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REVIEW

The second catalytic step of pre-mRNA splicing

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INTRODUCTION

A ubiquitous feature of eukaryotes is the presence of intervening sequences that interrupt the coding regions of genes. Nuclear pre-mRNA splicing is the process by which these intervening sequences (introns) in messenger RNAs are precisely removed and the functional coding sequences (exons) ligated. Splicing proceeds via two transesterification reactions. In the first reaction (step one), the 2′ hydroxyl group of an intron adenosine residue attacks the 5′ splice site phosphodiester bond, producing a branched lariat intermediate structure and a free 5′ exon. In the second reaction (step two), the 3′ hydroxyl group of the 5′ exon attacks the 3′ splice site phosphodiester bond, producing ligated exons and an excised lariat intron (Fig. 1).

The two catalytic steps of splicing are carried out by a large, ribonucleoprotein machine, the spliceosome. The main components of the spliceosome are five small nuclear RNAs (U1, U2, U4, U5, and U6 snRNAs), which are packaged as ribonucleoprotein particles (snRNPs). In addition, there are multiple non-snRNP proteins that interact transiently with the spliceosome. The discovery of snRNAs as essential components of the spliceosome and the fact that group II self-splicing introns use a similar two-step chemical pathway of intron excision has led to the hypothesis that pre-mRNA splicing and group II splicing share a common evolutionary origin. Indeed, it is now generally believed that the catalytic mechanism of pre-mRNA splicing will be largely, if not solely, RNA based.

Unlike group II introns, which are "hard wired" with the proper RNA structures required for catalysis, the spliceosome is assembled de novo onto each intron that is removed. It then carries out the catalytic reactions and is presumably disassembled. One role that spliceosomal proteins are thought to play is mediating the elaborate series of RNA conformational changes required during spliceosome assembly and catalysis (Madhani & Guthrie, 1994a). The dynamic nature of the spliceosome cycle is summarized briefly below. For comprehensive reviews and citations, the reader is referred elsewhere (Green, 1991; Guthrie, 1991; Rymond & Rosbash, 1992; Moore et al., 1993).

The first step in assembly is binding of U1 snRNP to the 5′ splice site via base pairing between U1 snRNA and the intron. Although this initial step is ATP-independent, each additional step in the assembly reaction requires ATP. Following U1, U2 snRNP binds the intron by base pairing to sequences that flank the branch site adenosine. The adenosine residue itself is not base paired, but bulges out of the U2 branch-site helix, allowing its utilization as a nucleophile in the first catalytic reaction (Query et al., 1994). After U2 snRNP binding, U4, U5, and U6 snRNPs enter the spliceosome, apparently as a triple snRNP particle. In this particle, U4 and U6 snRNAs are extensively base paired to each other. After binding of the U4/U5/U6 triple snRNP, the U4–U6 base pairing interaction is disrupted and U4 is destabilized from the spliceosome. U6 is then able to isomerize into a base pairing interaction with U2 that is mutually exclusive with its U4 interaction. The U1-5′ splice site base pairing interaction is also disrupted prior to the first catalytic step and is replaced by an interaction between the 5′ splice site and U5 and U6 snRNAs. The network of snRNA–snRNA and snRNA–intron interactions that is thus formed is thought to be the structural basis for the active site in the first catalytic step (Fig. 2) (Madhani & Guthrie, 1994a; Nilsen, 1994; Sun & Manley, 1995).

The focus of this review is events that occur subsequent to spliceosome assembly and the first catalytic step, namely 3′ splice site selection and the second catalytic step. Genetic, biochemical, and chemical data reviewed below demonstrate that the second catalytic step of splicing involves a unique set of RNA–RNA and protein–RNA interactions that is distinct from those occurring during spliceosome assembly and the first catalytic step. These data are discussed with an emphasis on their possible mechanistic implications for 3′ splice site selection and catalysis.

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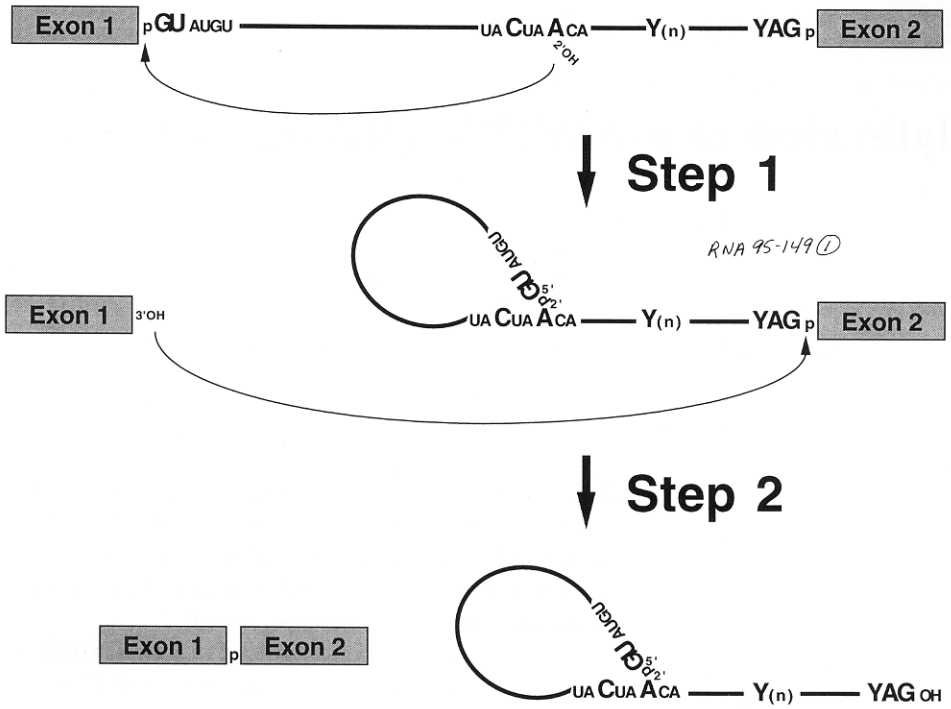

FIGURE 1. Two chemical steps of pre-mRNA splicing. Exons are depicted as shaded rectangles. Yeast consensus sequences are shown with nucleotides required for the second catalytic step in bold. Y represents a pyrimidine (C or U) and p represents a reactive phosphate.

CHEMICAL CONSIDERATIONS

Recently, powerful technological advances in constructing and manipulating RNAs have made it possible to ask questions about the nature of specific reactive groups (Moore & Sharp, 1992). Modification of the 2′ hydroxyl group in the ribose ring of either the first or last intron G residue appears to have little effect on the first catalytic step of the reaction. In contrast, a 2′-OCH_3, but not a 2′-H, in the first intron G residue slows the rate of the second step more than 10-fold. When these substituents are in the ribose ring of the last G residue, the rate of the second step is slowed 20- and 7-fold, respectively (Moore & Sharp, 1992). The magnitude of these effects suggests that the 2′ hydroxyl groups are not critical for the chemical mechanism of splicing per se, but might be involved in positioning of the reactive groups or in a recognition event unrelated to catalysis.

A second type of experiment with site-specifically modified RNAs involves the placement of stereochemical probes in the reactive 5′ and 3′ splice site phosphates. Inversion of the stereochemistry at phosphorothioates

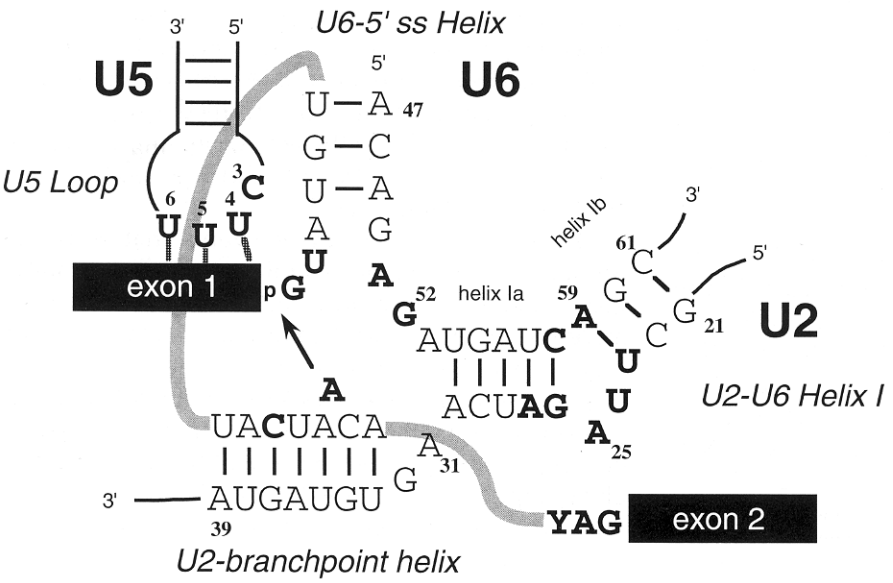

FIGURE 2. Network of RNA interactions prior to the first catalytic step. Numbering scheme for *S. cerevisiae* snRNAs is used. Depicted are the U2 branch site helix, the U6-5′ splice site helix, U2–U6 helix I, and the U5–exon 1 interaction. Exons are dark rectangles and the path of the intron is represented by the lightly shaded line. Residues that participate in the second catalytic step are highlighted in bold. Adapted from Madhani and Guthrie (1994a).

