RNA (1995), *1*:763–771. Cambridge University Press. Printed in the USA.
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REVIEW

Finding splice sites within a wilderness of RNA

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INTRODUCTION

The pre-mRNA splicing reaction has provided a great landscape of molecular interactions in need of survey and characterization. Although many features of this landscape are now known and in rough outline understood, important questions remain and presumably many interesting new molecular flora and fauna are still to be identified. One region of continued exploration encompasses the problem of how the splicing apparatus finds a splice site and pairs it with its correct partner across an intron. This review discusses our current picture of unregulated splice site selection in higher eucaryotes.

Some of the earliest explorers and missionaries to this part of the RNA world puzzled over the question of splice site choice (Lewin, 1980; Sharp, 1981). How is a splice site identified and paired with its correct partner? In most metazoan species, splice sites are defined by rather loosely conserved sequences at the junctions of introns and exons, the 5′ splice site at the 5′ end of an intron, and the 3′ splice site with its associated branch point and polypyrimidine tract at the 3′ end of the intron. These splice sites are recognized by the cellular splicing machinery that assembles the sites into a particle called the spliceosome and catalyzes the excision of the intron in two steps (Moore et al., 1993; Kramer, 1995). First, there is cleavage at the 5′ splice site with the concerted ligation of the intron 5′ end to the 2′ hydroxyl of a specific adenosine residue at the branch point. This step is followed by cleavage at the 3′ splice site and ligation of the two exons. The spliceosome contains five small nuclear ribonucleoproteins (snRNPs): U1, U2, U4, U5, and U6. Of these, only the U2, U5, and U6 snRNPs appear to be involved in the catalysis of intron excision. The components that initially recognize the splice sites are the U1 snRNP and an auxiliary protein factor U2AF. U1 contains an snRNA that base pairs with the 5′ splice site during the initial stages of spliceosome assembly. In *Schizosaccaromyces pombe*, U1 also base pairs to the 3′ splice site, but this has not yet been observed in other organisms (Nilsen, 1994a; Ares & Weiser, 1995; Madhani & Guthrie, 1995). The U2AF protein binds to the polypyrimidine tract within the 3′ splice site sequence and is required for the later binding of U2 to the branch point (Zamore & Green, 1991; Zamore et al., 1992).

In studies of spliceosome assembly using short vertebrate introns, U1 and U2AF are both found in the earliest splicing complex (Moore et al., 1993; Kramer, 1995). This is called the E complex (for early) or the commitment complex because, by this stage of spliceosome assembly, the splice sites have been chosen and the intron that is destined for excision has been defined (Séraphin & Rosbash, 1989; Michaud & Reed, 1991, 1993; Rosbash & Séraphin, 1991; Jamison et al., 1992). The U2 snRNP assembles onto the E complex, through base pairing to the branch point sequence, to form the pre-spliceosomal A complex (Moore et al., 1993; Kramer, 1995). The next step is the formation of the B spliceosome complex through the binding of the U4/U5/U6 tri-snRNP. The B complex undergoes a structural rearrangement where the interactions of U1 and U4 are broken and U6 is brought into interaction with the 5′ splice site and the U2 snRNA (Nilsen, 1994a; Ares & Weiser, 1995; Madhani & Guthrie, 1995). This mature spliceosome can then carry out the catalytic steps of the reaction. In these in vitro assembly studies, splice site choice was determined by the binding of U1 to the 5′ splice site and of U2AF to the 3′ splice site and the assembly of these two sites into an E or commitment complex. However, as described below, this E complex is not always a required way station on the path to a functional spliceosome. Other routes of assembly are possible.

Although the ultimate goal of a splice site is to be recognized by U1 or U2AF and to assemble into a spliceosome, there are features of many metazoan gene transcripts that make this recognition process complex. Yeast introns, with their scarcity, short length, and

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highly conserved splice sites, present few conceptual obstacles to understanding splice site choice. In contrast, in higher organisms, where the splice site sequences are less strictly conserved, messenger RNA precursors contain many copies of sequences that match the splice site consensus sequences equally as well as true splice sites. These cells need special mechanisms to distinguish a true splice site from all of the possible cryptic splice sites in the RNA jungle. Moreover, most genes in these organisms contain multiple exons, often separated by long introns of many thousands of nucleotides. There must also be mechanisms to ensure that a given 5′ splice site is paired with its correct 3′ splice site partner downstream and that splice sites or exons are not skipped. These are the two parts of the splice site choice problem—recognition of the true splice sites, and pairing of the proper sites across the intron. Significant progress has been made in understanding how true splice sites are recognized, although finding a splice site partner within the wilderness of a long RNA transcript still seems mysterious.

