrated in the B* complex, indicating that there are major RNP remodeling events involving these proteins during activation (Makarov et al., 2002, 2004).

Most of what we know about the dynamics of the spliceosome composition comes from studies in human cell extracts. Many known splicing factors are highly conserved between man and yeast. Based on their numbers, the evolutionarily conserved core machinery of the spliceosome likely consists of ~80 proteins. Due to the limited amount of regulated splicing in yeast, its splicing machinery is generally expected to be less complex than that of humans. However, answers to these questions await proteomic analyses of purified spliceosomal complexes from yeast. The number of proteins of the splicing machinery that are required for maintaining the catalytically active RNP network of the step 1 spliceosome (C complex) consists of about 35–40 proteins (Bessonov et al., 2008), almost all of which have highly conserved orthologs in yeast. Thus, the inner workings of the spliceosome also appear to be highly conserved across evolution.

A Flexible Source of Spliceosomal Proteins

In order to be prepared for the splicing of a wide variety of pre-mRNA introns and also to react better to changes in the state of the cell or its environment, the spliceosome appears to contain many proteins that are loosely associated and are only called into action or required in certain situations. A prime example is the regulatory protein RBM5 (RNA-binding motif protein 5), a critical player in the regulated splicing of the FAS pre-mRNA encoding the apoptotic factor FAS (Bonnal et al., 2008). RBM5 represses the splicing of Fas pre-mRNA by blocking the conversion of a cross-exon complex assembled on the pre-mRNA to a cross-intron complex. Consistent with its role early in spliceosome assembly, RBM5 dissociates from the spliceosome during the A complex to B complex transition, suggesting that other A complex proteins released at this stage may also play a role in regulating splicing. By analogy, it is also highly likely that other spliceosome-associated proteins, in particular those that are loosely associated or present in substoichiometric amounts, are utilized during the splicing of specific introns or under specific cellular conditions. Indeed, several proteins with apparently redundant functions (such as different SR proteins or U2AF65 and PUF60) are found in purified spliceosomes. U2AF65 and PUF60 are homologous proteins, and PUF60 can substitute for U2AF65 during early recognition of the 3′SS (Hastings et al., 2007). However, PUF60 plays a more important role in the recognition of introns with a weak 3′SS (Hastings et al., 2007). Recent knockdown experiments in flies and mutational analyses in yeast in which core components of the spliceosome were targeted revealed that depletion or mutation of different factors resulted in differential effects on global splicing patterns, with large defects in the splicing of some genes but moderate or little effect on other genes (Clark et al., 2002; Park et al., 2004; Pleiss

et al., 2007). These observations, in particular the findings in yeast where alternative splicing is extremely rare, suggest that pre-mRNA substrates differ even in their requirements for core components of the spliceosome. This may be due to different affinities between components of the spliceosome and the various pre-mRNAs or the redundant nature of splicing signals. Thus, the large compositional complexity of the spliceosome is likely due at least in part to the wide variety of pre-mRNA substrates it must engage and the widespread occurrence of regulated splicing events, at least in higher eukaryotes.

Extensive Remodeling of Spliceosomal Subunits

In contrast to the ribosomal subunits, spliceosomal subunits, in particular the U4/U6.U5 tri-snRNP, undergo massive remodeling during splicing. At the time of tri-snRNP interaction with the pre-mRNA, the U4/U6 and U5 snRNPs are stably associated with each other, predominantly through a number of protein-protein interactions (Figure 6A). The U4 and U6 snRNAs are extensively base paired with each other. The U4 snRNA is associated with the 15.5K/Snu13 protein and four other proteins (Liu et al., 2007; Nottrott et al., 1999), whereas the U6 snRNA is bound at its 3′ end by the LSm 2–8 proteins (Achsel et al., 1999; Mayes et al., 1999). Upon catalytic activation, the U4/U6 base-pairing interaction is disrupted, freeing the U6 snRNA to engage the pre-mRNA and U2 snRNA. The U4 snRNA and all U4/U6-associated proteins are destabilized or released from the spliceosome, along with proteins involved in tethering the U5 snRNP to the U4/U6 snRNP (Figure 6A). Whether these proteins are destabilized or released concomitantly or in discrete steps is not yet clear. In general, there is little known about how many discrete RNP remodeling events and thus how many structurally distinct spliceosome intermediates exist during splicing. Indeed, each rearrangement represents a step at which splicing can potentially be subjected to regulation. As the U6 snRNA has been ''stripped'' of all of its precatalytic binding partners, new partners must be provided. Anchoring of the U6 snRNA after activation likely involves the pre-mRNA substrate, U2 snRNA, and new protein interaction partners (Figure 6A). Yeast NTC complex (Prp19/CDC5 complex in metazoans) subunits and Prp19-related proteins clearly play an important role in this process, as has been demonstrated in yeast (Chan et al., 2003). It will be interesting for future studies to determine which of these proteins directly contacts the U6 snRNA at this stage.