in both steps of splicing reveals that the chemical steps involve two in-line S_N2 displacement reactions (Maschhoff & Padgett, 1993; Moore & Sharp, 1993). The fact that both reactions are inhibited by the R_p but not S_p phosphorothioate stereoisomer would suggest that the second step of splicing does not proceed as a reversal of the first catalytic step, as in group I self splicing introns, but involves a significant conformational rearrangement resulting in a different active site for step two (McSwiggen & Cech, 1989; Rajagopal et al., 1989; Suh & Waring, 1992; Moore & Sharp, 1993). However, a general two-metal ion mechanism has been proposed for group I, group II, and pre-mRNA splicing catalysis within a single active site (Steitz & Steitz, 1993). Even though the second step of splicing is a reversal of the first in this model, the mechanism can accommodate the stereochemical data from pre-mRNA splicing because a simple rotation around the scissile phosphate allows the pro Rp oxygen in step two to bind the catalytic magnesium ions in the same orientation as they are bound by the pro Rp oxygen in step one.

Even if there are minimal structural rearrangements between the chemical steps of pre-mRNA splicing, there must be at least two alterations to the active site. (1) The branch lariat must be displaced, and (2) the 3′ splice site phosphodiester bond must be positioned for attack by the hydroxyl group of the last residue in exon 1. The nature of the chemistry for each step (e.g., 2′-5′ versus 3′-5′ bond formation) further mandates that the substrate binding sites for each reaction cannot be identical, although there could be some overlap (e.g., a single exon 1 binding site used for both steps). The data reviewed below do indeed suggest that there is a significant structural change between the two steps of splicing. However, it remains to be determined whether this rearrangement reflects the creation of a different active site for step two or the minimal remodeling required for use of a step one-like active site.

RNAs REQUIRED FOR THE SECOND CATALYTIC STEP

Pre-mRNA sequences

Introns are defined by consensus sequences at the 5′ splice site, branch site, and 3′ splice site. The 5′ splice site consensus is R/**GU**AUGU in yeast (*Saccharomyces cerevisiae*) and G/**GU**RAGY in mammals. The branch site consensus is UA**C**UA**A**CA in yeast and YNYUR**A** CN in mammals. The 3′ splice site consensus sequence is **YAG**/ in yeast and **CAG**/ in mammals (/, cleavage site; R, purine; Y, pyrimidine; N, any nucleotide; the branch adenosine is underlined). In yeast, these sequences are highly conserved among introns, whereas in mammals they are more divergent (data from Rymond & Rosbash, 1992). Nucleotides in bold have been demonstrated to be required for the second catalytic step of splicing (Jacquier et al., 1985; Newman et al., 1985; Parker & Guthrie, 1985; Reed & Maniatis, 1985; Ruskin & Green, 1985; Ruskin et al., 1985; Aebi et al., 1986; Fouser & Friesen, 1986, 1987; Hornig et al., 1986; Vijayraghavan et al., 1986; Freyer et al., 1987; Query et al., 1994). The effects of branch site mutations on step two are particularly difficult to detect in mammalian systems, due to cryptic branch site activation, and in yeast because they tend to destabilize the lariat intermediate (Padgett et al., 1985; Ruskin et al., 1985; Reed & Maniatis, 1988; Burgess & Guthrie, 1993b; Query et al., 1994) Therefore, lack of boldface does not necessarily indicate that a nucleotide has no role in the second catalytic step of the reaction.

Besides these consensus sequences, a polypyrimidine tract is usually found between the branch site and 3′ splice site. Although the branch site and 3′ splice site are generally close together (15–40 nt), this spatial coupling is not obligatory (e.g., Schatz et al., 1986; Helfman & Ricci, 1989; Smith & Nadal-Ginard, 1989; Goux-Pelletan et al., 1990). The 5′ splice site and branch site sequences (and the pyrimidine tract in mammals) are required for both the first and second catalytic steps. The 3′ splice site YAG motif is dispensable for the first step of splicing and is only absolutely required for the second catalytic step (Frendewey & Keller, 1985; Reed & Maniatis, 1985; Ruskin & Green, 1985; Rymond & Rosbash, 1985; Rymond et al., 1987; Reed, 1989). In many mammalian and *Schizosaccharomyces pombe* introns, the YAG does appear necessary for the first step, but this requirement can be alleviated by strengthening the pyrimidine tract or by improving base pairing with U1 (see below) (Reed, 1989; Reich et al., 1992). Thus, the 3′ splice site can be recognized at least twice during splicing, with at least one recognition event occurring prior to the first step (Zhuang & Weiner, 1990).

It is not immediately obvious why the 5′ splice site and branch site sequences should be necessary for the second catalytic step of splicing after fulfilling their requirement in the first catalytic step. However, the dual role that these elements play may serve several purposes. First, although not obligatory, the close proximity of the branch site to the 3′ splice site may facilitate identification of a 3′ splice site after the first catalytic step. For this mechanism to operate, the branch structure must be identified and used as a marker to search for a downstream 3′ splice site. In mammalian in vitro splicing, there is evidence for a scanning mechanism that begins at the branch point (Smith et al., 1989; see below).

A second reason for participation of the 5′ splice site and branch site nucleotides in the second catalytic step is that they could serve to enhance the fidelity of splicing. After the first catalytic step, there may be inspection mechanisms that determine whether proper sequences were utilized to form the lariat intermediate. Incorrect

intermediates could be prevented from continuing the reaction or be eliminated. Indeed, such a mechanism has been found recently in yeast. The spliceosomal ATPase Prp16 (see below) controls a pathway that degrades lariat intermediates formed at mutant branch sites (Burgess & Guthrie, 1993b).

A final reason for requiring the 5′ splice site and branch site in the second catalytic step is that the nucleotides in the branch lariat structure may be direct participants in the reaction. This requirement would provide a means of ensuring coupling between the two steps of splicing because 3′ splice site cleavage and ligation would depend on proper 5′ splice site cleavage and lariat formation. A highly insightful experiment has provided evidence that this is the case. It had been known for some time that mutations in the first G of the intron allow the first step of splicing to proceed, albeit at a reduced rate. However, these mutations completely block the second step (Newman et al., 1985; Aebi et al., 1986; Fouser & Friesen, 1986; Vijayraghavan et al., 1986). Similarly, mutations in the last G residue also strongly block the second step of splicing (Vijayraghavan et al., 1986). Amazingly, when a G to A mutation in the first residue of the yeast actin intron is combined with a G to C mutation in the last intron residue, splicing is restored to relatively high levels (10% of wild-type message) (Parker & Siliciano, 1993). A change of G to A in the last position also functions to give mutual suppression in combination with the G to A change at position one, although to a lesser extent. The same interaction has also been demonstrated with the yeast rp51a intron (Chanfreau et al., 1994). The allele-specific nature of the suppression is strongly suggestive of a direct interaction between the first and last G residues. Thus, the 5′ splice site G residue in the branch lariat structure provides a specific recognition element for the G in the YAG motif at the 3′ splice site.

Similar results have now been obtained with the /A-C/ combination in a mammalian intron (Deirdre et al., 1995). Interestingly, it was also found that inosine, when substituted for guanosine in both the first and last intron positions, has little effect on the rate of the second catalytic step. This information, combined with possible noncanonical pairing schemes that accommodate I-I, G-G, and A-C with similar geometries, allows a reasonable guess as to the nature of the G-G interaction (Fig. 3) (Tinoco, 1993; Deirdre et al., 1995). Direct proof of this interaction will require a more thorough substitution analysis of the relevant guanosine ring substituents or perhaps identification of a crosslink between the first and last intron residues. To date, no further evidence has been obtained for other intron–intron interactions between conserved nucleotides (Chanfreau et al., 1994; Ruis et al., 1994; Deirdre et al., 1995).

