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RNA-RNA Interactions in Nuclear Pre-mRNA Splicing

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The precise removal of intervening sequences from nuclear pre-mRNA is catalyzed within massive, remarkably complicated, ribonucleoprotein complexes known as spliceosomes. Although significant progress has been made in understanding the mechanism of splicing, many fundamental aspects of the process remain poorly or marginally understood; indeed, the entire cast of required spliceosomal constituents has yet to be identified. Furthermore, despite intensive effort in a variety of systems, it is not entirely clear how splice sites are selected, the mechanism of catalysis is not known, and the nature of the catalytic entity(s) itself (RNA or protein) has not been established.

Several aspects of pre-mRNA splicing have been extensively reviewed in recent years, and the reader is referred to these sources for additional literature citations and alternative perspectives (Guthrie 1994; Madhani and Guthrie 1994a; Newman 1994; Nilsen 1994; Ares and Weiser 1995; Krämer 1995, 1996; Umen and Guthrie 1995). In this chapter, I summarize our current understanding of the role of U snRNAs in pre-mRNA splicing, concentrating on research published subsequent to the comprehensive review by Sharp and co-workers in 1993 in the *RNA World* (Moore et al. 1993). Particular emphasis is given to (1) the puzzling plasticity of RNA–RNA interactions required for splicing, (2) the remarkable discovery of a second spliceosome in mammalian cells, (3) the long-standing question of whether nuclear pre-mRNA splicing is evolutionarily related to the mechanistically analogous splicing of Group II introns, and (4) the case for RNA catalysis.

RNA–RNA INTERACTIONS IN THE SPLICEOSOME: A CONSENSUS VIEW

The First Catalytic Step

Several years ago, a coherent (if sketchy) view of snRNA–snRNA, as well as snRNA–pre-mRNA, interactions required for splicing of an

"ideal" intron emerged from combined genetic analysis in yeasts (both budding and fission) and biochemical analysis in a variety of systems (Fig. 1). Briefly, spliceosome assembly is initiated by the binding of U1 snRNP (via base-pairing) to consensus intronic nucleotides at the 5′ splice site. This early event is ATP-independent and "commits" the pre-mRNA to the splicing pathway. Subsequently, U2 snRNP engages the

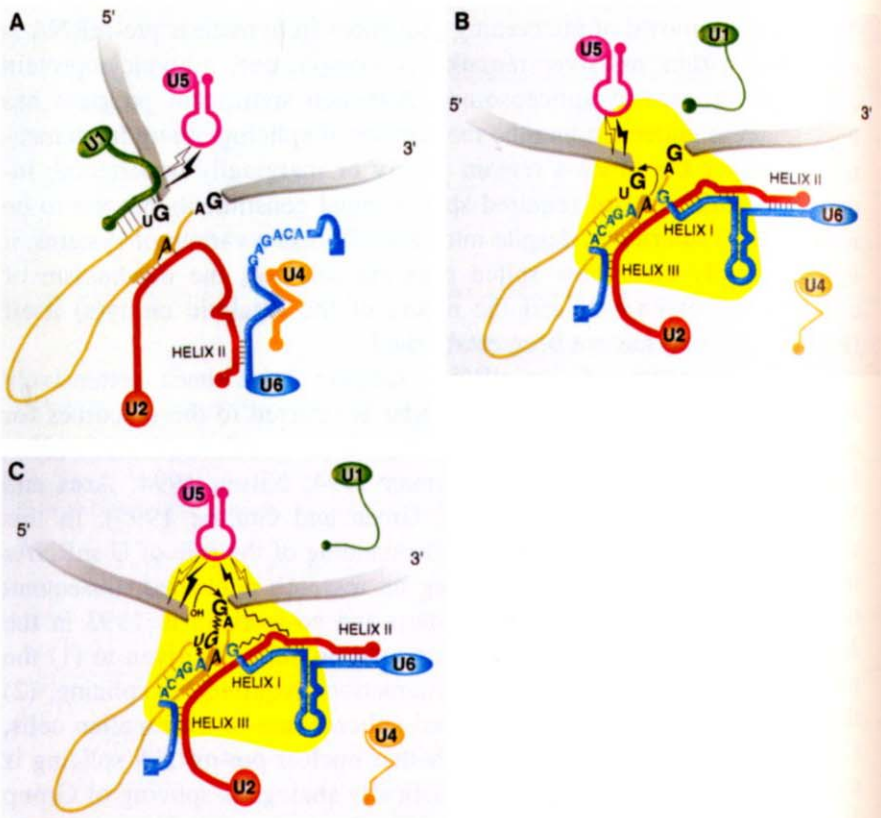

Figure 1 RNA–RNA interactions in pre-mRNA splicing. Three stages in spliceosome formation and catalysis are schematically depicted: (*A*) The fully assembled spliceosome prior to snRNA rearrangement. (*B*) The precatalytic spliceosome following snRNA conformational rearrangements. (*C*) The spliceosome following the first transesterification reaction just prior to execution of the second step. The putative catalytic center is enveloped in yellow. Watson-Crick base pairs are indicated by dashes. Non-Watson-Crick interactions are depicted as wavy lines. The contacts between U5 snRNA and exons (which may or may not be Watson-Crick) are indicated by lightning bolts. U snRNAs are designated at their 3′ ends. For detailed discussion, see text.

pre-mRNA by base-pairing to the branch point sequence. Once U1 and U2 are bound to the pre-mRNA, U4, U5, and U6 snRNPs join the spliceosome as a preformed tri-snRNP. It now seems clear that formation of the tri-snRNP is an obligatory prerequisite for association of these snRNAs with the pre-mRNA because depletion of tri-snRNP-specific proteins inhibits splicing at a stage prior to assembly of the complete spliceosome (for recent discussion, see Fetzer et al. 1997). Entry of the triple snRNP is still poorly understood, but it may be mediated through protein–protein interactions and/or a base-pairing interaction between the 5′ end of U2 snRNA and the 3′ end of U6 snRNA (helix 2, Fig. 1) (Datta and Weiner 1991; Wolff and Bindereif 1992; Roscigno and Garcia-Blanco 1995; Sun and Manley 1995). Photocrosslinking experiments indicate that, on entry into the spliceosome, U5 snRNP is positioned such that its invariant loop is tightly apposed to exon sequence just upstream of the 5′ splice site (Sontheimer and Steitz 1993; Wyatt et al. 1993; Newman et al. 1995). In sum, at least three regions of the pre-mRNA in the precatalytic spliceosome are contacted by snRNAs: exon and intron nucleotides at the 5′ splice site and the branch point. Notably absent among these interactions is contact with the 3′ splice site. Indeed, the mechanism and timing of 3′ splice site recognition is still, for the most part, mysterious.