EXON-BRIDGING INTERACTIONS

The simple answer to the recognition of true splice sites is that the cell uses more sequence cues to identify them than the splice site sequence itself. There can be auxiliary sequences that help to specify a correct splice site. A major advance in understanding how splice sites are recognized came from the discovery of exon-bridging interactions by the labs of Berget and Grabowski (Nasim et al., 1990; Robberson et al., 1990). These groups showed that the 5′ splice site on the downstream side of an exon can be a crucial determinant in the recognition and splicing of the upstream intron. More specifically, the binding of U1 at the downstream 5′ splice site will stabilize U2AF binding across the exon at the upstream 3′ splice site (Kuo et al., 1991; Hoffman & Grabowski, 1992). This exon-spanning interaction is mediated by as yet undefined bridging factors between U1 and U2AF (see below). Berget has called this process where both ends of the exon are recognized together "exon definition," and has pointed out that it explains many previously puzzling aspects of splice site choice (Robberson et al., 1990; Berget, 1995). For example, the most common phenotype of a single splice site mutation is the skipping of the entire exon, even though the splice site on the opposite side of the exon remains unaltered (Krawczak et al., 1992). In the exon definition model, the remaining wild-type splice site fails to be recognized by the splicing machinery because it has lost the enhancing effect of the splice site that has the mutation. The other common phenotype of a splice site mutation is the activation of a nearby cryptic splice site to replace the mutant site (Krawczak et al., 1992). It is thought that, in the presence of the mutation, these cryptic sites can be activated by the same exon-bridging interaction that normally is directed to the wild-type site. Without the mutation, the bridging interaction does not affect the cryptic site and the site is ignored. Thus, true splice sites can be defined by their ability to interact with the opposite splice site across the exon, prior to their pairing across an intron. Through exon-bridging interactions, each splice site on an exon serves as a buddy to prevent the splice site on the opposite side from becoming lost in the wilderness.

This buddy system can only occur on internal exons; 5′ and 3′ terminal exons must have other mechanisms to allow recognition of their single splice sites. Indeed, these predictions also have experimental support. There have been several results implicating the need for a 5′ cap structure in the splicing of single intron substrates in vitro. Most recently, a cap-binding protein complex was shown to be essential for splicing activity in vitro (Izaurralde et al., 1994). Even more interestingly, experiments on the in vitro splicing of a two-intron RNA showed that the absence of the 5′ cap nucleotide suppressed splicing of the 5′-most intron, while having little effect on the 3′ intron (Ohno et al., 1987). At the other end of the transcript, the 3′ terminal exon contains the poly-A site. Similar to the result above, mutating the poly-A site in a two-intron substrate inhibits the in vitro splicing of the 3′ intron but not the 5′ intron (Niwa & Berget, 1991). The U1 snRNP and its component proteins have also been observed binding in the region of poly-A sites; this may indicate that enhancement by a downstream poly-A site occurs by an equivalent mechanism to exon bridging in an internal exon (Wassarman & Steitz, 1993b; Lutz & Alwine, 1994). These results provide some confirmation of the idea that the terminal exons use their unique terminal structures to provide enhancement of splice site recognition in the absence of normal exon-bridging interactions.

Given the frequency of the exon-skipping phenotype of splice site mutations, exon-spanning interactions are likely to be involved in the recognition of most splice sites. However, these interactions are not always essential. In vitro splicing substrates containing strong splice sites, with good matches to the two consensus sequences, don't require enhancement by a downstream 5′ splice site (Wang et al., 1995). Very short introns also don't seem to require them (Reed & Maniatis, 1986; Talerico & Berget, 1994). In this case, complexes bound at the 5′ splice site presumably interact directly across the *intron* with a complex at the 3′ splice site.