Several U5 proteins are also released during the B complex to C complex transition (Figure 6B). Comparative proteomic analyses have been performed on spliceosomal complexes and a new 35S form of the U5 snRNP that was purified from HeLa nuclear extract under stringent conditions. These studies indicated that during spliceosome activation, Prp19/CDC5 complex subunits and Prp19-related proteins stably associate with the U5 snRNP (Makarov et al., 2002). The remodeled 35S form of the U5 snRNP probably represents a disassembly intermediate of the post-spliceosomal complex. Whether it initially dissociates as part of a larger complex containing U6, for example, is presently not clear as relatively little is known in detail about the dissociation phase of the spliceosome. In a later step, the 35S U5 snRNP must be converted back into a 20S form that reassociates with the U4/U6 snRNP prior to the next round of splicing (Figure 6B). Thus, spliceosome maturation, the generation of its active sites, and the catalytic steps of splicing all involve major RNP remodeling events.

Most of these events appear to be evolutionarily conserved. For example, the general tri-snRNP remodeling events observed in HeLa cell splicing extracts, including the loss or destabilization of U4/U6 and tri-snRNP-specific proteins during activation, have also been observed by mass spectrometric analyses of different spliceosomal complexes from the fly *Drosophila melanogaster* (Herold et al., 2009). In addition, endogenous yeast spliceosomes that likely represent a post-catalytic form of the splicing machinery also lack all U4/U6 proteins and several U5-specific proteins (Ohi et al., 2002). Finally, there is evidence in yeast that the LSm 2–7 proteins dissociate at the time of spliceosome activation (Chan et al., 2003). Remodeling of the tri-snRNP also appears to occur in vivo. Recent fluorescence resonance energy transfer studies in human cells indicated that during splicing, the tri-snRNP dissociates. In particular, these studies showed that after taking part in splicing, the U5 and U4/U6 snRNPs reassemble in Cajal bodies, away from the sites where splicing takes place (Stanek et al., 2008).

Mediators of Spliceosome Dynamics

The dynamic remodeling of RNA-RNA, RNA-protein, and protein-protein interactions during spliceosome assembly requires multiple driving forces and tight control of molecular switches. Enzymes such as DExD/H-type RNA-dependent ATPases/helicases and peptidyl-prolyl *cis*/*trans* isomerases (PPIases) function in conjunction with posttranslational modifications on spliceosome components to direct these remodeling events.

Major Enzymatic Driving Forces for Spliceosome Remodeling

DExD/H-type RNA-dependent ATPases/helicases that were discovered early on by elegant yeast genetic studies constitute one group of proteins that carry out the remodeling of the spliceosome interaction network. Eight of these DExD/H-box proteins (Sub2/UAP56, Prp5, U5-100K/Prp28, Brr2/U5-200K, Prp2, Prp16, Prp22, and Prp43) are conserved between yeast and humans. They act at specific steps of splicing (Staley and Guthrie, 1998) (Figure 2B) to facilitate transitions between mutually exclusive RNA-RNP interaction networks. By removing interaction partners (RNA or proteins), these proteins may allow a particular region of RNA to engage in new base pairing or protein binding (Pyle, 2008; Staley and Guthrie, 1998).