In this context, it may be relevant that introns in mammalian cells have been divided into two classes, AG-dependent and AG-independent (where AG is the highly conserved dinucleotide at the 3′ splice junction) (Reed 1989). In AG-independent introns, cleavage at the 5′ splice site does not depend on the integrity of the AG dinucleotide; AG-dependent introns, on the other hand, do not assemble functional spliceosomes if the AG dinucleotide is mutated (Reed 1989). The existence of AG-dependent introns indicates that recognition of the 3′ splice site can be a critical determinant for spliceosome formation. To date, biochemical approaches have not definitively revealed the AG recognition factor; early experiments suggested that a protein(s) may fulfill this role (for review, see Steitz et al. 1988), and more recently, site-specific photocrosslinking has provided evidence for sequential recognition of the AG by two distinct proteins (Chiara et al. 1996); these proteins await further characterization. It is still not clear whether an RNA is also involved (but see Chabot et al. 1985). Genetic strategies available in budding yeast are not useful for addressing the question of RNA involvement in early AG recognition, because all known introns in *Saccharomyces cerevisiae* are AG-independent. It seemed that fission yeast (where all introns appear to be AG-dependent) had provided an attractive scenario to explain early

recognition of the AG dinucleotide by a spliceosomal RNA. Here, genetic suppression experiments suggested a model in which the first step of splicing of an AG-dependent intron is rescued by base-pairing between the 3′ splice site and U1 snRNA (Reich et al. 1992). However, this approach has not been extended to AG-dependent mammalian introns and, moreover, restoring pairing to U1 does not rescue all AG-dependent introns in *Schizosaccharomyces pombe* (J.A. Wise, pers. comm.). Furthermore, the demonstration that 3′ splice site recognition in *S. cerevisiae* does not require base pairing with U1 snRNA (Séraphin and Kandels-Lewis 1993) and the fact that some mammalian AG-dependent introns can be efficiently spliced in the absence of U1 snRNP (Crispino et al. 1996) indicates that if 3′ splice site recognition is mediated, at least in part, by RNA–RNA interactions, they have yet to be discovered.

Once the spliceosome is fully assembled, a dramatic rearrangement of its snRNA constituents occurs. Although the nature and extent of the rearrangements have been extensively documented (Madhani and Guthrie 1994a; Nilsen 1994; Ares and Weiser 1995), an obligatory order of events has not been rigorously established. I first list the rearrangements and then suggest a tentative order for their occurrence. The base-pairing interaction (~20 bp) between U4 and U6 is dissolved and U6 establishes new base-pairing contacts, both with itself (the intramolecular stem) and with U2 (helices 1 and 3) (Madhani and Guthrie 1992; Sun and Manley 1995). Both the intramolecular stem-loop of U6 and the helix 1 interaction involve regions of U6 originally base-paired to U4; the bases of U6 that participate in the helix 3 interaction are thought to be single-stranded in the U4/U6 particle. In addition to these new contacts, U6 also makes contact with the pre-mRNA via base-pairing. This pairing occurs between U6 and a subset of the same nucleotides originally recognized by U1 snRNA (Wassarman and Steitz 1992; Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993).

Because the U6/pre-mRNA interaction is (at least in part) mutually exclusive with the U1/pre-mRNA interaction, U1 must be displaced from the 5′ splice site prior to or concomitant with U6 pairing (for discussion, see Konforti et al. 1993). Accompanying the rather dramatic remodeling of U6 snRNA's intra-and intermolecular interactions is a modest adjustment in the positioning of U5; site-specific cross-linking in mammalian and yeast cell extracts indicates that the invariant loop of U5 becomes tightly associated with exon sequence immediately upstream of the 5′ splice cleavage site (Sontheimer and Steitz 1993; Newman et al. 1995).

The net effect of the rearrangements preceding the first catalytic step is to juxtapose (at least in two dimensions) the nucleophile for the first step (the 2′ hydroxyl of an adenosine bulged from the branch point/U2 helix) and the scissile phosphodiester bond at the 5′ splice site. Furthermore, the remodeling of the precatalytic spliceosome must in some way position these reactive groups within the catalytic site for the first transesterification reaction. Whether the snRNAs left in the spliceosome (U2, U6, and U5) participate in catalysis per se is discussed below.

We do not know what triggers or catalyzes the RNA–RNA rearrangements that precede the first step of splicing; however, the order of some steps can be inferred. First, it seems likely that U1 displacement from the 5′ splice site is an early event. It has long been known that U1 is only tenuously associated with fully formed spliceosomes (for review, see Moore et al. 1993). More recently, it has been shown that model substrates (RNA oligoribonucleotides corresponding to a short 5′ exon and 5′ splice site) and certain pre-mRNAs engage U6 directly via base-pairing (Crispino and Sharp 1995; Konforti and Konarska 1995). Indeed, the presence of a functional U1 snRNP is inhibitory to spliceosome formation on these model oligoribonucleotides (Konforti et al. 1993; Konforti and Konarska 1995). Other experiments also indicate that pairing between U6 and the 5′ splice site can occur prior to the unwinding of U4 and U6 (for recent discussion, see Li and Brow 1996). How U1 is displaced from "normal" substrates is less clear. A possible hint has come from experiments carried out by Ast and Weiner (1996, 1997). Using cross-linking, they have observed an intimate association between the U5 and U1 snRNPs and have suggested that interactions between U5 and the pre-mRNA may facilitate destabilization of the U1 pre-mRNA interaction (Ast and Weiner 1997). Intriguingly, these same investigators have also observed a novel multi-snRNP complex containing U1, U4, and U5 snRNPs. Formation of this complex can be induced by a 2′Ome oligonucleotide complementary to U5, but the complex is also observed in the absence of the oligonucleotide, and its presence correlates with splicing (Ast and Weiner 1996). These observations in aggregate suggest that the U5 snRNP may in some way help to destabilize U4 and U1 within the precatalytic spliceosome. Although this is an attractive notion, it raises an interesting major question; i.e., if U5 snRNP is an entrenched constituent of the active spliceosome (see above), how can it be found in a distinct complex that lacks U2 and U6 snRNAs? Perhaps the U1/U4/U5 complex reflects a transient intermediate in snRNA organization prior to commitment to catalysis. An alternative, if heretical, explanation for the complex would be to posit that more than one U5 snRNP participates in splicing. At this time, there is no obvious way to exclude this possibility. Moreover, although it is generally assumed that

the U snRNAs are each present in a single copy in all spliceosomes, this has not been rigorously proven; indeed, demonstrating the stoichiometry of any splicing factor, especially those that may only transiently engage the spliceosome, is extremely difficult. Uncomfortable as it may be, a lack of one-to-one stoichiometry, even among the U snRNPs, cannot be dismissed out of hand.