Most vertebrate internal exons fall within a fairly narrow size range of 50–250 nt (Hawkins, 1988). The argument is made that this is to accommodate exon bridging (Robberson et al., 1990). However, there are many exons that are quite small (<20 nt) (Hawkins, 1988). It is difficult to envision how these could accommodate splicing complexes bound at both ends with

bridging factors in between. When deletions were made in an internal exon derived from the β-globin gene, exon skipping was observed when the exon length went below ~50 nt (Dominski & Kole, 1991). Conversely, regulated or other small exons that are normally skipped can be activated to splice efficiently by extending their length (Black, 1991; Sterner & Berget, 1993). This extra length is thought to allow for an exon-spanning interaction that enhances recognition of the splice sites. In fact, just making the splice sites strong matches to the consensus sequences induces splicing of a short exon (Dominski & Kole, 1992). The enhancement of some short-exon splice sites may come from an exon-bridging interaction spanning the entire adjacent intron and exon, if the distance is short enough (Sterner & Berget, 1993). However, in other examples, this seems unlikely (Rupp et al., 1992), and there must be other means of recognizing short-exon splice sites in the absence of exon bridging. Similarly, it is thought that long exons are uncommon because they would not allow for exon-bridging interactions (Robberson et al., 1990). Nevertheless, some long exons (>500 nt) can function perfectly well (Hawkins, 1988; Tacke & Goridis, 1991; Chen & Chasin, 1994; Humphrey et al., 1995). It is not known whether these have special features, such as secondary structure, that allow them to maintain communication between their two ends. Alternatively, they may use mechanisms other than exon bridging to ensure proper splice site recognition (see below).

These exceptions to the buddy system bring up the question of whether a pair of splice sites is truly defined as an exon or whether the recognition of an individual splice site simply needs enhancement, either by an especially good match to the splice site consensus sequence or by other sequences. There are several results that point to this latter possibility.

ENHANCEMENT BY OTHER MEANS

Experiments from a number of laboratories have shown that sequences other than downstream splice sites can enhance spliceosome assembly and the splicing of some introns (Fu et al., 1991; Watakabe et al., 1991; Lavigueur et al., 1993; Xu et al., 1993; Caputi et al., 1994; Dirksen et al., 1994; Tanaka et al., 1994; Tian & Maniatis, 1994; Humphrey et al., 1995; Wang et al., 1995). Most of these stimulatory sequences have been found in the exon downstream of the stimulated intron and contain predominantly adenosine and guanosine residues. They have thus been defined as purine-rich exonic splicing enhancers. These exonic splicing enhancers, when placed in the downstream exon, can activate the use of an otherwise weak 3′ splice site, even in the absence of an exon-bridging interaction (Lavigueur et al., 1993; Sun et al., 1993; Watakabe et al., 1993; Tian & Maniatis, 1994; Wang et al., 1995). In one case, a purine-rich enhancer activates splicing at an upstream 5′ splice site (Humphrey et al., 1995). Interestingly, although their effects are not tissue-specific, these sequences have predominantly been found in regulated exons. Enhancer sequences may provide a stimulating effect that is more controllable by regulatory proteins than the enhancement by exon bridging.

The similarities between the effects of exonic enhancers and exon-bridging interactions goes beyond their stimulatory effect on splicing; these sequences assemble some of the same components of the general splicing apparatus. A splicing enhancer from the IgM M2 exon will crosslink to the U1 snRNA in cell extracts (Watakabe et al., 1993). Moreover, characterization of the complexes that assemble onto either a 5′ splice site or an exonic splicing enhancer sequence showed them to be very similar (Staknis & Reed, 1994). Interestingly, both types of complex contained the U1 snRNP as well as an intensely studied class of factors called the SR proteins.

SR proteins exhibit a wide array of properties (see Fu, 1995 for a complete review). Members of this tribe of proteins contain one or more RNP-CS type RNA-binding domains and a domain rich in serine and arginine residues (hence "SR" [Roth et al., 1991]). The prototype of this family, ASF/SF2, was identified as a protein required for splicing activity in vitro and that altered 5′ splice site choice in vitro (Ge & Manley, 1990; Krainer et al., 1990, 1991; Ge et al., 1991; Horowitz & Krainer, 1994). Other SR family members can have similar effects in in vitro assays (Fu et al., 1992; Mayeda et al., 1992), although splice sites can differ in the SR proteins they require (Fu, 1993; Tian & Maniatis, 1993; Zahler et al., 1993; Ramchatesingh et al., 1995; Screaton et al., 1995; Tacke & Manley, 1995; Zahler & Roth, 1995). These proteins are potential regulators of alternative splicing patterns and are also likely targets for more specific regulatory proteins (Tian & Maniatis, 1993, 1994; Caceres et al., 1994; Screaton et al., 1995; Wang & Manley, 1995).