The YAG motif is the only 3′ splice site sequence element that is absolutely required for the second catalytic step. Mutations in these three nucleotides inhibit the second step of splicing in the following order of increasing severity Py < A < G (Vijayraghavan et al., 1986; Fouser & Friesen, 1987; Parker & Siliciano, 1993; Chanfreau et al., 1994). Therefore, besides the aforementioned /G-G/ interaction, there must be other recognition interactions involving these nucleotides during the second step. Base pairing of the 3′ splice site AG dinucleotide with the 5′ end of U1 snRNA has been shown to be required for the splicing of some *S. pombe* introns, but only for the first step of splicing (Reich et al., 1992). A test of the same base pairing scheme in *S. cerevisiae* failed to turn up evidence for this interaction in either step of splicing (Seraphin & Kandels-Lewis, 1993; J.G. Umen & C. Guthrie, unpubl.).

Besides the YAG trinucleotide, an upstream pyrimidine tract contributes to 3′ splice site recognition during the second step of splicing in both mammals and yeast. In mammals, this requirement is easily obscured

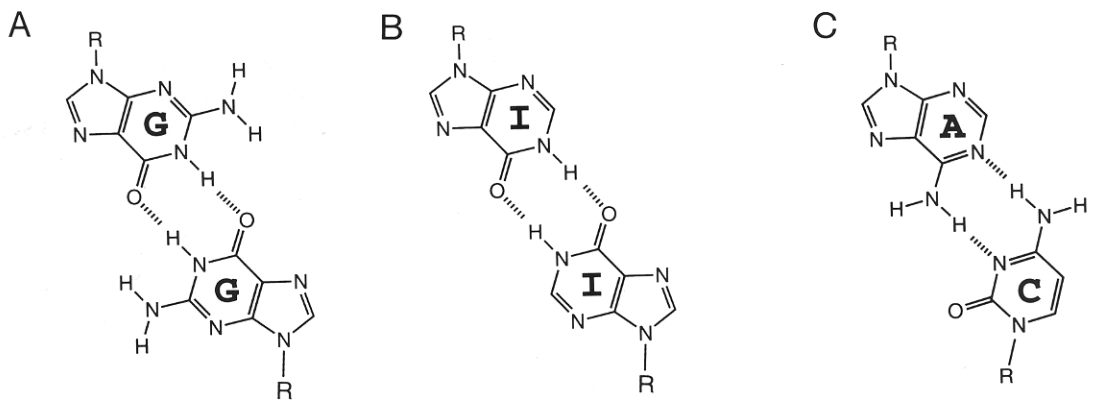

FIGURE 3. Non-Watson–Crick interaction between the first and last intron residues. **A:** *G-G* hydrogen bonding thought to occur in wild-type introns. **B:** Wild-type interaction is not disrupted when guanosine *G* is replaced with inosine *I*. **C:** Adeonsine-cytosine *A-C* configuration is accommodated with some backbone distortion (note position of R-groups in A and B versus C). Adapted from Tinoco (1993).

by the strong requirement for the pyrimidine tract in spliceosome assembly prior to the first catalytic step (Frendewey & Keller, 1985; Reed & Maniatis, 1985; Ruskin & Green, 1985). However, with the appropriate substrate, a role for the pyrimidine tract in the second catalytic step has been uncovered (Reed, 1989). In yeast, the pyrimidine tract is less conserved than in mammals and is restricted to mostly uridine residues. Analysis of the yeast intron database reveals a strong enrichment for uridine residues preceding the YAG motif in yeast 3′ splice sites. One position in particular, −9 with respect to the G at the 3′ cleavage site, is conserved as a uridine in over 80% of yeast introns and could be considered as a 3′ splice site consensus element (Parker & Patterson, 1987; Rymond & Rosbash, 1992). The functional role of the pyrimidine tract in yeast was demonstrated by making use of a sensitive 3′ splice site competition assay. When two 3′ splice sites are linked in *cis*, if one contains a uridine-rich tract, it has a strong competitive advantage over the uridine-poor 3′ splice site (Patterson & Guthrie, 1991).

In addition to the YAG motif and pyrimidine tract, there appear to be other elements that can contribute to 3′ splice site recognition. One of these, discussed below, is an interaction with U5 snRNA and exon positions adjacent to the 3′ splice site. There are also "context" effects that appear when the 3′ splice site sequence is suboptimal. For example, when the actin intron 3′ splice site is mutated from UAG/ to UUG/, most of the splicing still occurs at this mutated cleavage site and not at a UUG sequence upstream in the intron, even though the upstream UUG is in what appears to be a favorable location. Furthermore, a second, cryptic 3′ splice site with the sequence AUG/ is activated by the UUG/ mutation (Parker & Siliciano, 1993); this site is 5 nt upstream of the utilized UUG/ 3′ splice site. Why is this double-mutant cryptic site chosen over the more upstream UUG sequence, and how is the spliceosome able to choose the "correct" UUG? Use of these 3′ splice sites does not correlate with U5 snRNA base pairing potential at the exon sequences (see below). Therefore, there must be additional information in the pre-mRNA that can specify possible splice sites. This information does not appear to be encoded in consensus sequence elements. Instead, it might be correlated with overall sequence content. Yeast intron sequences are more AU rich than exons (Parker & Patterson, 1987); therefore, the intron/exon borders could be crudely defined by the changes in sequence content flanking the splice junction. A striking example of a role for sequence context in splicing is found in plants. Plant introns can be visually identified by a very strong difference in AU content between introns and exons. Furthermore, AU richness (versus a specific sequence motif) has been shown to play a prominent functional role in the splicing of plant introns (Goodall & Filipowicz, 1989). More work is necessary to determine whether such a mechanism is utilized in other organisms.

U2 and U6 snRNAs

Only three of the spliceosomal snRNAs, U2, U5, and U6, appear to be required for the catalytic steps of splicing. U1 and U4 are both destabilized from the spliceosome prior to the first catalytic step, and splicing catalysis has been observed in their absence (Yean & Lin, 1991; Crispino et al., 1994; Tarn & Steitz, 1994). As mentioned above, during spliceosome assembly, U2 and U6 snRNAs isomerize into a base pairing interaction that is required for the first catalytic step of splicing. This interaction consists of two short helices (Ia and Ib) interrupted by a 2-nt bulge in U2 (see Fig. 2) (Madhani & Guthrie, 1992). The accumulation of lariat intermediates that is seen when helix I is disrupted indicates that it is required for the second catalytic step. In mammals, there are additional base pairing interactions formed between U2 and U6, but their role in step two has not been determined (Datta & Weiner, 1991; Wu & Manley, 1991; Sun & Manley, 1995).

Two clusters of individual nucleotides in U2 and U6 snRNAs are necessary specifically for the second catalytic step. The first set lies just upstream of helix I in the phylogenetically conserved ACAG**AG** sequence in U6. Alterations in the first four bases of this sequence cause a block to the first step of splicing, whereas mutations in the last two (boldface) bases block the second catalytic step in vitro and in vivo (Fabrizio & Abelson, 1990; Madhani et al., 1990; Vankan et al., 1992; Datta & Weiner, 1993; Yu et al., 1993; Wolff et al., 1994). The other set of nucleotides that are required for the second step of splicing are in the bulge region between helices Ia and Ib (Fabrizio & Abelson, 1990; Madhani et al., 1990; Madhani & Guthrie, 1992, 1994b; McPheeters & Abelson, 1992; Wolff et al., 1994). These include U6 residues C58 and A59 (from yeast) opposite the bulge and their U2 base pairing partners U23 and G26. Alterations in U2 A27 cause a modest decrease in the second catalytic step. Some discrepancies between data from yeast and mammalian systems suggests that the step-two nucleotides in U2 and U6 are not always rate limiting (Vankan et al., 1992; Datta & Weiner, 1993; Wolff et al., 1994).

Although part of the second step splicing block caused by mutations around the bulge region of helix I is due to disruption of helices Ia and Ib, there is a phenotypic asymmetry resulting from alterations in U6 at position 59 versus its U2 pairing partner U23 that cannot be explained by disruption of helix Ib. In vivo, mutations in yeast U6 position 59 lead to temperature sensitive growth or lethality, whereas position 23 mutations in U2 cause no growth phenotypes (Madhani

& Guthrie, 1992). This asymmetry suggests that A59 in U6 plays an additional role in the second catalytic step besides base pairing with U2. There is also evidence suggesting that the bulged structure itself in helix I is important for the second step. Although the U2 nucleotides in the bulge can be substituted with other bases without greatly affecting splicing efficiency (McPheeters & Abelson, 1992; Madhani & Guthrie, 1994c), altering the spacing of the bulge by insertion or deletion of nucleotides is highly deleterious (Madhani & Guthrie, 1994c; Wolff et al., 1994).