Returning to the precatalytic spliceosome (and assuming stoichiometric participation of the U snRNPs), it is not clear how U4 is displaced from U6. In this regard, it has long been known that a number of RNA-dependent ATPases bearing striking similarities to proteins with demonstrable RNA helicase activity are required for splicing (for review, see Wassarman and Steitz 1991; Burgess and Guthrie 1993a). The existence of these factors has led to the notion that they might directly catalyze RNA rearrangements within the spliceosome. However, despite much effort, none of these proteins has been shown to possess helicase activity. It is possible that the definitive substrates for the putative helicases exist only in the context of the spliceosome itself. Alternatively, the RNA-dependent ATPases may have "proofreading" functions independent of any direct role in catalyzing RNA–RNA rearrangements (see below), or they may expend energy to keep the RNA–RNA interactions "fluid" within the spliceosome until the most stable subset of helices is formed or "trapped" by additional protein factors (see below). In the latter case, the actual RNA–RNA rearrangements may be driven by the RNAs themselves. Indeed, the feasibility of such RNA-driven reactions involving snRNAs has been recently demonstrated in vitro (Brow and Vidaver 1995).

Although the gross nature of remodeling of the spliceosome prior to the first step has been established, we do not know what triggers catalysis or how the scissile bond at the 5′ splice site is identified. It is clear that release of U4 is not sufficient to initiate the catalytic process (Yean and Lin 1991; Kim and Lin 1996). This fact was established in budding yeast carrying a temperature-sensitive allelle for a specific RNA-dependent ATPase. Spliceosomes demonstrably devoid of U4 are not active but will go on to splice upon addition of the ATPase and an additional (as yet not fully characterized) protein factor (Kim and Lin 1996). It will be of significant interest to determine whether these proteins induce structural changes within the U snRNAs resident in the spliceosome.

The extensive literature on identification of the scissile bond at the 5′ splice junction contains many unresolved inconsistencies. Under certain conditions (i.e., 5′ splice sites mutant in the GU dinucleotide), the invariant loop of U5, presumably in collaboration with other factors, unambiguously has a hierarchical role in determining the site of cleavage (Newman and Norman 1991; also see Cortes et al. 1993). Thus, it was generally assumed that U5 would play an analogous role in normal splicing. Remarkably, however, Newman and colleagues recently showed that accurate 5′ splice site cleavage occurs in the absence of the U5 loop (O'Keefe et al. 1996). It will be interesting to see whether and where cleavage occurs in a GU-mutant pre-mRNA in reactions reconstituted with U5 snRNAs lacking the invariant loop. An integral protein of the U5 snRNP (Prp8) has also been implicated in 5′ splice site recognition; the protein can be cross-linked to the GU dinucleotide, and cross-linking is perturbed by mutations in the dinucleotide (Reyes et al. 1996). However, accurate cleavage still occurs in pre-mRNAs with mutations in the 5′ splice site dinucleotide predicted to severely disrupt this interaction (Aebi et al. 1986, 1987). If the U5 loop is not necessary for 5′ splice site identification, what is? An obvious candidate is U6 snRNA; however, for some introns, the potential base-pairing between U6 and the 5′ splice site can be totally disrupted, yet accurate cleavage occurs (Yu et al. 1993). The situation is further complicated by the lack of a unifying model for activation of cryptic 5′ splice sites in mammalian cells. In many cases, mutation of authentic splice sites leads to aberrant cleavage at sites that bear no obvious resemblance to bona fide splice sites. Although these phenomena have not been rigorously investigated, the activation of at least some cryptic sites does not appear to correlate with known RNA–RNA interactions.

Finally, in SL RNA-mediated *trans*-splicing (see below), a specific 4-base block substitution mutation in U6 snRNA results in a remarkable phenotype; that is, the authentic 5′ splice site is ignored and the branch-point adenosine attacks U6 itself (Yu et al. 1993). The region of U6 altered in this mutant had not previously been shown to be involved in 5′ splice site identification (but see Sawa and Abelson 1992). Are all of these disparate phenomena related, and what do they tell us about the first catalytic step? In general, it seems obvious that there are no hard and fast sequence requirements within pre-mRNAs that specify the site for nucleophilic attack by the branch point. Second, once catalytic activation has occurred, the spliceosome is remarkably versatile in carrying out the first step. A facile and convenient rationalization of these findings is to assume that, in naturally occurring introns, multiple (perhaps redundant) known functions are sufficient to specify the correct 5′ splice cleavage site. However, this fails to explain some of the reactions observed to date, and it thus seems more probable that additional interactions

(presumably non-Watson-Crick) remain to be discovered. In this regard, it is well established that 5′ splice site recognition in Group I introns requires multiple tertiary interactions, and the effect of mutations remote from the splice site itself can have rather dramatic effects on splice site positioning (for discussion, see Downs and Cech 1994). The positioning of the 5′ splice site in Group II introns with respect to the catalytic center is currently not well understood (for review, see Michel and Ferat 1995).

The Second Catalytic Step

The foregoing discussion indicates that there is much to be learned about RNA–RNA interactions involved in the first step of splicing. The second step is perhaps better understood (for review, see Umen and Guthrie 1995), but even here many questions remain. A prerequisite to the second step is a conformational change in the spliceosome. This dynamic reorganization has been revealed by a variety of techniques, including pre-mRNA accessibility to oligonucleotides and changes in cross-linking patterns (for review, see Umen and Guthrie 1995). Notably, the changes discovered to date are quite subtle in comparison to the extensive shifts in pairing that accompany catalytic activation. Moreover, it seems unlikely that major base-pairing rearrangements accompany the transition to the second step, since such rearrangements would have been detected by now, and many interactions established prior to the first transesterification persist through the second. It is clear that, following the first step, the conformation of U5 changes such that the invariant loop makes a new contact with exon sequence immediately downstream from the 3′ splice site (Sontheimer and Steitz 1993; Newman et al. 1995). Mutationally sensitive nucleotides in U2 can also be cross-linked to the pre-mRNA near the 3′ splice site and the (nearly) invariant U of the 5′ splice site GU becomes closely apposed to a highly conserved nucleotide in U6 snRNA (Sontheimer and Steitz 1993; Newman et al. 1995; Kim and Abelson 1996). Finally, the bulged dinucleotide of U2 in the U2/U6 helix I interaction region interacts via tertiary contacts with an invariant sequence in U6 (Fig. 1c) (Madhani and Guthrie 1994b).

In the second step spliceosome, the free 3′ hydroxyl of the 5′ exon must be positioned to attack the 3′ splice site. On the basis of numerous lines of evidence, it was thought that contacts made between the invariant loop of U5 snRNA and the 5′ exon prior to the first step (see above) would be necessary for 5′ exon anchoring and perhaps positioning (Newman and Norman 1991; Sontheimer and Steitz 1993; Wyatt et al. 1993). This appears to be true, since loopless U5 (although it allows the first step to proceed, see above) does not support the second step (O'Keefe et al. 1996), and indeed, the association of the 5′ exon with spliceosomes formed with such U5 snRNPs appears to be labilized (O'Keefe et al. 1996).