There is also evidence that SR proteins mediate the stimulatory effects of both exonic enhancers and downstream 5′ splice sites. Several exonic enhancer sequences have been shown to bind SR proteins and SR proteins are required for these enhancer's effects in vitro. Different enhancer sequences apparently bind to different subsets of SR proteins (Lavigueur et al., 1993; Sun et al., 1993; Heinrichs & Baker, 1995; Ramchatesingh et al., 1995; Tacke & Manley, 1995; Wang & Manley, 1995; Wang et al., 1995). ASF/SF2 also binds directly to 5′ splice sites in vitro (Zuo & Manley, 1994), and several SR proteins, including ASF/SF2, can stabilize the binding of the U1 snRNP to a 5′ splice site (Kohtz et al., 1994; Zahler & Roth, 1995).

The SR proteins also engage in protein/protein interactions through their SR domains. ASF/SF2 was shown

to interact with the 70-kDa U1 snRNP protein immobilized on membrane filters (Kohtz et al., 1994). In addition, several SR proteins interact with the 35-kDa subunit of U2AF in yeast genetic two-hybrid assays (Wu & Maniatis, 1993). The SR protein SC35 was shown to bind to components at both the 5′ and 3′ splice sites during spliceosome assembly (Fu & Maniatis, 1992). SR proteins can thus interact with the 5′ splice site, the U1 snRNP, splicing enhancer sequences, and the U2AF protein.

These many interactions of SR proteins lead to the appealing model that these proteins provide the bridge between 5′ and 3′ splice sites, both across the intron during E complex formation and in exon-bridging interactions (Fu & Maniatis, 1992; Wu & Maniatis, 1993; Staknis & Reed, 1994; Fu, 1995). In both cases, U2AF binding to the 3′ splice site is thought to be stabilized by SR proteins that in turn are bound by U1 at the 5′ splice site. This 5′ splice site can either be upstream of the 3′ splice site, forming a pre-spliceosomal E complex, or downstream, forming an exon-bridging complex. Apparently, an exonic splicing enhancer can replace the downstream 5′ splice site by binding the SR proteins directly and stabilizing the binding of U2AF to the upstream 3′ splice site (Wang et al., 1995). Although the U1 snRNP is present in some enhancer assembled complexes, it is not clear whether U1 is binding directly to the enhancer sequence, as it does to a 5′ splice site, or whether it is tethered to the enhancer through the SR protein.

PATHS TO A FUNCTIONAL SPLICEOSOME

The relationship between the complexes assembled on exons and the actual spliceosomes assembled on introns is puzzling. Assembly studies on short introns in vitro have indicated that the commitment or E complex is a precursor to the A complex, and seems to contain U1 bound to the 5′ splice site and U2AF bound to the 3′ splice site (Michaud & Reed, 1991, 1993; Jamison et al., 1992). However, it is also known that the U2 snRNP will assemble onto a 3′ splice site in the presence of U2AF and U1 snRNP, but the absence of a 5′ splice site (Konarska & Sharp, 1986). This A complex formation without a prior E complex requires SR proteins and is stimulated by exonic enhancers or downstream 5′ splice sites (Robberson et al., 1990; Hoffman & Grabowski, 1992; Staknis & Reed, 1994; Crispino & Sharp, 1995; Tarn & Steitz, 1995; Wang et al., 1995).