Interestingly, substitution of U2 position 25 in the bulge affects 3′ splice site utilization. Changes at this position slightly decrease the in vivo efficiency of step two for wild-type substrates, and an A to G change in U2 25 can suppress the effects of alterations at the 3′ splice junction AG dinucleotide. The suppression is not allele specific, but could be taken to suggest that the U2 mutation is altering the active site to decrease its selectivity (Madhani & Guthrie, 1994c). There is a mutation in yeast U6, G52U, that results in similar nonspecific suppression of 3′ splice site AG alterations (Lesser & Guthrie, 1993). Moreover, these two residues in U2 and U6 are implicated in a possible tertiary interaction involving a noncanonical G-A pairing. Evidence for the tertiary interaction comes from a covariation seen between these nucleotides after randomization and selection for functional variants of U2 and U6 in vivo (Madhani & Guthrie, 1994c). As with U6 A59/U2 U23, there is a phenotypic asymmetry observed between U2 position 25 mutations and U6 position 52 mutations, indicating that participation of U6 G52 in the proposed tertiary interaction is secondary to a more critical role for this nucleotide. Nevertheless, the tertiary interaction is capable of bringing together the two clusters of U2 and U6 residues that are required for the second catalytic step and is supported by crosslinking data (see below).

Intriguingly, in mammalian extracts, a crosslink can be isolated between the nucleotide adjacent to the tertiary interaction in mammalian U6 (underlined adenosine residue in the U6 ACAG**A**G motif) and position two in the intron (underlined U in G/**GU**RAGY at the 5′ splice site) (Sontheimer & Steitz, 1993). Moreover, a different crosslink has recently been identified between yeast U2 residue U23 and the first nucleotide of exon 2 (A. Newman, pers. comm.). These crosslinks both occur in the lariat intermediate and suggest that all the nucleotides important for the second catalytic step in U2 and U6 can be simultaneously juxtaposed with critical substrate residues in the intron prior to catalysis (Fig. 4).

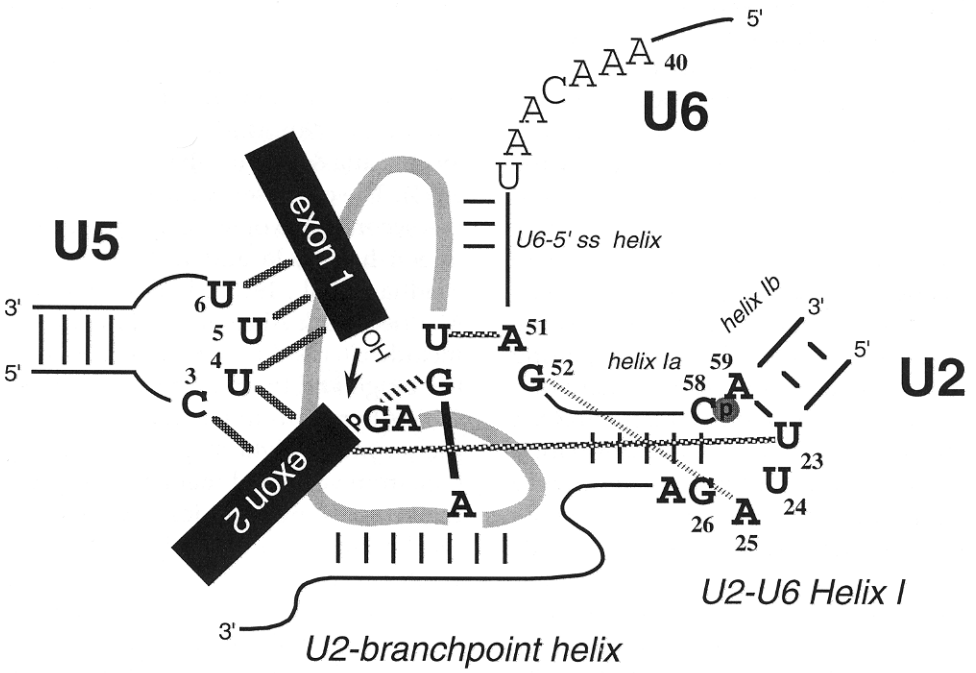

FIGURE 4. Network of RNA interactions prior to the second catalytic step. Numbering scheme for *S. cerevisiae* snRNAs is used. Depicted are the U2 branch site helix, U6-5′ splice site helix, U2–U6 helix I, and U5–exon 1/exon 2 interactions after lariat intermediate formation. Exons are dark rectangles and the intron is represented by a lightly shaded thick line. Nucleotides and phosphates that participate in step two are shown in bold. U6 heptanucleotide sequence that interacts genetically with *PRP16* is also shown. The 2′-5′ A-G bond at the lariat branch is depicted as a thick dark line. Noncanonical G-G interaction between the first and last intron residues is depicted as a coarsely striped line, and tertiary interaction between U2 A25 and U6 G52 is represented as a finely striped line. Crosslinks between U6 A51–intron +2 or U2 U23–exon two +1 are depicted as dark stippled lines. Phosphate whose nonbridging oxygen is important for step two is indicated by the shaded gray circle. Adapted from Madhani and Guthrie (1994b). See text for individual citations.

A second type of analysis with U6 snRNA has involved substitution of phosphates with phosphorothioates. These experiments were performed by in vitro reconstitutions of U6 in both nematodes and yeast. The results of the two studies are highly congruent in their identification of Rp phosphorothioates that block each step of splicing. In particular, the phosphate between positions 58 and 59 in yeast U6 (positions 48 and 49 of *Ascaris lumbricoides*) is required specifically for the second step of splicing (Fabrizio & Abelson, 1992; Yu et al., 1995). Notably, this phosphate is positioned in the "kink" of the bulge region that is critical for the second catalytic step (see Fig. 4). An interesting possibility is that this phosphate is responsible for binding a catalytic magnesium ion in the active site. Although the inability to rescue the defect with manganese does not rule out this possibility (Fabrizio & Abelson, 1990; Yu et al., 1995), there is, as yet, no direct evidence for specific magnesium binding sites in the spliceosome. Identification of such sites is of obvious importance because it is likely that magnesium ions participate in RNA-mediated catalysis (Piccirilli et al., 1993; Steitz & Steitz, 1993).

U5 snRNA

The role of U5 snRNA in either step of splicing has until recently seemed enigmatic. The finding that depletion of U5 can block the second step of splicing in vivo and in vitro suggests that U5 is not absolutely required for the first step (Patterson & Guthrie, 1987; Winkelmann et al., 1989). However, this interpretation runs counter to the standard view of spliceosome assembly and to other reports that demonstrate a stringent U5 snRNP requirement for spliceosome assembly and for the first catalytic step (Lamm et al., 1991; Seraphin et al., 1991; Segault et al., 1995). This discrepancy cannot be explained solely by the extent of U5 depletion in the respective experiments. That is, if any spliceosome that can carry out the first step of splicing contains U5 snRNP, then all spliceosomes that undergo the first catalytic step must contain U5 and therefore should be able to carry out the second step. One resolution of this paradox is possible if U5 is absolutely required for both steps of splicing but is exchangeable between spliceosomes and can dissociate after the first catalytic step. A more conventional explanation invokes the existence of exchangeable U5 snRNP proteins that are required for the second step of splicing. If the second-step U5 proteins must compete for binding with other U5 proteins, then depletion of U5 might increase the concentration of free U5 proteins to the point where the second step proteins are outcompeted. Interestingly, there is one yeast U5 snRNP protein, Prp18, that behaves as an exchangeable factor and is required for the second catalytic step (see below) (Horowitz & Abelson, 1993b).

The most conserved portion of U5 snRNA is a 9-nt loop at the top of a stem structure (Guthrie & Patterson, 1988; Frank et al., 1994). A breakthrough in deciphering the function of U5 has come through the isolation of loop mutants that can suppress splice site mutations or affect cryptic splice site usage in yeast. Careful genetic analysis has shown that 4 nt in the loop (in boldface; 5′ $G_1C_2\mathbf{C_3U_4U_5U_6}U_7A_8C_9$ 3′) can base pair with both exons adjacent to the 5′ and 3′ splice sites (Newman & Norman, 1991, 1992) (Figs. 2, 4). Activation of cryptic 5′ splice sites can occur in yeast when the normal 5′ splice site is mutated and the UU residues at loop positions 5 and 6 can base pair with positions −2 and −3 with respect to the cryptic cleavage site. Genetic experiments with the U5 loop in mammals have yielded similar results and possibly demonstrated an extension of the base pairing to include an interaction between loop position 4 and position −1 of the first exon (Cortes et al., 1993). With respect to the second step of splicing, the effects of alterations in the 3′ splice site AG dinucleotide can be partially suppressed when complementarity is created between positions 4 and 5 of the U5 loop (CU) and exon 2 positions +1 and +2 (Newman & Norman, 1992). It is notable that the two U5–exon interactions described are out of register with one another in terms of forming a continuously base paired structure.