Sub2/UAP56 and Prp5 act during early spliceosome assembly stages, where they facilitate the exchange of SF1 for the U2 snRNP at the BPS. The transition from the B to the B* complex, where catalytic activation of the spliceosome takes place, requires the action of three additional DExD/H-box proteins as well as the GTPase, Snu114. The Prp28 protein is involved in mediating the transfer of the 5′SS from the 5′ end of the U1 snRNA to the ACAGAG motif in U6 snRNA (Figure 4C). This event initiates the catalytic activation of the spliceosome. Brr2 is subsequently required for the unwinding of the U4/U6 duplex (essentially unpacking part of the ''cutting tool'') and to allow annealing of U6 with U2. The activities of Prp28 and Brr2 must be coordinated, and Brr2 must be tightly regulated to prevent

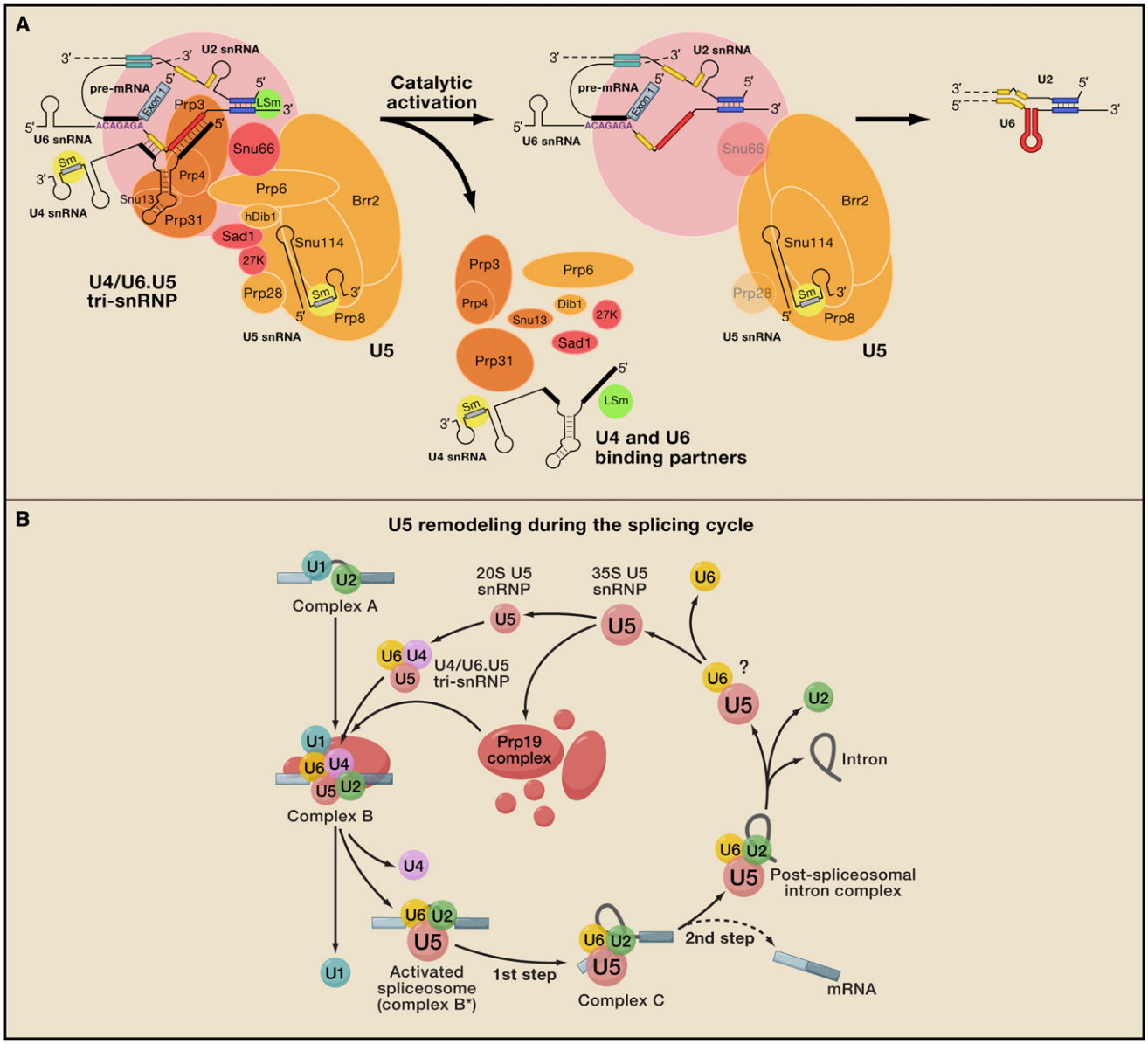

Figure 6. The U4/U6.U5 tri-snRNP Is Extensively Remodeled during Splicing

(A) Disruption of U4/U6 base-pairing interactions and loss of all U4/U6 proteins, as well as some U5 and tri-snRNP proteins, during catalytic activation of the spliceosome. The regions of U4, U6, and U2 snRNAs that engage in new base-pairing interactions after spliceosome activation are highlighted in yellow or red. Only the 5′ end of U2 and the 5′ end of the pre-mRNA (exons, blue box; intron, black line) are depicted. After the loss of all known precatalytic U6 snRNA-binding partners, additional proteins (pink) that include the subunits of the nineteen complex (NTC) in yeast (Chan et al., 2003) and the Prp19/CDC5 complex and Prp19-related proteins in humans help to tether the U6 snRNA to the activated spliceosome. Proteins are depicted in relative size to each other and are denoted by yeast nomenclature except for the human 27K protein, which has no yeast homolog.

(B) Remodeling of the U5 snRNP (red) during the splicing cycle, as proposed by Makarov et al. (2002). During activation, the U5 snRNP appears to be remodeled so that it interacts stably with the Prp19/CDC5 complex and Prp19-related proteins. U5 is thought to dissociate after splicing in the form of a 35S particle and must then be converted to a 20S form before it can associate with the U4/U6 snRNP and partake in another round of splicing. The size of the U5 snRNP reflects its compositional complexity at various stages of the splicing process.

premature cleavage of the pre-mRNA. Furthermore, Brr2 is a resident subunit of the spliceosome and its activity is required again during spliceosome disassembly (Small et al., 2006). Thus, it requires a control mechanism that can be switched on and off repeatedly.

A number of very recent findings suggest that Prp8, the largest and most highly conserved protein of the spliceosome (Grainger and Beggs, 2005), may provide much of this regulation for Brr2. A fragment in the C-terminal quarter of the protein was shown to adopt an RNase H-like fold that is augmented by other