Recent results now make clear that the two splice sites of an intron can be paired after the formation of the A complex at the 3′ splice site. The prior formation of the E complex is not required. It was shown that a spliceosomal A complex, containing U2 bound to the branch point, will *trans*-splice to a 5′ splice site supplied on a separate transcript (Bruzik & Maniatis, 1995; Chiara & Reed, 1995). *Trans*-splicing at the 3′ splice site requires the presence of either a splicing enhancer or a downstream 5′ splice site (Bruzik & Maniatis, 1995; Chiara & Reed, 1995). A 3′ splice site alone will form a U2-containing A complex, but does not *trans*-splice to other transcripts. This difference between the A complex assembled with just a 3′ splice site and the "enhanced A complex" indicates a role for exonic splicing complexes that goes beyond stabilizing U2 binding to the 3′ splice site. Indeed, the *trans*-splicing complexes assembled with a downstream 5′ splice site (rather than a splicing enhancer) will *trans*-splice to each other—joining the downstream 5′ splice site of one transcript to the upstream 3′ splice site of another (Chiara & Reed, 1995). These results indicate that both *intronic* splice sites need not be recognized and paired to initiate functional spliceosome assembly, as had been observed previously studying yeast and short vertebrate introns in vitro. Instead, a preassembled spliceosomal A complex can recruit a 5′ splice site to carry out the splicing reaction. This is important in understanding the pairing of splice sites across long introns. The cell can apparently identify or even commit to splicing at an enhanced 3′ splice site before it has found its upstream partner. This enhanced spliceosomal A complex at the 3′ splice site may then be used as a base camp in exploring for upstream 5′ splice sites.

These artificial mammalian *trans*-splicing reactions are similar to the natural *trans*-splicing observed in trypanosomes and nematodes (Nilsen, 1993, 1994b). In the latter organisms, a specific spliced leader RNA (SL RNA) is spliced onto a 3′ splice site at the beginning of each message. This reaction requires a specific RNP structure of the SL RNA, but does not require the U1 snRNP. Several studies by Bruzik have shown that an SL RNA from trypanosomes can be spliced by mammalian spliceosomal components (Bruzik & Steitz, 1990; Bruzik & Maniatis, 1992, 1995). This indicates that the 3′ splice site complex with which the SL RNA associates is likely to be very similar to the enhanced mammalian A complex.

The phenomenon of splicing without E complex formation is probably also related to results where high levels of SR proteins allowed splicing in vitro under conditions where the U1 snRNP was absent or blocked from interacting with a 5′ splice site (Crispino et al., 1994; Tarn & Steitz, 1994, 1995; Crispino & Sharp, 1995). Under similar conditions of inactive U1, the U6 snRNP has been shown to bind to a 5′ splice site (Konforti et al., 1993; Konforti & Konarska, 1994). High levels of SR proteins presumably stabilize U6 binding to the 5′ splice site, which then interacts with the U2 complex at the 3′ splice site. This assembles a functional spliceosome without the services of U1.

A *trans*-splicing reaction has also been observed at the 3′ splice site (M. Moore, pers. comm.). Spliceosomes

that have carried out the first step of splicing (5′ splice site cleavage and lariat formation) can *trans*-splice to a transcript containing a lone 3′ splice site without an associated branch point. This result agrees with earlier analyses of the role of the polypyrimidine tract in the 3′ splice site. These studies showed that the first step of splicing in vitro requires the poly-pyrimidine tract for proper branch point recognition, but does not always require the AG dinucleotide at the 3′ cleavage site (Reed, 1989; Smith & Nadal, 1989; Smith et al., 1989; Zhuang & Weiner, 1990). Thus, the cell may be able to commit to a specific branch point without committing to the exact 3′ splice site downstream.

FINDING A PARTNER

None of these results really answer the question of how Stanley finds Livingston. That is, how does a splice site with its assembled factors find its correct *intronic* partner across a large jungle of other RNA sequence? For short introns, the two splice sites may find each other through random collisions or the direct bridging of proteins and snRNA across the intron. This seems impossible for long introns many kilobases in length. Although the fidelity of the splicing process in linking exons in order may not be as high as was thought before the advent of PCR (Nigro et al., 1991; Cocquerelle et al., 1992), there still must be a system for consistently finding the correct partner across a long stretch of intronic RNA.