Importantly, the interactions between the exons and the loop of U5 have been demonstrated for a wild-type intron that lacks the potential to base pair with the loop nucleotides (Wyatt et al., 1992; Sontheimer & Steitz, 1993). 4-Thiouridine placed at position −1 in exon 1 of a mammalian intron crosslinks to U5 loop nucleotides 4 and 5 prior to step one, and this U5-pre-mRNA crosslink can be "chased" through both steps of splicing, indicating its functional relevance (Sontheimer & Steitz, 1993). Both exon 1 and exon 2 crosslinks are in the proper register with respect to the genetic interactions described for each exon (Sontheimer & Steitz, 1993). 4-Thiouridine at exon 2 position +1 crosslinks to loop nucleotides 3 and 4 only after the first step of splicing. In summary, the loop nucleotides of U5 are in close proximity to the reactive groups that participate in the second catalytic step (see Fig. 4).

Despite the wealth of detailed information on the U5 loop, its specific function is still unclear. Because exon sequences are not well conserved, the general function of the U5 loop nucleotides cannot be to specify splice site location through base pairing with exons. It has been proposed that the function of the loop is to hold exon 1 after the first step of splicing and to help align the exons for the second step of splicing (Newman & Norman, 1992; Sontheimer & Steitz, 1993). Nonetheless, the limited and variable extent of this interaction suggests that other factors are required for its stabilization.

It is important to note that the above sections have focused on nucleotides and phosphates that are re-

quired only for the second catalytic step. It is highly likely that there is also some overlap with residues involved in the first catalytic step. Knowledge of which residues are specific to step one and step two and which are required for both catalytic steps will be invaluable for understanding the relationship between the step one and step two active sites. Even without this information, however, the interactions that have already been inferred from genetic and biochemical experiments allow an impressive number of constraints to be imposed on the architecture of the putative active sites. These constraints must be viewed with some caution, of course, because they are often predicated on the assumption that the RNA–RNA interactions described are occurring simultaneously. Nucleotides and RNA–RNA interactions required for the second catalytic step are summarized in Figure 4.

PROTEINS REQUIRED FOR THE SECOND CATALYTIC STEP

Just as there are RNA sequences required specifically for the second step of splicing, there is a set of proteins that is also required for step two (see Table 1). In addition, at least one protein, Prp8, is required for both steps of splicing. Because more information is available on these proteins in yeast, the yeast data will be discussed first, followed by a summary of mammalian proteins involved in the second step of splicing.

Prp16

PRP16 was first identified from a mutant, *prp16-1*, that suppresses the splicing defect of an A to C change at the intron branch nucleotide (Couto et al., 1987; Burgess et al., 1990). Subsequently, it was identified as a temperature-sensitive mutant, *prp23-1*, blocked at the second catalytic step (Vijayraghavan et al., 1989). In vitro, Prp16 is required only for the second step (Schwer & Guthrie, 1991). The sequence of the gene reveals it to be a member of the DEXH family of putative RNA helicases (Burgess et al., 1990; Schmid & Linder, 1992). Indeed, Prp16 has been shown to be an RNA-dependent ATPase, although no RNA helicase activity has been found associated with the purified protein (Schwer & Guthrie, 1991). *PRP16* shares sequence homology with

TABLE 1. Proteins required for the second step of splicing.

Protein	MW (kDa)	Essential?[a]	Sequence motifs	ATP requirement?[b]	Functions	References
Yeast proteins						
Prp16	120	Yes	DEAH	Yes	RNA-dependent ATPase; branch site suppressor; induces conformational change at 3′ splice site; crosslinks to 3′ splice site	Couto et al., 1987; Burgess et al., 1990; Schwer & Guthrie, 1991, 1992; Schmid & Linder, 1992; Burgess & Guthrie, 1993; Umen & Guthrie, 1995c
Prp17	52	No	WD	Yes		Neer et al., 1994; Jones et al., 1995
Prp18	28	No		No	U5 snRNP associated	Horowitz & Abelson, 1993a, 1993b
Slu7	44	Yes	Zinc knuckle	No	3′ splice site selection; crosslinks to 3′ splice site	Frank & Guthrie, 1992; Ansari & Schwer, 1995; Jones et al., 1995; Umen & Guthrie, 1995c
Prp8	280	Yes	Proline-rich domain	nd	U5 snRNP protein; 3′ splice site selection; crosslinks to 5′ splice site, branch site and 3′ splice site; required for first step; required for U4/U5/U6 snRNP stability	Lossky et al., 1987; Jackson et al., 1988; Brown & Beggs, 1992; Hodges et al., 1995; Teigelkamp et al., 1995; Umen & Guthrie, 1995a, 1995b, 1995c
Ssf1	~200	nd		nd	Functions after Prp16	Ansari & Schwer, 1995
Mammalian proteins						
PSF	100	na	RGG; RRM; proline/ glutamine-rich	nd	Pyrimidine tract binding	Patton et al., 1993; Gozani et al., 1994
SF3/HLF	nd	na		Yes	Heat labile	Krainer & Maniatis, 1985; Sawa & Shimura, 1991
SF4a	nd	na		nd		Krainer & Maniatis, 1985
CMFT[c]	nd	na		No		Lindsey et al., 1995
DEAE-I[c]	nd	na		Yes		Sawa & Shimura, 1991
Ia	nd	na		nd		Perkins et al., 1986

[a] Indicates whether the encoded gene product is essential for viability in yeast. na, not applicable. nd, not determined.

[b] Indicates the order of protein function with respect to the ATP requirement during step two. Yes indicates that the protein functions at or before an ATP-requiring step. No indicates that the protein functions in the absence of ATP. nd, not determined.

[c] These proteins are complex mixtures that have been given no designation by the authors. Column fractions from which they are derived are used as a provisional means of nomenclature.

two other splicing factors, *PRP2* and *PRP22*. All three belong to the DEAH subgroup and are tripartite in structure with a unique N-terminal domain followed by homologous "helicase" and C terminal domains (Chen & Lin, 1990). Prp2 is required just prior to the first catalytic step, and Prp22 is required for the release of mature message from spliceosomes (Company et al., 1991; Kim & Lin, 1993).

Prp16 functions by binding to spliceosomes after lariat intermediate formation; it then hydrolyzes ATP and exits (Schwer & Guthrie, 1991). Part of its spliceosomal binding site may be the 3′ splice site, because Prp16 can be specifically crosslinked to this region of the intron (Umen & Guthrie, 1995c). Moreover, ATP hydrolysis by Prp16 results in a conformational change that leads to protection of the 3′ splice site from oligonucleotide-directed RNase H cleavage (Schwer & Guthrie, 1992a). This conformational change is correlated with 3′ splice site crosslinking of two other proteins, Slu7 and Prp8 (see below) (Umen & Guthrie, 1995c). The conformational change could reflect a function for Prp16 in bringing a potential 3′ splice site into the spliceosomal active site or in altering the spliceosome for the second catalytic step.

If Prp16 is involved in remodeling the spliceosome for step two, then it would be expected to interact with spliceosomal snRNAs required for this step. Interestingly, U2 and U6 snRNAs are among the strongest stimulators of the RNA-dependent ATPase activity of Prp16 in vitro (Y. Wang, pers. comm.). Moreover, a genetic interaction with U6 snRNA was found by isolating U6 suppressors of dominant negative, cold-sensitive *PRP16* alleles (Madhani & Guthrie, 1994b). These dominant negative alleles are thought to block a reaction after spliceosomal binding of Prp16, e.g., release of the protein after ATP hydrolysis (Schwer & Guthrie, 1992b). In this model, the U6 suppressors would act to partially disrupt the Prp16 binding site, thus allowing the protein to exit the spliceosome after carrying out its function. The strongest of these suppressors are single nucleotide deletions in U6 just upstream of the ACAGAG motif in a heptanucleotide sequence AAACAAU (nt 40–46; see Fig. 4) (Madhani & Guthrie, 1994b). Because these nucleotides are not known to be critical for either step of splicing, they may form a secondary or redundant binding site for Prp16. Disruption of the site would then provide enough destabilization for suppression but not enough to interfere significantly with wild-type functions of U6 or Prp16.