Prp8-specific elements (Pena et al., 2008; Ritchie et al., 2008; Yang et al., 2008). The nature of many genetic and physical interactions (reviewed in Grainger and Beggs, 2005) that map to this RNase H-like domain in Prp8 suggest that it acts together with Prp28 to provide a *trans*-helicase activity for the robust handover of the 5′SS from U1 to U6 (Pena et al., 2008) (Figure 4B). The RNase H domain in Prp8 is followed by an expanded Jab1/MPN domain that extends to the C-terminal end of the protein (Pena et al., 2007; Zhang et al., 2007). Strikingly, a C-terminal fragment of Prp8 comprising the RNase H and Jab1/MPN domains interacts with Brr2 and stimulates its helicase activity (Maeder et al., 2009). Thus, a platform within Prp8 appears to organize the activities of both Prp28 and Brr2 (Figure 4C).

Additional levels of regulation are also present to tightly control the activity of Brr2. In yeast, Prp8 is reversibly ubiquitinylated and its ubiquitinylated form represses Brr2 activity (Bellare et al., 2008). The Jab1/MPN domain of Prp8 binds ubiquitin (Bellare et al., 2006), suggesting that upon deubiquitinylation of Prp8, this domain could be liberated to stimulate Brr2 activity. Furthermore, Brr2 is under the control of the Snu114 GTPase (Bartels et al., 2002; Small et al., 2006). GDP-bound Snu114 blocks Brr2′s helicase activity, whereas its GTP-bound form promotes helicase activity (Small et al., 2006). Interestingly, Snu114 interacts with the same region of Prp8 as Brr2 (Liu et al., 2006; Pena et al., 2007; Zhang et al., 2007) (Figure 4C). Therefore, the C-terminal region of Prp8 may additionally coordinate Snu114 control of Brr2, thus representing a hot spot for the regulation of spliceosome catalytic activation. Moreover, Prp8, Brr2, and Snu114 form a salt-stable complex that can be detached from the U5 snRNP (Achsel et al., 1998), suggesting that these proteins are organized as a functional unit or a micro-machine within the spliceosome.

Another safeguard against ill-timed pre-mRNA cleavage may be provided by Prp2, the least understood helicase of the spliceosome. Prp2 activity is required after U4/U6 unwinding by Brr2 and before the first step of the splicing reaction (Kim and Lin, 1996). It may also control yet another rearrangement that changes an activated spliceosome (characterized by the removal of U1 and U4) to a particle that is catalytically competent for the first transesterification step (Figure 2B). Prp16, Prp22, and Prp43 also facilitate structural changes in the spliceosome during later stages of splicing. Prp16 (Schwer and Guthrie, 1992) and Prp22 (Schwer, 2008) act prior to and during the second transesterification step, respectively, whereas Prp43 (Arenas and Abelson, 1997) functions during spliceosome disassembly (Figure 2B).