Early models for this problem invoked tracking mechanisms through the intron from one splice site to the next (Lewin, 1980; Sharp, 1981). Experimental results have ruled out the simplest versions of this idea, although tracking could occur between pre-assembled exonic complexes (Kuhne et al., 1983; Lang & Spritz, 1983; Aebi & Weissmann, 1987). Another early idea was that the order of transcription of specific sites determines the order of spliceosome assembly and hence the order of ligated exons (Aebi & Weissmann, 1987). Indeed, in chromatin spreads from *Drosophila* embryos and in the Balbiani ring 1 transcript of *Chironomus tentans*, the splicing of upstream introns can be observed before the completion of transcription (Beyer & Osheim, 1988; Bauren & Wieslander, 1994). Moreover, in some very long introns of *Drosophila*, the splicing of the transcript as it is being synthesized does limit the splicing options of sites further downstream (LeMaire & Thummel, 1990; Bomze & Lopez, 1994; Lopez, 1995). However, in many transcripts, the upstream introns are not the first to be excised (Tsai et al., 1980; Lang & Spritz, 1987; Shiels et al., 1987; Gudas et al., 1990; Kessler et al., 1993). Although it is possible that upstream introns are the first to assemble a spliceosome and are committed to splicing before other splice site choices present themselves, multiple exon RNAs spliced in vitro often do not show substantial exon skipping, even though they are introduced to the splicing apparatus as full-length transcripts (Lang & Spritz, 1987). In yeast, base pair interactions between sequences near the 5′ splice site and the branch point can affect splice site choice (Goguel & Rosbash, 1993; Libri et al., 1995). A similar interaction has also been observed in certain metazoan introns (B. Chabot, pers. comm.).

The packaging of the pre-mRNA as it is being transcribed is also likely to affect splice site pairing. Nascent RNA transcripts are coiled into hnRNP particles around the core hnRNP proteins (hnRNP A1, A2, B1, B2, C1, and C2) (Dreyfuss et al., 1993). An hnRNP $(C1)_3C2$ protein tetramer is thought to wrap approximately 230 nt of RNA around itself. Three of these C tetramers then assemble into a larger triangular complex containing ~700 nt of RNA and three $(A2)_3B1$ or $(A1)_3B2$ tetramers (Huang et al., 1994). These variable lengths of wrapped RNA give the hnRNP complexes a certain flexibility in bringing distant sequences close together. For example, if hnRNP core proteins are excluded from the exonic splicing complexes but not from intron sequences, then an 800-nt intron would assemble a single three-tetramer triangular complex, leaving just 100 nt of intron sequence unwrapped. Longer introns might assemble into still higher-order structures that essentially loop out the intron sequences not bound by exonic splicing complexes. By the selective packaging of intron sequences, long introns could appear shorter to the splicing machinery. After this hnRNP packaging, splice sites might find their partners by random collisions or the direct bridging of factors across the now short intron sequence. Electron microscopic analysis of a purified hnRNP packaged transcript, the Balbiani ring granule of *Chironomus*, indicates that it does indeed have a specific higher-order structure, but it is not clear yet whether this structure could help juxtapose splice sites (Wurtz et al., 1990; Kiseleva et al., 1994).

In general, the interaction of hnRNP particles with the splicing machinery is poorly understood. Several of the core hnRNP proteins have affinity for poly-pyrimidine tracts in 3′ splice sites (Swanson & Dreyfuss, 1988). In another study, crosslinking of core proteins to the pre-mRNA was dependent on intact U1 and U2 snRNPs (Mayrand & Pederson, 1990). It is interesting that one of the core proteins, the A1 protein, is known to affect which of two duplicated 5′ splice sites is chosen by the splicing apparatus (Mayeda & Krainer, 1992; Cáceres et al., 1994). High levels of A1 can shift the 5′ splice site to the choice further away from the 3′ splice site—in effect making the intron longer. It is not known whether the A1 protein is assembling with the other core proteins on these transcripts. The development of biochemical assays for the interaction of hnRNP core particles with the spliceosomal components will be

needed to understand any relationship between hnRNP packing and splice site pairing.