How do these data fit with the original identification of *prp16-1* as a suppressor of an intron branch site mutation? The *prp16-1* allele was found to contain a single substitution near a conserved NTP-binding motif in the helicase domain (Burgess et al., 1990). Not surprisingly, this allele causes a severe defect in RNA-dependent ATPase activity (Schwer & Guthrie, 1992b). Subsequent work in which additional branch site suppressor alleles were isolated and analyzed demonstrated that these alleles all map to the helicase domain and cause a decrease in RNA-dependent ATPase activity. It was also determined that the suppressors do not function by accelerating the rate of the second catalytic step with mutant branch lariats. Instead, the suppressor alleles inhibit the degradation of these aberrant lariats (Burgess & Guthrie, 1993b). The mechanism of suppression can be explained if there is a rate-limiting step (LI → LI*) for productive splicing of mutant lariat intermediates, and this rate-limiting step is in kinetic competition with ATP hydrolysis by Prp16 (Burgess & Guthrie, 1993a, 1993b).

The rate-limiting step might be the aforementioned conformational change that results in 3′ splice site protection (Schwer & Guthrie, 1992a). This idea is appealing given that the branch lariat structure itself could be part of a binding site for the 3′ splice site and would thus contribute energetically to the conformational change. ATP hydrolysis by Prp16 would lock the lariat-bound 3′ splice site into place and allow splicing to proceed. However, mutant branch sites would impair 3′ splice site binding and prevent the LI → LI* transition from taking place before ATP hydrolysis, thus dooming the lariat intermediate to degradation. In this model, some spliceosomal binding of the 3′ splice site can take place independently of ATP hydrolysis by Prp16. This facet of the model is supported by the finding of 3′ splice site crosslinks to a spliceosomal protein, Prp8, in the absence of Prp16 (see below) (Umen & Guthrie, 1995c).

One important and unresolved issue is how many rounds of ATP hydrolysis are required to complete the Prp16-dependent (and other ATP-dependent) steps of splicing. Whether there are multiple rounds of hydrolysis or a single event would have a large impact on how we think about the mechanism by which Prp16 functions. For example, 3′ splice site selection may involve several rounds of ATP hydrolysis accompanied by the binding and release of one or more potential 3′ splice sites. ATP hydrolysis by Prp16 (or another ATPase) could prevent the binding reaction from reaching equilibrium and thereby enhance the fidelity of splice site selection in a manner akin to kinetic proofreading during translation (Hopfield, 1974; Ninio, 1975).

Slu7

SLU7, like *PRP16*, encodes an essential function and is required for the second step of splicing, both in vivo and in vitro (Frank & Guthrie, 1992; Frank et al., 1992; Ansari & Schwer, 1995; Jones et al., 1995). This gene was isolated as *slu7-1* in a screen for mutations that are *s*ynthetically *l*ethal with loop mutations in *U5* snRNA.

The *SLU7* sequence contains a short stretch of homology to a family of retroviral capsid proteins (Frank & Guthrie, 1992). This sequence motif ($CX_2CX_4HX_4C$), termed a "zinc knuckle," is implicated in RNA packaging by retroviruses (Rein, 1994). Thus, Slu7 is predicted to be an RNA binding protein. Although Slu7 does not appear to be stably associated with U5 snRNP (D. Frank & C. Guthrie, unpubl. data), a clue to a possible RNA binding site came from experiments using splicing substrates with tandem 3′ splice sites competing in *cis*. The *slu7-1* allele causes a selective defect in utilization of 3′ splice sites that are greater than ~12 nt downstream of the branch site (Frank & Guthrie, 1992). Therefore, Slu7 is likely to be involved in utilization of 3′ splice sites that are distal to the branch site. Supporting the idea of a direct role for Slu7 in 3′ splice site selection is the finding of a Slu7-3′ splice site crosslink during the second step of splicing (Umen & Guthrie, 1995c). It has not been determined whether complete absence of the protein differentially affects distal 3′ splice sites or whether this is a special property of the *slu7-1* allele. Despite predictions from sequence homology, mutations in the zinc knuckle motif of *SLU7* cause only a modest decrease in cell growth and splicing and do not appear to affect 3′ splice site selection or 3′ splice site crosslinking significantly (Frank & Guthrie, 1992; J.G. Umen & C. Guthrie, unpubl. data).

In vitro experiments with Slu7 have demonstrated that this protein does not require ATP to carry out its function in the second step of splicing (Ansari & Schwer, 1995; Jones et al., 1995). This finding suggests that Slu7 acts after the Prp16-dependent step, which does require ATP. A more direct test of this idea utilized purified spliceosomes lacking both Prp16 and Slu7. Adding back Prp16, Slu7, or ATP in a specified order confirmed that Prp16 functions prior to Slu7 and that Slu7 functions in the absence of ATP. Additional fractionation experiments suggest the existence of a novel second-step factor, SSF1, that is required in addition to Prp16 and Slu7. The biochemical properties of SSF1 indicate that it is probably not identical to Prp17 or Prp18 (see below) (Ansari & Schwer, 1995).

Consistent with its function after ATP hydrolysis by Prp16, Slu7 crosslinks most strongly to the 3′ splice site after the Prp16-dependent step of the reaction. This crosslinking also depends on the functions of other known second-step splicing factors, Prp17 and Prp18. Thus, Slu7 interacts with the 3′ splice site at a time very close to the second catalytic event (Umen & Guthrie, 1995c). One of its functions could be to mediate or stabilize binding of the 3′ splice site to the spliceosome before or after Prp16 exits. 3′ splice sites that are distal to the branch site might be particularly sensitive to loss of Slu7 function because they are expected to have a slower "on" rate for spliceosome binding due to their greater distance from the binding site.

Prp17 and Prp18

Both *PRP17* and *PRP18* were identified from temperature-sensitive mutants, *prp17-1* and *prp18-1*, that specifically block the second step of splicing (Vijayraghavan et al., 1989; Vijayraghavan & Abelson, 1990). *slu4-1*, which is synthetically lethal with a loop mutation in U5 snRNA, is also a mutant allele of *PRP17* (Frank et al., 1992). The *PRP18* sequence reveals no homologies to known proteins. *PRP17* contains four copies of the WD motif, a segment of approximately 40 amino acids that is thought to mediate protein–protein interactions (Neer et al., 1994). Interestingly, the genes that encode Prp17 and Prp18 are not essential and, as expected, absence of either protein causes only a partial block to the second step of splicing in vitro (Horowitz & Abelson, 1993b; Jones et al., 1995). Prp18 can be found associated with the U4/U5/U6 triple snRNP, probably as a U5 snRNP component (Horowitz & Abelson, 1993b). Because depletion of Prp18 from extracts does not result in a first-step splicing block, co-immunodepletion of U4/U5/U6 must only be limited. Thus,

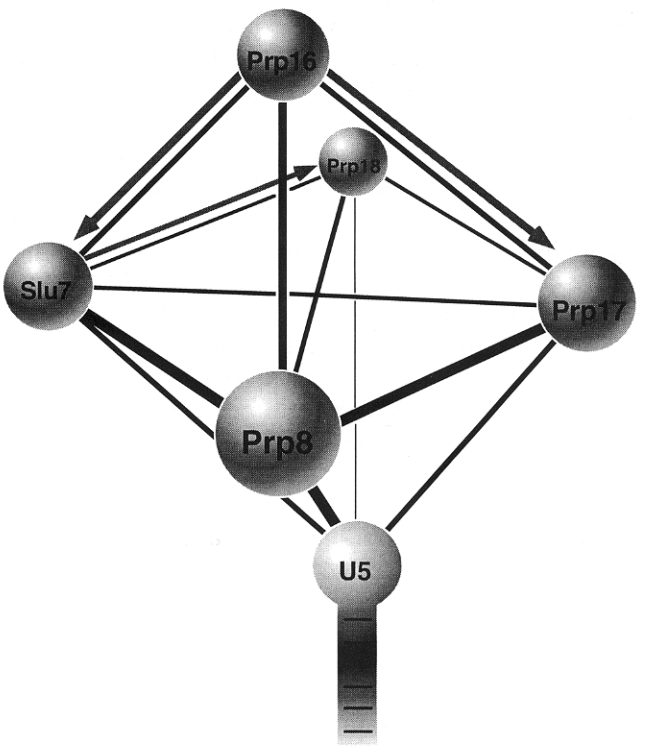

FIGURE 5. Genetic interactions between yeast second-step splicing factors. Proteins are depicted as spheres and U5 snRNA as a sphere with a stem structure. Solid dark lines between spheres indicate that alleles of the genes that encode the splicing factors are synthetically lethal. Line thickness is used only to represent perspective. Lightly colored arrows indicate genetic suppression by overexpression. Overexpression of the factor at the beginning of the arrow results in partial suppression of the growth phenotype caused by a mutation in the factor at the arrow head. See text for citations.

Prp18 associates with these snRNPs relatively weakly (i.e., in an exchangeable manner) or with only a subset of triple snRNPs (Horowitz & Abelson, 1993a).