Most of the spliceosomal helicases seem to be geared toward the local reorganization of RNA-RNA or RNA-protein interactions, reminiscent of canonical DEAD-box proteins (Sengoku et al., 2006). Several of these enzymes, including Prp5 (Xu and Query, 2007), Prp16 (Burgess et al., 1990), and Prp22 (Mayas et al., 2006), also act as timers for kinetic proofreading to ensure accuracy in the splicing process. These factors mediate swift transitions along the splicing pathway if a suitable substrate is presented and allow disposal of suboptimal substrates that are not rapidly converted. The activities of such enzymes have therefore been likened to the role played by the elongation factor eEF1A/EF-Tu during translation (Burgess et al., 1990). The recent observation that spliceosomes can reverse both transesterification reactions (Tseng and Cheng, 2008) suggests that additional possibilities for proofreading may exist.

Regulation of the DExD/H-box enzymes is not very well understood. Most of the RNA helicases join the spliceosome transiently at the stage at which their actions are required (Figure 2B), comparable to the association of elongation factors with the ribosome during translation elongation. Similar to the elongation factors, some of these helicases may exhibit overlapping binding sites, thereby explaining in part their sequential recruitment. In addition, specific protein partners may mediate recruitment of a helicase (for example, Spp2 in the case of Prp2; Roy et al., 1995) or modulate its activity (for example, Ntr1 in the case of Prp43; Tsai et al., 2005).

Although the above DExD/H-box proteins are evolutionarily conserved, demonstrating the conserved nature of the remodeling events they facilitate, at least four other DExD/H-box proteins without obvious counterparts in yeast are found in human spliceosomes (p68, KIAA0560, Abstrakt, DDX35). Thus far, a functional role has only been ascribed to p68, which appears to act during catalytic activation (Liu, 2002). It remains to be determined whether the remaining three proteins are also involved in additional remodeling steps that may be specific to the human spliceosome.

Additional Enzymatic Driving Forces?

Additional proteins associated with the spliceosome also possess enzymatic activities that could modulate protein conformations and thereby steer spliceosome assembly and catalysis. Human spliceosomes contain at least eight peptidyl-prolyl *cis*/*trans* isomerases (PPIases) that are recruited at distinct stages during splicing. The precise functions of the spliceosomal PPIases, all of which are members of the cyclophilin family, remain to be established. As most of these proteins have no obvious orthologs in yeast where there is little occurrence of alternative splicing, these PPIases might function specifically in higher eukaryotes to enhance the flexibility of the spliceosome during processes such as alternative splicing. The isomerization of a peptide bond preceding a proline residue can be the rate-determining step in protein folding (reviewed in Nagradova, 2007). Thus, spliceosomal PPIases may mediate structural transitions that otherwise might slow down pre-mRNA splicing. This is consistent with the observation that the cyclophilin inhibitor, cyclosporin A, affects both steps of the splicing reaction (Horowitz et al., 2002). Also consistent with a remodeling function for these proteins, at least some of these PPIases appear to associate with the spliceosome in a way that leaves their PPIase active sites unobstructed. For example, the PPIase cyclophilin H (CypH) associates with the U4/U6-Prp4 complex through a region that is located opposite its active site (Reidt et al., 2003). Many other spliceosomal PPIases also appear to harbor additional domains for mediating spliceosome association that are distinct from those containing their active sites. These include an RNA recognition motif in CypE, a WD40 domain in Cyp64, and an RS domain in PPIase G (PPIG).

The above enzymes are potentially involved in at least one protein conformational switching event during splicing. Considering that Brr2, Snu114 (Small et al., 2006), and Prp22 (Mayas et al., 2006) act at more than one step of the splicing reaction

(Figure 2B), a number of these proteins may also have more than one function. Snu114 also exhibits striking sequence homology with the ribosomal translocase eEF2/EF-G (Fabrizio et al., 1997). Though this similarity suggests that Snu114 may function like a motor protein and facilitate mechanical movements during spliceosome assembly or catalysis, more recent data indicate that Snu114 acts more like a molecular switch, transducing signals to Brr2 (Small et al., 2006). Nonetheless, more spliceosome maturation states than the limited number identified to date must exist.