Although the pre-mRNA and snRNA nucleotide sequence requirements for the first step of splicing are surprisingly loose (see above), it has been known for some time that the requirements for the second step appear to be considerably more stringent. Efficient execution of the second catalytic step depends on a number of elements in the pre-mRNA; minimally, the sequence (GY) of the 5′ splice site dinucleotide, the branch point nucleotide (A), and the trinucleotide YAG that comprises the 3′ splice site. In addition to those snRNA sequences required for the first step, the second catalytic step requires the invariant loop of U5, certain nucleotides and backbone phosphate oxygens in U6 snRNA, and specific nucleotides in U2 snRNA (Fabrizio and Abelson 1990, 1992; Madhani et al. 1990; McPheeters and Abelson 1992; Yu et al. 1995; O'Keefe et al. 1996). The second step sequence requirements within the pre-mRNA have yet to be adequately explained. For example, we do not know why a pyrimidine is required at position two of the intron. As noted above, this nucleotide becomes tightly associated with a highly conserved sequence in U6 (Sontheimer and Steitz 1993; Kim and Abelson 1996) after the first step; however, it has not been determined whether the same contact occurs if the plus-two pyrimidine is changed to a purine. It also is possible that this requirement reflects its contact with Prp8 (Reyes et al. 1996).

It does seem clear that the requirement for a guanosine at position plus one reflects a non-Watson-Crick hydrogen-bonding interaction between the first and last nucleotides of the intron. As discussed by Parker and Siliciano (1993), the two terminal guanosines can engage in a non-Watson-Crick interaction that is nearly isosteric with a specific adenosine cytosine conformation. Indeed, these workers demonstrated that the effect of an adenosine substitution at position one could be partially suppressed by co-substitution of the 3′-terminal guanosine with cytosine. Subsequently, several lines of experimentation, as well as the occurrence of natural introns with A-C terminal nucleotides (see below), have provided strong support for the necessity of "compatible" intronic termini. Although the requirement seems clear, an obvious mechanistic rationale for it is not. At one extreme, the noncanonical interaction be-

tween terminal nucleotides might contribute positively (i.e., by providing functional groups) to second-step catalysis. At the other extreme, these combinations of nucleotides may be the only ones tolerated by the active site of the spliceosome; other combinations would then be sterically excluded. In between, appropriate combinations of nucleotides may confer a kinetic advantage to the conformational rearrangement of the spliceosome between the first and second steps, or they may be the only ones that can pass a "proofreading" test (see below).

Regardless of its mechanistic basis, the proposed noncanonical interaction between intronic termini is not an absolute requirement for the second step. In fact, mutations at the 3′ splice site (in the presence of a wild-type 5′ splice site) can be suppressed to varying degrees by changes in U6 (Lesser and Guthrie 1993), or by a combination of changes in U6 and U2 (Madhani and Guthrie 1994b). Perhaps most surprisingly, alterations in the invariant loop of U5 snRNA that improve the complementarity of U5 with 3′ *exon* nucleotides downstream from the 3′ splice site (Newman and Norman 1992) also suppress 3′ splice site mutations. Because we lack a detailed picture of the active site(s), it is impossible to unambiguously interpret the suppression phenomena in a mechanistic way. Furthermore, as discussed above for the interaction between intron termini, suppression of 3′ splice site alterations may reflect a variety of underlying causes.

In this regard, studies of branch site suppression are particularly illuminating. Some time ago, Guthrie and colleagues convincingly showed that an A to C alteration in the branch point adenosine (a mutation that results in a second step block) can be suppressed by a mutation in one of the spliceosomal RNA-dependent ATPases (see above) (Burgess and Guthrie 1993b). Surprisingly, the suppressor allele of Prp16 (the ATPase) interfered with shunting of two-thirds intermediates containing C-G branches into a discard pathway. Strikingly, the suppressor allele was debilitated in its ATPase activity (Burgess and Guthrie 1993b). As discussed by these authors, the above results provide strong evidence that at least one of the spliceosomal RNA-dependent ATPases actively proofreads the structures formed in the spliceosome (Burgess and Guthrie 1993a,b). It will be of obvious interest to determine the phenotypes of analogous mutations introduced into the other "helicase homologs" known to be necessary for splicing. Furthermore, the dissection of branch point suppression clearly demonstrates that suppressor phenomena must be interpreted with caution, or at least with an open mind. Even when "direct" effects are observed (i.e., compensatory base substitutions, etc.), it is impossible to exclude kinetic considerations or

subtle changes in elements recognized by proofreading activities. Such considerations may help to explain the multiplicity of alterations that demonstrably influence 3′ splice site utilization and other examples of branch point suppression (see above and McPheeters 1996).

Finally, we do not know in detail how the 3′ splice site itself is recognized for the second catalytic step. Recent experiments by Anderson and Moore (1997) may help to resolve this long-standing problem. Briefly, these investigators have studied a truncated AG-independent pre-mRNA lacking a 3′ splice site which, as discussed above, can undergo the first step of splicing, but obviously cannot proceed through the second step. Remarkably, if a 3′ exon and its accompanying 3′ splice site are added in *trans* subsequent to incubation of the 5′ half-molecule (which presumably has completed the first step), correct exon ligation is observed. Strikingly, the sequences required within the 3′ half molecule are minimal: Any RNA with a 5′ CAG appears to serve as a 3′ exon in these reactions. Moreover, if the 5′-most CAG is mutated, the next available AG is selected, suggesting a "scanning" model of 3′ splice site selection (but see Umen and Guthrie 1996; and Luukkonen and Séraphin 1997). Although it remains to be shown how these 3′ half-molecules are recruited to the spliceosome, the results suggest the provocative notion that a spliceosome that has undergone the first step is "primed" to accept the first CAG/exon it encounters (for further discussion, see Anderson and Moore 1997). It will be of considerable interest to find out how closely the requirements (snRNA and pre-mRNA) for this peculiar reaction parallel those of "normal" splicing; regardless, this model system will undoubtedly provide insight into the recognition and use of 3′ splice sites in much the same way as studies using oligoribonucleotides containing a 5′ splice site have contributed to a "minimalist" understanding of 5′ splice recognition and use (Konforti et al. 1993; Konforti and Konarska 1994, 1995; Ghetti and Abelson 1995; Reyes et al. 1996).

In addition to pre-mRNA constraints, certain sequences and/or backbone positions in U2, U6, and U5 are required for the second step. Because these observations are generally invoked in the context of the RNA catalysis question, they are dealt with in more detail below. However, it is worth noting that interpretation of second-step effects elicited by altering snRNAs is subject to the same litany of ambiguities listed above; i.e., are the effects solely kinetic, do they affect recognition by protein factors, do they produce inappropriate conformations, or do they retard conformational changes? The answers to these and related questions await a much more thorough understanding of the snRNA configurations in the wild-type spliceosome.