BLANK SPOTS ON THE MAP

In addition to the large question of how exon order is achieved, there are many interesting questions regarding the assembly of the exonic splicing complexes. The role of the U1 snRNP at the 5′ splice site in these complexes is particularly intriguing. The presence of an upstream 3′ splice site does stabilize the binding of factors, presumably including U1, to the downstream 5′ splice site across the exon (Robberson et al., 1990). However, it is not clear whether this upstream 3′ splice site can also stimulate splicing downstream. In one system, a full exonic splicing enhancer complex on an upstream exon did not stimulate the splicing of the downstream intron (Cooper, 1992; Xu et al., 1993). In the *trans*-splicing assay, a single exon transcript carrying an exon-bridging complex could act as a 5′ splice site to *trans*-splice to a 3′ exon, but it did so no better than a transcript containing just a 5′ splice site (Chiara & Reed, 1995). Moreover, what is the role of the U1 in an exonic splicing enhancer complex? Is it used to bind the upstream 5′ splice site across the intron? Perhaps the exonic complex can stabilize an otherwise weak U1/5′ splice site interaction. The SR proteins clearly play a role in stabilizing U1/5′ splice site interactions (Kohtz et al., 1994; Zahler & Roth, 1995). On the other hand, when splicing is enhanced by a downstream 5′ splice site, a single U1 is presumably not binding both the upstream and downstream 5′ splice sites. In this case, it does not appear that the upstream 5′ splice site needs to be part of an exonic splicing complex (Chiara & Reed, 1995), although SR proteins may still be involved. Also interesting is how individual SR proteins can specify binding only to certain splice sites. Finally, is U1 always necessary, or are there natural examples of introns where U6 rather than U1 is used to recognize the 5′ splice site (Konforti et al., 1993; Crispino et al., 1994; Konforti & Konarska, 1994; Tarn & Steitz, 1994; Crispino & Sharp, 1995)? Although not involved in catalysis, U1 is proving to be the most enigmatic of the spliceosomal snRNPs.

There are also questions of higher-order splicing complexes. Do multiple exonic complexes interact across their separating introns to form concatenated spliceosomes? There are several reports of very large complexes containing spliceosomal components associated with specific transcripts (Wassarman & Steitz, 1993a; Miriami et al., 1995). Alternatively, are the exon-spanning interactions lost once the splice sites are paired across an intron? When two exons are ligated, does a new exonic splicing complex assemble using the ends of the now larger exon? There is an interesting example of this in a pair of exons of the preprotachykinin gene (Nasim et al., 1990). However, what happens as more exons are spliced and the distance across the ligated exons becomes large? Many new experiments will be needed to fill in some of these blank areas of the map.

The above discussion has focused on splice site choice under general conditions. There are many examples of regulated splicing choices, and a great diversity of mechanisms have been uncovered that affect splice site choice (Rio, 1993; Inoue et al., 1995). Splice sites can be blocked by specific factors, by secondary structure, or by the general splicing machinery binding at sites nearby the repressed sites (Inoue et al., 1990; Clouet et al., 1991; Guo et al., 1991; Libri et al., 1991; Eperon et al., 1993; Horabin & Schedl, 1993; Gooding et al., 1994; Siebel et al., 1994; Lin & Patton, 1995; Singh et al., 1995). Positive regulatory sequences can be exonic and work through specific regulatory factors and SR proteins (see Inoue et al. [1995] and Lynch & Maniatis [1995] and references therein). There are also positive-acting intronic sequences that are not purine rich, and it is not known whether they act through SR proteins (Black, 1992; Huh & Hynes, 1994; Del Gatto & Breathnach, 1995). Because most alternative splicing patterns involve the choice of pairing a given splice site with one of several competing partners, any mechanism that alters the rates of factor binding to splice sites could be used to alter the splicing pattern. Thus, in the regulation of splicing, there are likely to be many mechanisms to control splice site choice.

Exonic splicing complexes, splicing enhancer sequences, and the stabilization of splicing complexes by SR proteins are new features in the landscape of pre-mRNA splicing. Their discovery is akin to the discovery of the large equatorial African lakes. The 19th century explorers who discovered these lakes were correct in thinking that here was the source of the river Nile. This did not prevent certain adventurers from choosing the wrong lake and finding themselves after many months in the Atlantic Ocean rather than the Mediterranean Sea (Moorehead, 1960). The origins of splice site choice may show a similarly confounding geography.

ACKNOWLEDGMENTS

I thank Steve Smale, Robin Reed, Melissa Moore, Joan Steitz, Paula Grabowski, Susan Berget, and members of my laboratory for comments on the manuscript and for interesting discussions. I am supported by NIH grant R29 GM49662-02 and as an Investigator of the Howard Hughes Medical Institute and a Packard Foundation Fellow.

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