Molecular Switches during Spliceosome Assembly and Catalytic Activation

Several spliceosomal proteins are posttranslationally modified. Many of these modifications play key regulatory roles in the progression of splicing. The spliceosome harbors a number of enzymes that introduce or remove posttranslational modifications. At least four protein kinases (SR protein kinases 1 and 2, Prp4 kinase, and Clk/Sty kinase) have been shown to phosphorylate spliceosomal proteins. Reversible protein phosphorylation of spliceosomal proteins plays decisive roles during both spliceosome assembly and splicing catalysis (Mathew et al., 2008; Shi et al., 2006 and references therein). For instance, the phosphorylation of SR proteins is essential for their activity. Phosphorylation of Prp28, a component of the U5 snRNP, is required for tri-snRNP addition during spliceosomal B complex formation (Mathew et al., 2008). The U1 snRNP-associated 70K protein is also a phosphoprotein and its dephosphorylation is required for the first step of splicing. Finally, the U2-associated SF3b155 protein is specifically hyperphosphorylated just prior to or during the first step of splicing (Wang et al., 1998) and appears to be dephosphorylated by PP1/PP2A phosphatases concomitant with the second step of splicing (Shi et al., 2006). Spliceosomal proteins also appear to undergo other types of modifications. These include ubiquitination, as in the case of Prp8 (Bellare et al., 2008), and possibly also acetylation (Kuhn et al., 2009). More detailed characterization of the modification status of spliceosomal proteins using mass spectrometry will likely greatly expand our knowledge of the number and nature of modified proteins. These analyses are likely to reveal a plethora of molecular switches that contribute to the fine tuning of the splicing process and provide additional opportunities for regulation.

Traditionally, the functions of biological macromolecules are thought to intimately depend on stable structures, as is the case for most RNP machines. However, a large number of proteins that are either natively disordered (intrinsically unstructured proteins, IUPs) or bear sizeable regions that lack stable tertiary structure (intrinsically unstructured regions, IURs) do not follow this structure-function paradigm (reviewed in Dyson and Wright, 2005). A well-studied example of such proteins in the spliceosome is SF3b155. This protein exhibits a C-terminal array of 22 tandem helical repeats that form a major scaffolding element for the heteromeric SF3b complex (Golas et al., 2003). However, the N-terminal 450 amino acid region of SF3b155 is intrinsically unstructured (Cass and Berglund, 2006). Binding of the SF3b14a/p14 protein to the end of the unstructured N-terminal region induces a folding transition in SF3b155 (Schellenberg et al., 2006; Spadaccini et al., 2006). The SF3b155 N-terminal region also associates with several other spliceosomal proteins such as U2AF65 (Figure 3), SPF45, and PUF60, which may similarly induce structural changes in this region of SF3b155.

SF3b155 further provides an example of how posttranslational modifications can lead to structural transitions in modified intrinsically unstructured proteins or regions to provide new interaction platforms. The NIPP1 protein, which contains a phosphothreonine/serine-binding forkhead-associated domain, interacts with SF3b155 in a manner that depends upon the phosphorylation of specific threonine-proline motifs in the unstructured SF3b155 N-terminal region (Boudrez et al., 2002). Furthermore, NIPP1 appears to communicate the SF3b155 phosphorylation state to protein phosphatase-1 (Tanuma et al., 2008). Interestingly, the juxtapositioning of prolines with phosphorylated amino acids in SF3b155 is reminiscent of the C-terminal domain of the largest RNA polymerase II subunit. There, phosphorylation marks, whose configurations are further modulated by the PPIase PIN1, constitute an intricate code for the recruitment of mRNA processing factors (reviewed in Lu and Zhou, 2007). It will be interesting to see whether similar strategies involving the spliceosomal PPIases are implemented in the spliceosome.

Other distinguishing aspects of intrinsically unstructured proteins or regions include their conformational malleability, which allows them to contact diverse interaction partners, and their immobilization or folding upon complex formation, which enables the formation of specific complexes with limited thermodynamic stability. Intrinsically unstructured proteins or regions also are able to accelerate interaction kinetics by allowing a greater radius of target binding site capture (fly-casting mechanisms) and are readily inactivated through protein degradation. These attributes make the intrinsically unstructured proteins or regions appear ideally suited to promote the extraordinary plasticity of the spliceosome. Therefore, we expect that they likely play a central role in maintaining the dynamic nature of the splicing machinery.