RNA-RNA Interactions Idiosyncratic to SL-addition *trans*-Splicing

As discussed above, a variety of experimental approaches have been used to dissect the *cis*-splicing process. One useful approach (with many variations, see above) has been to study reactions in which the pre-mRNA is experimentally divided into half-molecules. Under appropriate conditions, the exons of such half-molecules are efficiently joined by the splicing machinery. Although it has been possible to systematically analyze the contributions of 5′ and 3′ halves (see above; for further discussion, see Bruzik and Maniatis 1995; Chiara and Reed 1995), none of these bimolecular *trans*-splicing reactions is known to occur in nature.

Certain organisms do process their nuclear pre-mRNAs by snRNP-mediated *trans*-splicing. In these organisms (kinetoplastid protozoa, nematodes, euglenoids, and certain trematodes) (Blumenthal 1995; Davis 1996; for recent review, see Nilsen 1997), the 5′ exon (the spliced leader) is delivered to the spliceosome in the form of a *trans*-splicing-specific snRNP, the SL RNP. Unlike the snRNPs, which are required cofactors for *cis*-splicing, the SL RNP is consumed during the *trans*-splicing reaction. In contrast to *cis*-splicing (of most introns), SL-addition *trans*-splicing does not require U1 snRNP, but does require the four other spliceosomal U snRNPs (Maroney et al. 1996). Extensive analysis, both in vivo in kinetoplasts, and in vitro in nematodes, has shown that snRNA–snRNA interactions required for *cis*-splicing are also required for *trans*-splicing (for review, see Nilsen 1997). Notably, however, the SL RNP in nematodes engages in an extensive base-pairing interaction with U6 snRNA (Hannon et al. 1992). This interaction is thought to facilitate association of the SL RNP with the U4/U5/U6 triple snRNP, thereby allowing the SL RNP to enter the *trans*-spliceosome as a preformed quadruple snRNP (for discussion, see Maroney et al. 1996). The aforementioned base-pairing interaction of the SL RNP and U6 involves sequences in U6 not known to be engaged in other pairing interactions within the *cis*-spliceosome. Thus, almost the entire length of U6 is engaged in Watson-Crick contacts at some point in the nematode *trans*-splicing pathway. Although numerous questions remain regarding the phylogenetic distribution of SL-addition *trans*-splicing and the mechanism of splice site selection in organisms that carry out this reaction (Nilsen 1997), the existence of a *trans*-splicing-specific snRNP raises interesting questions regarding the origin and evolution of the spliceosomal snRNAs. Furthermore, studies of SL-*trans*-splicing suggest that much of the spliceosome may be prefabricated prior to engaging the pre-mRNA (see above). It seems likely that an analogous situation may pertain to

cis-spliceosomes in vivo; i.e., precatalytic spliceosomes may well exist as preformed structures (see Konarska and Sharp 1987). If this were the case, the apparent stepwise formation of spliceosomes observed in vitro might reflect the reestablishment of contacts disrupted during extract preparation.

The Apparent Plasticity of U snRNAs

It is well established that certain regions of the spliceosomal U snRNAs are extremely well conserved in primary sequence as well as secondary structure. Furthermore, with the exception of budding yeast, the overall lengths of the spliceosomal RNAs are also well conserved. Certainly, some U snRNAs (most notably U6, see below) are more conserved than others, but in general, the sequence elements known to be involved in the RNA–RNA interactions portrayed in Figure 1 are nearly invariant. In this light, it is surprising that few, if any, nucleotides are absolutely required for splicing. Indeed, if one examines all of the mutational data available in all systems, it is difficult to define, by consensus, any specific interaction that must occur for splicing to take place. Do these observations indicate that the current view of RNA–RNA interactions is wrong (it is probably oversimplified, see above) or that phylogenetically conserved nucleotides are not important?

Before these questions can be answered in a definitive way, it is worth considering some of the experimental limitations inherent in the analysis of pre-mRNA splicing. First, the current picture of RNA–RNA interactions in the spliceosome is compiled from experiments performed in many organisms under a wide variety of assay conditions. Overall, the preceding discussion in this chapter has been intended to be organism-neutral. Perhaps this catholic approach is too cavalier since there has been much debate about whether snRNA requirements for splicing are distinct in various creatures, most notably yeast and mammalian cells (see, e.g., Datta and Weiner 1993; Sun and Manley 1995, 1997; Field and Friesen 1996; Yan and Ares 1996). Many examples could be cited, but the several proposed interactions between U6 and U2 are particularly notable. In this regard, the helix-2 interaction was thought to be required in mammalian cells but dispensable in yeast. Similarly, the helix-3 interaction has only been demonstrated for a single intron in mammalian cells, and has not received experimental support in *S. cerevisiae* (see references cited above). The potential to form each interaction is conserved in both mammals and yeast. Recently, it was demonstrated that

helix 2 can be required in yeast if the helix-1b interaction is perturbed via mutation (Field and Friesen 1996). It seems likely that under specific conditions (as yet unknown), the helix-3 interaction will also be important. My own view is that phylogenetic conservation of a potential interaction will be a more reliable predictor of importance than mutational analysis. From this perspective, the lack of a phenotype when highly conserved nucleotides are changed probably reflects the assay conditions rather than the unimportance of the nucleotides. In this regard, it should be noted that we simply do not know what is rate-limiting for splicing under most conditions. A dramatic example of how important it is to understand the nature of the rate-limiting step comes from studies with the *Tetrahymena* group I intron. Here, a deoxy substitution at the 5′ splice site has only a modest effect on overall splicing efficiency, yet the rate of the chemical step of the reaction is reduced by three orders of magnitude (for review, see Cech 1993). It will be quite some time before snRNA-mediated splicing is reduced to elemental rate constants, but only then will the results of mutational analyses be truly interpretable.

Furthermore, as noted above, much of our understanding of the biochemistry of pre-mRNA splicing has per force been derived from studies on an extraordinarily limited subset of introns. It seems likely that intron-specific requirements will account for some of the constraints imposed on those conserved regions of snRNAs that currently appear to be dispensable or mutationally flexible. A clear example of intron specificity comes from the recent demonstration that certain introns can be spliced in the absence of a functional U1 snRNP (Crispino et al. 1994, 1996; Tarn and Steitz 1994). However, it is not yet known whether such U1-independent splicing occurs in vivo (a hard experiment at best).

In addition, I have not dealt explicitly with the concept of "functionally redundant" interactions. As noted above, a requirement for helix 2 in yeast can be demonstrated only if helix 1b is compromised (Field and Friesen 1996). Similarly, an extension of the intramolecular stem of U6 can compensate for the lack of helix 1b for at least one mammalian intron (Sun and Manley 1995). Presumably, other interactions may be dispensable (or become important) in the presence of second or third site mutations. However, there are practical constraints on combinatorial mutagenesis in most experimental systems (but see Madhani and Guthrie 1994b), and thus it is unlikely that the absolute degree of functional redundancy within snRNA-snRNA interactions in the spliceosome will be determined soon.