Like Prp16 and Slu7, Prp17 and Prp18 have been functionally ordered with respect to the ATP requirement during the second step of splicing. Prp17 acts before or concomitant with an ATP-dependent reaction, whereas the function of Prp18 is ATP-independent (Horowitz & Abelson, 1993a; Jones et al., 1995). Thus, a strict ordering would place the Prp16/Prp17-dependent functions prior to the Slu7/Prp18-dependent functions (see Fig. 6). Perhaps the nonessential partner in each of these pairs facilitates the function of the essential factor. The observation that overexpression of Prp16 suppresses a *PRP17* mutation and that overexpression of Slu7 suppresses a *PRP18* mutation support this idea (Jones et al., 1995). Using information on ATP requirements, protection, and crosslinking, some of the events that take place during the second step of splicing can be tentatively ordered with respect to protein factor requirements (Fig. 6).

The formal ordering of these factors described above may actually prove misleading for understanding their functions. In the strictest sense, the "upstream" factors should not be affected by the "downstream" ones. This relationship is violated, however, by the finding that release of Prp16 from the 3′ splice site partly depends on a "downstream" protein, Slu7 (Umen & Guthrie, 1995c). Genetic experiments, in particular, suggest close functional connections that are independent of ATP requirements (Fig. 5). Alleles of *SLU7*, *PRP17*, and *PRP18* are all synthetically lethal with each other and with loop mutations in U5. Alleles of *PRP16* are synthetically lethal with alleles of *PRP17* and *SLU7* but not *PRP18* (Frank et al., 1992). Overexpression of *PRP16* can suppress mutations in *SLU7* as well as in *PRP17* (Jones et al., 1995). Finally, alleles of all four genes, *PRP16*, *PRP17*, *PRP18*, and *SLU7*, are synthetically lethal with an allele of *PRP8*, *prp8-101* (see below; Fig. 5) (Umen & Guthrie, 1995c). Extensive genetic interactions such as these are often indicative of physical interactions. To date, the best evidence of direct associations are between U5 snRNP and the proteins Prp18 and Prp8 (Lossky et al., 1987; Horowitz & Abelson, 1993b). Preliminary results suggest that Prp8 directly contacts U5 snRNA (C. Collins, pers. comm.).

Prp8

Unlike the previous four proteins, Prp8 was first identified from a temperature-sensitive mutant, *prp8-1*, that blocks splicing prior to the first catalytic step of the reaction in vivo and in vitro (Jackson et al., 1988; Brown & Beggs, 1992). Removal of the protein by genetic de-

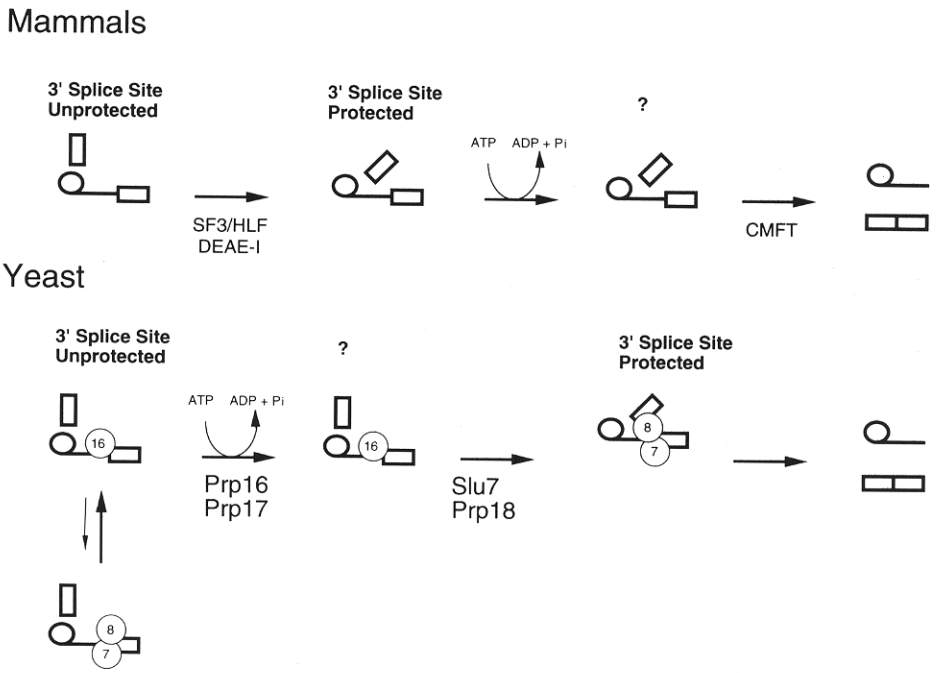

FIGURE 6. Conformational rearrangements at the 3′ splice site during the second catalytic step. The lariat intermediate is drawn with the 3′ splice site in an unprotected (exons perpendicular) or protected (exons at acute angle) state. Protein factor and ATP requirements are listed under the arrows that show the transitions between stages. A question mark indicates that no information is available. 3′ splice site crosslinking is shown as spheres for yeast proteins Prp16 (16), Slu7 (7), and Prp8 (8). Equilibrium depicted in the unprotected state for yeast represents strong 3′ splice site crosslinking of Prp16 and weaker crosslinking of Prp8 and Slu7. Protein factors are described in Table 1.

pletion also inhibits the first step of splicing. The consequence of removing Prp8 is destabilization of the U4/U5/U6 triple snRNP which, in turn, blocks spliceosome assembly (Brown & Beggs, 1992). Immunoprecipitations indicate that the protein is present in U5 snRNP, U4/U5/U6 snRNP, and spliceosomes (Lossky et al., 1987; Whittaker et al., 1990; Brown & Beggs, 1992). Anti-Prp8 antibodies were shown to cross-react with a mammalian U5 snRNP protein of similar size (220 kDa), giving a first indication of the remarkable evolutionary conservation of *PRP8* (Anderson et al., 1989; Pinto & Steitz, 1989). Subsequent sequence comparisons revealed that the yeast and *C. elegans* homologues are approximately 68% identical and 80% similar over most of the entire length of both genes. The cloned regions of *PRP8* homologues from other species show similar levels of identity and similarity (Hodges et al., 1995).

Although the conservation and large size of Prp8 indicate that it likely plays critical roles in the splicing reaction, its sequence reveals no conserved motifs in common with other proteins that give a clue to its function. Crosslinking experiments in yeast and mammals have demonstrated that Prp8 or its mammalian homologue p220 directly contacts the pre-mRNA, lariat intermediate, and excised lariat intron (Garcia-Blanco et al., 1990; Whittaker & Beggs, 1991; Teigelkamp et al., 1995b). Subsequently, one contact point was found at position −2 of the first exon during mammalian in vitro splicing. This interaction takes place prior to the first catalytic step (Wyatt et al., 1992).

A specific functional role for Prp8 was first identified in 3′ splice site selection. A novel allele, *prp8-101*, was found to block recognition of the uridine tract preceding the 3′ splice site. As expected from its impaired 3′ splice site recognition phenotype, this allele inhibits the second catalytic step of splicing. It also strongly exacerbates the phenotypes of mutations in the YAG motif at the 3′ splice junction (Umen & Guthrie, 1995b). Crosslinking of Prp8 to the 3′ splice site suggests that its role in mediating 3′ splice site selection is direct (Teigelkamp et al., 1995a; Umen & Guthrie, 1995b).

Further crosslinking of Prp8 to the splicing substrate has identified contacts with the first exon in positions −1, −2, and −8 prior to the first step of splicing. After the first step, Prp8 contacts the branch site, 3′ splice site, and part of the second exon (Teigelkamp et al., 1995a). Thus, Prp8 is a good candidate for a protein that assists the U5 loop in binding to exon sequences (see above). Detailed kinetic analysis of Prp8-3′ splice site crosslinking indicates that it occurs weakly prior to hydrolysis of ATP by Prp16 and strengthens afterward. Strong crosslinking is also dependent upon the functions of Prp17, Prp18, and Slu7. Slu7-3′ splice site crosslinking follows a similar profile to that of Prp8 (Umen & Guthrie, 1995c). Comparison of these crosslinking kinetics to those of Prp16 suggests that the 3′ splice site is recognized in at least two distinct stages during the second catalytic step. The first stage is characterized by strong crosslinking of Prp16 and the second by strong crosslinking of Prp8 and Slu7. This two-stage binding regime may reflect a proofreading mechanism that ensures proper 3′ splice site selection. The crosslinking kinetics are also consistent with the presence of Prp8 and Slu7 at or near the 3′ splice site during the second catalytic step. This result, combined with the high degree of sequence conservation of Prp8 in particular, is suggestive of a role for this protein at the active site.