Regulatory Decisions of the Spliceosome

Several of the design principles of the spliceosome, including its extensive compositional dynamics and its flexible intermolecular RNA-RNA, RNA-protein, and protein-protein interactions, ensure that it is highly responsive to regulation. These characteristics are in part a consequence of its assembly from a large number of smaller subunits. Many alternative splice-site choices are made during the very early phases of spliceosome assembly. As previously discussed, these choices are determined by the combined actions of both positive and negative regulatory proteins and the contribution of splicing enhancer and silencer sequences (reviewed in Smith and Valcarcel, 2000). Recent results indicate that regulatory decisions are also made at later stages of the splicing process, including during the transition from a cross-exon A-like complex to a cross-intron B complex (Bonnal et al., 2008; House and Lynch, 2006; Sharma et al., 2008) and even after the first step of splicing (Lallena et al., 2002). Furthermore, recent data have unexpectedly revealed that the spliceosome mediates the maturation of the 3′ end of telomerase RNA (the catalytic subunit of the telomerase RNP enzyme) by cleaving the RNA from a longer

precursor through a reaction identical to the first catalytic step of splicing (Box et al., 2008). However, the spliceosome does not perform the second step of splicing on the telomerase RNA, indicating that its activity in this context is negatively regulated after the first catalytic step. Indeed, there are likely to be a multitude of checkpoints throughout the splicing cycle where the spliceosome can make decisions that alter the outcome of the splicing process.

The Spliceosome's Active Site

The spliceosome qualifies as an RNP enzyme due to the extensive interplay of RNA and protein functions during assembly and catalytic activation. Yet, the question remains whether cooperation between RNA and protein extends to the chemical catalysis of splicing. The chemistry of nuclear pre-mRNA splicing recapitulates the self-splicing that is catalyzed by group II introns. Furthermore, the short sequences of some snRNAs that resemble catalytic portions of group II introns. Analysis of the effects of thioester substitutions in the pre-mRNA has established that the spliceosome is a metalloenzyme (Sontheimer et al., 1997). Phosphorothioate-suppression experiments further demonstrated that the U6 intramolecular stem loop mediates the positioning of a catalytically or structurally important metal ion (Yean et al., 2000). Intriguingly, the recent crystal structure of a hydrolytic group IIC intron revealed that its U6-like intramolecular stem loop might mediate the transesterification reactions by coordinating two metal ions for catalysis (two-metal-ion mechanism) (Toor et al., 2008). Furthermore, engineered RNAs that contain only portions of U6, U2, and the pre-mRNA can carry out a reaction resembling the first step of splicing (Valadkhan and Manley, 2001). However, it remains unclear to what extent this minimal system represents the situation in the spliceosome. Taken together, these findings leave no doubt that snRNAs and the pre-mRNA form major parts of the spliceosome's active sites. However, it is presently still unclear whether group II introns and the spliceosome represent divergent or convergent evolutionary solutions to the same chemical problem and whether they share the same chemical catalytic principles (Weiner, 1993).

Biochemical and genetics analyses place at least two spliceosomal proteins directly at or near the spliceosome's active sites. One of these proteins is the U2-specific SF3b14a/p14 protein, which contacts the BPS adenosine during the early stages of spliceosome formation and also after the first catalytic step (Will et al., 2001). The other protein is the U5-specific Prp8 protein, which can be crosslinked to the 5′SS, the BPS, and the 3′SS after association of the U5 snRNP during B complex formation. Prp8/U5-220K has also been suggested to function as a scaffold for active site RNAs or proteins (reviewed in Grainger and Beggs, 2005). Recent crystal structures of a 5′SS-interacting region of Prp8 revealed an RNase H-like domain containing a truncated catalytic center (Pena et al., 2008; Ritchie et al., 2008; Yang et al., 2008). Together with the effects of single point mutants targeting both the apparent active site residues of the RNase H-like domain and the surrounding area in Prp8 (Pena et al., 2008; Ritchie et al., 2008; Yang et al., 2008), these results point to the possibility that Prp8 may directly participate in the chemical catalysis of splicing and that the active sites of the spliceosome may be true collaborations between RNA and protein (Abelson, 2008).