Finally, the potential role of proteins in stabilizing (or compensating for) weakened RNA-RNA interactions cannot be ignored. Under certain conditions (e.g., introns optimized for efficient splicing) a multiplicity of weak protein-protein interactions might render some RNA-RNA interactions dispensible. Such considerations may serve to explain what appear to be anomalous results obtained in the *Xenopus* oocyte system. In this system, splicing activity can be restored to U2-depleted oocytes with U2 snRNAs lacking the entire helix-1 interaction region (Hamm et al. 1989). In the same system, U6 snRNAs altered in mutationally inflexible regions (in other organisms) restore splicing (Vankan et al. 1990).

In summary, although we know a great deal about RNA–RNA interactions in the spliceosome, major uncertainties still exist, and one must ask how reliable the current model is. Perhaps the best validation of this model comes from a completely unexpected source, the mechanism whereby noncanonical introns are processed in mammalian cells.

SPLICING OF AT-AC INTRONS: DISCOVERY OF A PARALLEL SPLICEOSOME

Clearly, the most stunning development in recent years has come from the recognition and characterization of noncanonical introns and the subsequent identification of the snRNPs required for their excision. It has been known for some time that certain pre-mRNA introns possess termini (AU-AC) that deviate from the GU-AG consensus (for review, see Tarn and Steitz 1997). Because a 5′ terminal A is (at least partially) compatible with a 3′ terminal C for conventional intron removal (Parker and Siliciano 1993), it was generally assumed that AU-AC introns were rare examples of naturally occurring mutants. However, as more examples of AU-AC introns were characterized, it became obvious that they share diagnostic sequence elements in addition to their terminal nucleotides. In particular, AU-AC introns have an extended and apparently rigid 5′ splice site sequence, as well as a conserved sequence a short distance upstream of the 3′ splice site. Padgett and colleagues compared these conserved elements with the known sequences of U snRNAs and found a striking complementarity between the AU-AC 5′ splice site and the 5′ end of U11 snRNA. In addition, they noted that a region of U12 snRNA could interact via base-pairing with the conserved sequence upstream of the AU-AC 3′ splice site (Hall and Padgett 1994). Provocatively, this potential interaction was predicted to bulge an adenosine residue in a manner analogous to the U2-branch point sequence interaction (Hall and Padgett 1994). U11 and U12 snRNAs had previously been characterized as low-abundance Sm snRNPs in the

Steitz laboratory (Montzka and Steitz 1988) and had no firmly established function (but see Gontarek et al. 1993). The possible pairings of U11 to the 5′ splice site and U12 to the putative branch point region of noncanonical introns led to the hypothesis that U11 and U12 function in a manner analogous to U1 and U2 in the splicing of conventional (GU-AG) introns (Hall and Padgett 1994). Although similarity between the predicted secondary structures of U11 with U1 and U12 with U2 had been noted (Montzka and Steitz 1988), the notion that alternative snRNAs could participate in splicing seemed farfetched. An onslaught of new results, however, has unequivocally proven that U11 and U12 are essential for AT-AC intron splicing. Even more surprisingly, these U snRNPs do not collaborate with standard spliceosomal U snRNAs in the splicing of AT-AC introns, but instead are part of a new spliceosome comprising four snRNAs distinct from those present in the canonical spliceosome; only U5 snRNP appears to be a common constituent of both spliceosomes (for review, see Nilsen 1996; Tarn and Steitz 1996a,b, 1997).

The evidence for a new spliceosome comes both from in vivo genetic suppression experiments and in vitro biochemical analysis. In vivo analysis has revealed a required Watson-Crick base-pairing interaction between U12 and the branch site, as well as pairing between U11 and the 5′ splice site (Hall and Padgett 1996; Kolossova and Padgett 1997). In vitro experiments have shown that excision of an AT-AC intron occurs in a complex analogous to the GU-AG spliceosome, and that AT-AC intron splicing proceeds through a two-step transesterification pathway identical to that in the GU-AG spliceosome (Tarn and Steitz 1996a). These in vitro analyses have also confirmed an essential role for U12 in AT-AC splicing, and most recently, U11 has been shown to cross-link to an AT-AC 5′ splice site (Yu and Steitz 1997). In early experiments, it seemed that neither U6 nor U4 was required for AT-AC excision, because targeted degradation of these snRNAs did not inhibit splicing. Affinity purification of AT-AC spliceosomes resolved this anomalous finding by revealing the presence of two novel snRNAs designated U4atac and U6atac RNAs. These new RNAs unambiguously carry out the respective roles of U4 and U6 in the splicing of canonical introns (Tarn and Steitz 1996b). The fact that both of these RNAs have heretofore escaped detection in HeLa cells (arguably the most intensively studied system in eukaryotic biology) is remarkable, and one has to wonder how many other functional RNAs remain uncharacterized.

After the fact, it is possible to reconcile the failure to observe U4atac and U6atac because they are of low abundance. Furthermore, at first glance they bear only passing resemblance to the GU-AG spliceosomal U4 and U6 snRNAs, and accordingly would never have been detected by hybridization. With knowledge of their function, however, the similarities of U4atac and U6atac to their major spliceosomal counterparts are just as striking as the differences. The predicted stability of the U4atac-U6atac complex is nearly identical to that of U4 and U6 (Tarn and Steitz 1996b). Furthermore, it is apparent that the U4atac–U6atac interaction must be dissolved prior to catalysis (Tarn and Steitz 1996b). The existence and required role of U4atac in an otherwise distinct spliceosome provides compelling evidence that U4-like function is necessary for nuclear pre-mRNA splicing.

U6atac itself is a remarkable molecule. At the primary sequence level, it is less similar to human U6 snRNA than budding yeast U6 is to human U6. Nevertheless, a comparison of U6 and U6atac reveals that the patches of sequence that are conserved between the two molecules correspond precisely to those regions of U6 that have been shown to be critical for its function (perhaps catalytic, see below) in canonical splicing. Furthermore, the presumptive secondary structure of U6atac within the AT-AC spliceosome is identical to that of U6 in the major spliceosome (Tarn and Steitz 1996b, 1997; also see Nilsen 1996).

Perhaps most relevant to our current view of RNA–RNA interactions involved in splicing are the interactions formed (some inferred, some proven) between the AT-AC spliceosomal snRNAs. Indeed, there appears to be a one-to-one correspondence between interactions formed in both spliceosomes despite the incredible degree of sequence variation between the RNAs involved (Fig. 2). Briefly, U11 engages the 5′ splice site via base-pairing and U12 pairs to the branch point (see above). Following entry of the AT-AC (presumptive) tri-snRNP, U11 is displaced, as is U4atac, and U6atac pairs to intronic nucleotides downstream from AU dinucleotide at the 5′ splice site (Tarn and Steitz 1996b; Kolossova and Padgett 1997; Yu and Steitz 1997). Most remarkably, the interactions formed between U6atac and U12 are congruent with those formed between U2 and U6. A pairing analogous to helix 1a and 1b has been demonstrated using psoralen cross-linking (Tarn and Steitz 1996b); helix 2 cannot form because U12 is truncated at its 5′ end relative to U2. Significantly, U12 and U6atac can also interact to form an analog of helix 3. The correspondence of RNA–RNA interactions in the AT-AC and GU-AG spliceosomes provides compelling validation of the current view of RNA–RNA interactions involved in snRNP-mediated pre-mRNA splicing and (at least in my mind) strengthens the case for RNA catalysis (see below).