Support for the idea that Prp8 may be involved in utilization of the YAG motif at the 3′ splice junction comes from a novel class of *PRP8* alleles that is distinct from *prp8-101*. In contrast to *prp8-101*, which exacerbates the phenotypes of YAG alterations at the 3′ splice site, these new alleles suppress YAG alterations without affecting uridine tract recognition (Umen & Guthrie, 1995a). This suppression is highly reminiscent of that seen when presumptive active-site RNA residues, yeast U6 G52 or U2 A25, are mutated (see above). Although these *PRP8* alleles can suppress a wide spectrum of YAG alterations, the complex pattern of allele preference displayed suggests a direct interaction with the YAG trinucleotide and/or the active site.

Mammalian second step splicing factors

Study of the second step in mammalian splicing extracts has lagged somewhat compared to yeast, possibly because of the difficulty involved in reproducibly blocking this step in vitro or the low relative abundance of second-step proteins. Nonetheless, there has been progress recently in isolating such proteins in mammals.

Early biochemical experiments yielded two fractions that contained essential second-step activities. These were termed SF3 and SF4a (Krainer & Maniatis, 1985). SF3, a heat-labile activity, was later shown to function at or prior to an ATP-dependent step, making it analogous to Prp16 and Prp17 (Sawa et al., 1988). No further characterization of SF3 or SF4a has been reported. A fraction termed Ia, which also contains an essential second-step activity, was independently isolated (Perkins et al., 1986). A more recent fractionation experiment has identified at least one other factor that appears to be distinct from SF3. The purest preparation of this fraction, the flow-through from a CM-sepharose column (CMFT), was shown to act only at the second step of splicing and to function after the requirement for ATP. Thus, this activity might be analogous to Slu7 or Prp18 (Lindsey et al., 1995).

An alternative approach for identifying putative second-step factors in mammals has been to purify large quantities of spliceosomes that are blocked for step two. This block is achieved by using a mutant intron that is missing the AG dinucleotide at the 3′ splice site. The proteins associated with this substrate are ex-

pected to be enriched for second-step factors (Gozani et al., 1994). Indeed, at least one mammalian second-step protein, PSF (see below), is present in these spliceosomes, along with 14 novel proteins. It is not yet known at which steps of splicing the novel proteins act.

To date, only a single identified mammalian protein, PSF, has been shown to play a role in step two. PSF (*P*TB associated *s*plicing *f*actor) is a pyrimidine tract binding protein that is found complexed with another pyrimidine tract binding protein, PTB (Garcia-Blanco et al., 1989; Patton et al., 1993). PTB does not appear essential for splicing in vitro. Although originally characterized as a first-step splicing factor, depletion of PSF also results in lariat intermediate accumulation. The second-step block can be complemented with purified recombinant PSF (Gozani et al., 1994). The ATP requirement of PSF during the second step has not been reported.

A different line of experimentation has yielded the interesting conclusion that protein dephosphorylation also plays a role in the second step of splicing. It was found that okadaic acid, an inhibitor of type 2A (and to a lesser extent type 1) protein phosphatases, can specifically block the second step of splicing (Mermoud et al., 1992; Tazi et al., 1992). This result correlates with the finding that ATPγS also inhibits the second step, presumably by rendering a target protein phosphatase resistant (Tazi et al., 1992). Thus, it appears that one or more proteins must be dephosphorylated prior to the second catalytic step. These two inhibitors should prove useful for developing assays to identify the relevant phosphatase and its substrate(s).

MECHANISMS OF 3′ SPLICE SITE SELECTION

Some aspects of the mechanism of 3′ splice site selection in yeast and mammals are worth comparison. It should be noted that for both systems, part of 3′ splice site choice is dictated by the site of branch lariat formation during the first catalytic step. Here we will focus only on the mechanism of 3′ splice site localization after lariat intermediate formation.

Using oligonucleotide-directed RNAse H cleavage as an assay, 3′ splice site protection has been probed in both systems. In yeast, the 3′ splice site is accessible to cleavage after the first catalytic step, but becomes protected in an ATP- and Prp16-dependent reaction (see above). This protection is correlated with strong cross-linking of Slu7 and Prp8 to the 3′ splice site. In mammals, the 3′ splice site is also accessible to cleavage after the first catalytic step, but can be converted to a protected form with the addition of two fractions necessary for step two. However, this protection occurs in the absence of ATP. Subsequent addition of ATP to these spliceosomes is sufficient to allow the second step to proceed (Sawa & Shimura, 1991). The difference in ATP requirements may be due to the nature of the assay systems. The yeast experiments were in Prp16-depleted extracts and utilized a mutant 3′ splice site, whereas the mammalian experiments were performed with spliceosomes immobilized on a solid support. In both systems, the 3′ splice site undergoes at least one conformational change, from unprotected to protected, prior to the second catalytic step. As suggested above, the protection might arise from direct interaction of the 3′ splice site with its spliceosomal binding site. The 3′ splice site protection patterns seen in yeast and mammals are shown in Figure 6.

A difficult issue has been the mechanism of 3′ splice site localization in yeast and mammals after the first catalytic step. As mentioned above, there is evidence for a 5′ to 3′ scanning mechanism in mammals that chooses the first AG dinucleotide downstream of the branch site. The scanning "rule" is broken only in exceptional circumstances where the first AG is either very close to the branch site (and probably sterically occluded) or directly adjacent to a downstream AG (Smith et al., 1989, 1993). In yeast, despite an inherent preference for branch site-proximal 3′ splice sites, several lines of evidence argue against a simple scanning mechanism (Langford et al., 1984; Patterson & Guthrie, 1991). First, even in relatively poor sequence context (i.e., preceded by purines, not pyrimidines), a downstream AG can be somewhat competitive with an upstream AG (Patterson & Guthrie, 1991). Second, interposing secondary structures can be accommodated by the yeast splicing machinery (Halfter & Gallwitz, 1988; Deshler & Rossi, 1991). Finally, the sequence context of a downstream splice site in yeast can affect utilization of an upstream competitor (Patterson & Guthrie, 1991). Even if a "leaky" scanning mechanism were invoked, it would not explain the ability of a downstream splice site to be utilized at the expense of an upstream competitor.

How can these observations be resolved? First, it should be noted that, besides the difference in organism, the yeast data are based on in vivo observations, whereas the mammalian data are from in vitro experiments. A recent attempt to look at competition between 3′ splice sites in yeast extracts yielded the result that only branch site-proximal AG dinucleotides were utilized (Teigelkamp et al., 1995a). Thus, in vitro 3′ splice site selection may involve different dynamics than the in vivo reaction. Competition between AGs has been observed in mammalian extracts in apparent violation of the abovementioned scanning rules (Reed, 1989). This experiment utilized a different splicing substrate than that used to infer the scanning mechanism. Therefore, the particular sequence context of the substrate or the splicing extract preparation may affect whether or not scanning takes place. In both yeast and mammals, branch site proximal 3′ splice sites are favored, suggesting that if scanning is not used, then a

diffusion-collision mechanism is employed. That is, 3′ splice sites that are closer to the branch site would have a higher rate of interaction with the spliceosome and would be used more frequently than distal 3′ splice sites. In summary, the two systems may utilize both scanning and diffusion-collision mechanisms for 3′ splice site localization.

CONCLUSIONS

A growing amount of information is now available on proteins and RNAs that contribute to the second step of splicing. Besides their predicted role in modulating RNA rearrangements, second-step proteins also mediate splice site selection and may have a role in forming the spliceosomal active site. Our focus must now turn to questions regarding details of how these proteins interact with each other and with RNAs required for the second step. For example, do the second-step proteins interact physically to form a complex? Do Prp16 and Prp8 bind active-site snRNAs? If so, what are the binding kinetics? With the answers to these questions comes the the exciting prospect of integrating proteins into the ever more intricate network of RNA–RNA interactions that is formed during the second catalytic step.

Even without information on proteins, characterization of spliceosomal RNAs is bringing us tantalizingly close to a possible active-site model for step two involving RNA (Fig. 4). Yet, one largely missing piece of information from this network is the set of interactions responsible for binding the 3′ splice site itself. The data available are consistent with YAG recognition taking place within the step-two active site (as opposed to a binding site distinct from the site of catalysis). As already evidenced by the aforementioned G-G interaction, YAG recognition may be complicated, involving multiple noncanonical interactions between the 3′ splice site and active site RNAs. However, determining the nature of this binding site could be especially rewarding. The additional spatial constraints that this information would add to current models may allow us to start thinking on a new level about the three-dimensional architecture of the active site and about possible catalytic mechanisms.

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