Toward Monitoring Dynamic RNPs in Action

A full mechanistic understanding of an RNP machine requires structural information about each of its numerous functional stages, as has been demonstrated impressively with the ribosome (reviewed in Korostelev et al., 2008; Steitz, 2008). The most fundamental requirement to attain comparable structural knowledge for the spliceosome is the availability of homogeneous preparations of the RNP particle in defined functional stages. Presently, the most successful techniques of spliceosome isolation combine kinetic control of spliceosome assembly with affinity purification steps and glycerol gradient centrifugation (Bessonov et al., 2008 and references therein). The resulting preparations are amenable to structural investigation, including mapping of specific spliceosomal components by cryo-electron microscopy (reviewed in Stark and Lührmann, 2006). However, these preparations will probably require greater homogeneity for high-resolution studies.

The control of particle homogeneity may be aided by small-molecule inhibitors that stall the splicing machinery at specific steps. This approach has been particularly successful in the study of the ribosome, for which nature has provided a large number of antibiotics that allow stalling of this RNP at different functional stages. No comparable natural inhibitors of pre-mRNA splicing are known. Because spliceosomal enzymes embody the driving forces for spliceosome assembly and remodeling, inhibitors that specifically block the activities of individual enzymes should be useful for stalling the spliceosome at specific stages. Indeed, recent screening efforts have unearthed a number of chemicals that block pre-mRNA splicing at different stages in vitro (reviewed in Jurica, 2008). In the future, it will be of primary importance to test whether these substances can generate more homogeneous spliceosome preparations that are amenable to high-resolution ultrastructural analyses. However, reconstitution of spliceosomal complexes at defined stages using purified subcomplexes and recombinant proteins may ultimately be required to achieve this goal. Because of the apparent lower number of spliceosome-associated proteins in yeast and also the availability of yeast spliceosomal proteins harboring temperature-sensitive mutations, the yeast system may prove particularly useful for determining, at high resolution, the molecular structure of the spliceosome.

Due to the ease with which the structural context of RNA molecules can be probed biochemically, we possess a relatively detailed picture of RNA transactions during pre-mRNA splicing. In contrast, little is known about the structural rearrangements that involve spliceosomal proteins because no comparable protein footprinting techniques are available. Hydrogen-deuterium exchange monitored by mass spectrometry (Maier and Deinzer, 2005) has proven useful for delineating molecular surfaces that are buried upon complex formation or during a folding transition. Moreover, radiolytic footprinting in combination with mass spectrometry constitutes a promising approach to revealing dynamic interaction networks in protein complexes (Takamoto and Chance, 2006).

Site-specific labeling of individual subunits with spectroscopic or biochemical probes and reconstitution of functional complexes bearing such reporter groups would be tremendously useful to allow tracking of the changing molecular

environments during pre-mRNA splicing. Possible approaches include the introduction of fluorescent probes for fluorescence resonance energy transfer measurements or for tracking the dynamics of RNA/RNP remodeling events using single-molecule approaches. They also include the introduction of hydroxyl-radical generators that allow mapping of RNAs in the vicinity of the probe. Pioneering work was again carried out in the study of ribosomes (reviewed in Wilson and Noller, 1998), and first steps in this direction have been undertaken with the spliceosome (Crawford et al., 2008; Donmez et al., 2007; Rhode et al., 2006; Kent and MacMillan, 2002).

In order to decipher the combinatorial control involved in splice-site selection and pairing, as well as the principles underlying the cooperative activities of multiple splicing regulatory proteins, thermodynamic and kinetic parameters that describe their molecular interactions are required. Again, studies of the ribosome teach us that every step must be described kinetically in order to achieve a full mechanistic understanding (Wintermeyer et al., 2004). Surprising results may lie ahead with the attainment of a more detailed kinetic description of spliceosome assembly. Indeed, Nilsen and colleagues have recently characterized a new type of exonic splicing silencer that modulates the kinetics with which a U1 snRNP-bound 5′SS engages in splicing (Yu et al., 2008). Interestingly, these new regulatory sequences did not appear to require binding by auxiliary splicing factors.

Many approaches discussed here, as well as classical biochemical techniques such as crosslinking approaches, should provide useful information regarding the spatial relationship of components within the spliceosome at different stages of the splicing cycle. These disparate pieces of data would provide spatial constraints for integrative modeling techniques to generate congruent three-dimensional pictures of the splicing machinery, as has recently been pioneered with the nuclear pore complex (reviewed in Alber et al., 2008). With this arsenal of techniques, it may prove possible to unravel the conformational and compositional dynamics of the spliceosome over the course of the splicing cycle at near-atomic resolution.

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