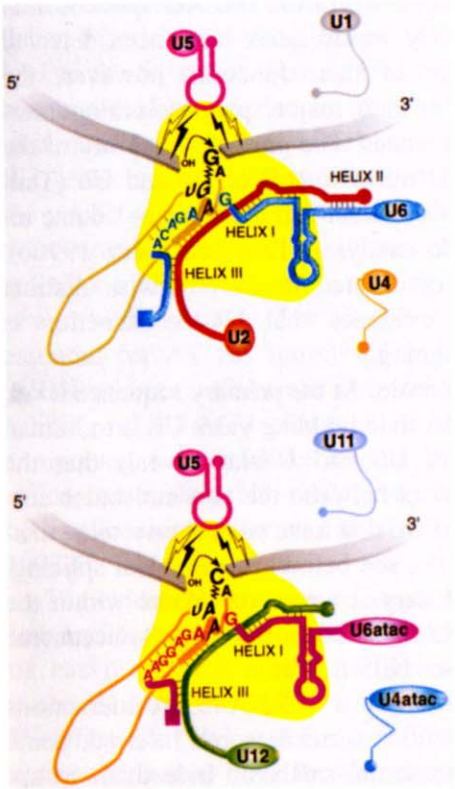

Figure 2 Schematic comparison of RNA–RNA interactions in GU-AG (*top*) and AT-AC (*bottom*) spliceosomes. Designations are as in Fig. 1. For discussion, see text.

Curiously, only U5 snRNP is common to both the AT-AC and GU-AG spliceosomes, although we do not yet know whether a specific variant (Sontheimer and Steitz 1992) of U5 is involved in AT-AC splicing. It is of obvious interest to determine if such a variant exists or, alternatively, how the same U5 snRNP can interact both with U4/U6 and with U4atac/U6atac. Furthermore, studies on the AT-AC spliceosome should provide some insight into tri-snRNP entry into the spliceosome in the absence of a helix-2-like interaction.

The existence of an alternative spliceosome raises a mind-boggling array of questions, only some of which are mentioned here. From a biochemical point of view, what proteins are required for AT-AC splicing? Although this question is certainly technically nontrivial (AT-AC spliceosomal components may be of quite low abundance), it seems particularly relevant. It is difficult to imagine that an entirely distinct array (perhaps a hundred or more) of proteins will participate in AT-AC splicing. If, as is perhaps more likely, common proteins play similar roles in both types of splicing, the AT-AC spliceosome will be invaluable in elucidating the substrates for protein factors.

Evolutionary questions are also obvious. Where did AT-AC introns and their splicing machinery come from? The existence of presumptive AT-AC introns in plants suggests an ancient origin (for review, see Tarn and Steitz 1997; also see Wu and Krainer 1997), but our current knowledge of the phylogenetic distribution of these introns is minimal. Unfortunately, there is as yet no evidence for AT-AC introns in *S. cerevisiae* (M. Ares, pers. comm.); whether they were once present and subsequently lost is unclear. It is also unclear whether the GU-AG and AT-AC spliceosomal RNAs evolved from a common ancestral splicing apparatus or arose independently. Speculation seems pointless at this time; however, the existence of two spliceosomes clearly impinges on the oft (and perhaps endlessly) discussed question of whether spliceosomal snRNAs descended from a primordial group II-like intron (see below).

Furthermore, just as a glimmer of hope emerged that splice site selection is at least in part understood (for discussion, see Horowitz and Krainer 1994; Berget 1995; Black 1995), we are confronted with another layer of complexity; i.e., how does the AT-AC spliceosome find the right exon/intron boundaries and why are these sites ignored by the GU-AG machinery? Already, there is evidence that the GU-AG and AT-AC spliceosomal machineries may interact in processing the same pre-mRNA, although this may not always be the case (Kohrman et al. 1996; Wu and Krainer 1996). Finally, as if things were not complicated enough, it is clear that certain introns with GU-AG termini are processed via the AT-AC pathway, and similarly, a subset of introns with AU-AC termini are processed via the GU-AG pathway (Wu and Krainer 1997; R. Padgett, pers. comm.).

THE RELATIONSHIP BETWEEN PRE-mRNA AND GROUP II SPLICING

It has been just over a decade since the demonstration that certain group II introns can excise themselves autocatalytically (for review, see Michel and Ferat 1995). The fundamental similarities in reaction pathways between group II autoexcision and pre-mRNA splicing immediately fueled speculation that the two processes were related evolutionarily (for review, see Moore et al. 1993; Guthrie 1994). In the intervening years,

this topic has received much attention and the arguments pro and con have been extensively documented (see, e.g., Weiner 1993; Wise 1993). At present, we simply do not know whether the relationship between the two splicing processes is meaningful in an evolutionary sense. This is an important question, but it may never have a satisfactory answer. In this regard, an unambiguous demonstration (should such exist) of evolutionary relatedness would go a long way toward proving the tacit assumption of many (or most) in the field that pre-mRNA splicing is an RNA-catalyzed reaction. However, it is important to note that the converse result (i.e., proof of unrelatedness) is not meaningful with regard to the mechanism of snRNP-mediated pre-mRNA catalysis. There is no doubt that the quest for commonality between the two types of splicing has been and will continue to be a driving force for many interesting experiments; however, such experiments should not be overinterpreted.

Recently, a provocative correspondence between the positions of backbone phosphates in U6 snRNA required for pre-mRNA splicing and those phosphates within domain 5 required for group II splicing was noted (Yu et al. 1995). In addition, a similarity between the phenotypes of mutations within highly conserved nucleotides in U6 and the phenotypes of mutations in possibly related nucleotides in domain 5 has been documented (i.e., Boulanger et al. 1995; Peebles et al. 1995). These observations led to the speculation that the intramolecular stem of U6 may be functionally analogous (homologous?) to domain 5. Although this is an interesting notion, the evidence is far from convincing. We do not know the "real" conformation of U6 within the spliceosome, and mutational phenotypes are subject to numerous interpretations (see above). In the same light, it was recently shown that human U5 snRNA could substitute in *trans* for a subdomain of a specific group II intron (Hetzer et al. 1997). As discussed recently by Newman (1997), the functional parallels between U5 in pre-mRNA splicing and the EBS1 stem-loop (the subdomain) in group II splicing are striking. Accordingly, the complementation observed by Hetzer et al. (1997) is intriguing. It will be of considerable interest to determine the nature of the contacts that serve to align the subdomain within the overall group II structure and whether analogous contacts exist in the spliceosome.

Although, as noted above, the continued comparison of group II and pre-mRNA splicing will undoubtedly yield interesting results, other experimental systems may prove more fruitful for defining an evolutionary relationship. It is well established that most group II introns are not autocatalytic (i.e., catalysis is not observed in the absence of protein cofactors). However, the nature and mechanism of action of the required

proteins is underinvestigated and largely obscure. A thorough study of such proteins might reveal homologs of spliceosomal proteins, a result that would have obvious significance. Finally, the machinery for excision of so-called group III introns (to date found only in euglenoid organelles) is completely mysterious (Copertino and Hallick 1993). These introns, thought to be degenerate group-II-like introns, must require *trans*-acting factors for their excision. Identification of such factors (RNA or protein) should help to clarify the relationship of group II and group III introns and might prove significant for pre-mRNA splicing as well.

Whatever the outcome of comparisons between group II and GU-AG pre-mRNA splicing, we now have to contend with two spliceosomes (see above). In addition, U4 function must be accounted for. No element analogous to U4 appears to be required in group II introns, yet no one has been able to observe pre-mRNA splicing in the absence of U4. Clearly, the existence and stability of the U4/U6 helices are crucial for nuclear pre-mRNA splicing, but the reasons for this interaction are not yet defined. It has been proposed that U4 acts as an antisense repressor of U6 activity; alternatively, it may be necessary for appropriate folding of U6 in the precatalytic spliceosome (for discusson, see Guthrie 1991; Nilsen 1994). Unfortunately, both of these notions are difficult to prove.

THE CASE FOR RNA CATALYSIS IN PRE-mRNA SPLICING

As discussed above, it has long been thought (assumed, presumed) that nuclear-pre-mRNA splicing is catalyzed by some combination of the spliceosomal U snRNAs. By now, the arguments in favor of RNA catalysis have been extensively documented and discussed. These include, but are not limited to, the following: (1) snRNAs are absolutely required for splicing; (2) the two catalytic steps proceed through in-line transesterification reactions analogous to those observed in catalytic RNAs (Moore and Sharp 1993); (3) certain U snRNA mutations and backbone substitutions have effects on catalysis, including specific second-step effects (Fabrizio and Abelson 1990, 1992; Madhani et al. 1990; Madhani and Guthrie 1992; McPheeters and Abelson 1992; Yu et al. 1995); (4) there are analogies to other catalytic RNAs, particularly group II introns (see above and Padgett et al. 1994) (for discussion of similarities to non-group-II ribozymes, see Tani et al. 1992; Sun and Manley 1995, 1997); and (5) the first catalytic step of pre-mRNA splicing occurs through a metal-ion-dependent pathway suggestive of RNA catalysis (Sontheimer et al. 1997), consistent with a previously proposed model for the spliceosomal active site(s) (Steitz and Steitz 1993).

Collectively, these arguments are convincing, even compelling; but they are not definitive. The question of RNA (or protein) catalysis is important because the answer has obvious implications for the origin(s) of introns and the machinery that excises them. Clearly, an unambiguous demonstration that snRNAs alone could catalyze pre-mRNA splicing would be definitive. However, given the huge number of proteins known to be involved in the process, it seems highly unlikely that simple mixing experiments will work. An alternative approach involves randomization-selection schemes using snRNAs as starting points. Acknowledging the power of such techniques and the fact that many novel RNA catalysts have emerged from various selection strategies, such approaches are likely to yield RNA molecules that can catalyze at least a subreaction of pre-mRNA splicing. The relevance of these (presumptive) catalysts to true pre-mRNA splicing will depend on their final similarity to the natural snRNAs and their sensitivity to functional group modification.

Perhaps the most conceptually appealing approach (for discussion, see Guthrie 1994) is to use strategies analogous to those employed by Noller and colleagues in the demonstration that rRNA (almost certainly) catalyzes peptide-bond formation (for review, see Noller et al. 1995). In such an approach, one would prepare inactive precatalytic spliceosomes that have undergone the first snRNA conformational rearrangement, and then gently deproteinize. This strategy would depend on reasonable stability of the preformed RNA–RNA contacts, perhaps enhanced by strategic cross-links and a mechanism to trigger catalysis. Metal rescue of thiophosphate substitutions in the pre-mRNA or U6 snRNA might conceivably provide such a trigger.

Even if it is conclusively demonstrated that pre-mRNA splicing is RNA-catalyzed (my own, perhaps misguided, view is that the circumstantial evidence leaves no reasonable doubt), the requirement for multiple proteins must be confronted. In this regard, considerable progress has been made in elucidating the ways in which proteins can function in RNA-mediated reactions. It is well established that proteins, both nonspecific and specific, can have profound effects on RNA folding by acting as chaperones (for a detailed review, see Herschlag 1995). Additionally, proteins can act by stabilizing weak (or compromised) RNA–RNA interactions, e.g., the binding of tyrosyl tRNA synthetase to the catalytic core of a group I intron (for recent discussion, see Caprara et al. 1996). Perhaps most intriguingly, Cech and coworkers have shown that proteins can function by "trapping" transient tertiary interactions, and in so doing, greatly enhance the overall rate of catalysis (Weeks and Cech 1995, 1996; for review, see Woodson 1996). As discussed thoroughly in the work cited above, it seems very likely that many of the spliceosomal proteins will prove to have analogous functions. Nevertheless, the stepwise dissection of protein function in pre-mRNA splicing is going to take considerable time and effort; such studies are still in their infancy.

SUMMARY AND PERSPECTIVES

Combined biochemical and genetic approaches in a variety of systems have led to a working model of RNA–RNA interactions involved in nuclear pre-mRNA splicing. Several considerations indicate that the model is useful and most likely accurate, but much refinement is still needed. It is particularly unclear how both of the rearrangements within the spliceosome are initiated and catalyzed, and the mechanism by which the nucleophiles are activated is still obscure. Our current understanding of the catalytic sites themselves is hazy at best, although it has become clear that significant remodeling of the catalytic site must occur between the first and second steps (Moore and Sharp 1992, 1993; Sontheimer et al. 1997).

It is hoped that the entire catalog of required factors will be completed soon; however, it is not clear where significant mechanistic breakthroughs will develop. Continued mutagenesis of U snRNAs may not be fruitful, unless efficient strategies of introducing multiple mutations in independent RNAs are developed (perhaps the AT-AC spliceosome is the perfect multiple mutant). Cross-linking, both site-specific and random, will undoubtedly continue to provide useful information; however, these techniques are laborious and give a static picture.

It would be of obvious significance to apply biophysical techniques to purified spliceosomes; nevertheless, despite considerable progress in the characterization of large macromolecular complexes via these approaches, their application to the spliceosome does not appear to be imminent.

In the absence of a major technical advance, it seems that the highest priority must be to establish the network of protein–protein and protein–RNA contacts in the spliceosome. Given the complexity of the players, this will undoubtedly prove to be a formidable and time-consuming task.

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