

# SR proteins and splicing control

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Twenty years have passed since the discovery of pre-mRNA splicing (for review, see Sharp 1994). The studies leading to this discovery were carried out on the adenovirus late transcript, which undergoes complex alternative splicing, and, therefore, the concept of alternative splicing is also 20 years old. In the intervening time, intervening sequences, or introns, have been found in a vast majority of higher eukaryotic genes, and a large fraction of intron-containing transcripts have been shown to be subject to alternative splicing. Furthermore, modulation of pre-mRNA splicing is now known to be a widespread mechanism of gene control. But despite considerable advances in our understanding of the splicing reaction per se (for review, see Moore et al. 1993; Madhani and Guthrie 1994), insights into how alternative splicing is controlled have been slower to emerge.

Several factors have been responsible for our somewhat limited understanding of how splicing can be regulated. First, the combined use of biochemical and genetic methodologies available in yeast has greatly accelerated understanding of the basic splicing reaction. But true alternative splicing (i.e., selection of alternative splice sites) does not appear to occur in *Saccharomyces cerevisiae*, and studies on this process are therefore limited to higher eukaryotes. With the important exception of the *Drosophila* sex determination pathway (for review, see Baker 1989), what we know about alternative splicing comes almost entirely from in vitro biochemical approaches without the aid of genetic approaches. Second, again with a limited number of exceptions (for review, see Rio 1993), the identification of *cis*-acting sequences responsible for modulating specific splicing events has been difficult, and, therefore, the isolation of sequence-specific RNA-binding splicing factors has been slow. Indeed, whether gene-specific regulators play a major role in splicing control, as sequence-specific DNA-binding proteins do in transcription, constitutes an important, unresolved question in the field. Finally, the number of regulated splicing events that have proven amenable to in vitro analysis is small, adding to the difficulties in the identification of regulatory molecules and mechanisms. Despite these limitations, significant progress has been made in the identification and analysis of a family of proteins, the SR proteins, that likely play important roles in splicing control. (The name SR reflects the presence of a characteristic serine/arginine-rich domain; see Fig. 1.) The purpose of this review is to summarize our knowledge of these proteins, concentrat-

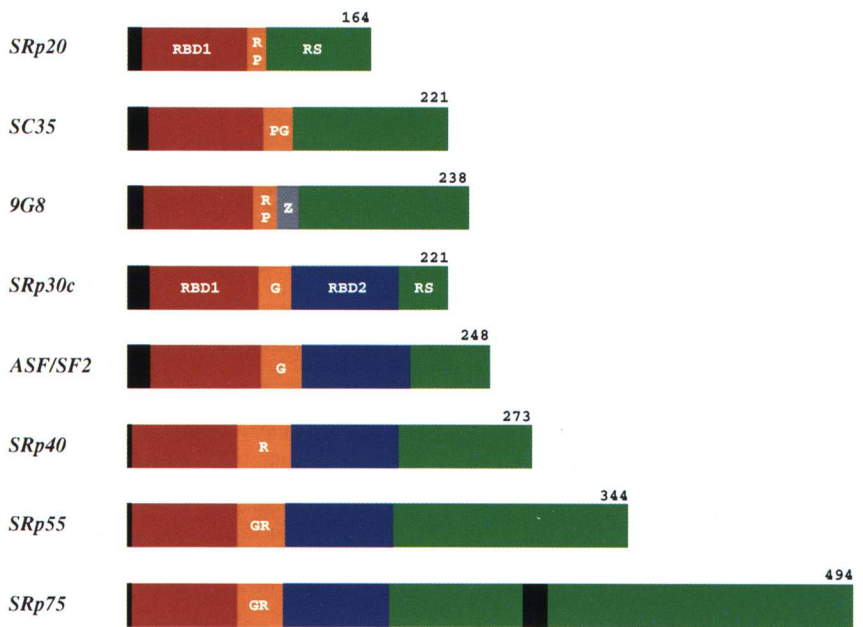
ing on how they might participate in splicing regulation. A related review, including discussion of what appears to be an extended superfamily of SR-type proteins, has been published recently (Fu 1995).

## ASF/SF2, SC35, and the SR protein family

The first two SR proteins were discovered as a result of biochemical studies of mammalian splicing. The prototypical SR protein, ASF/SF2 (also referred to as SF2/ASF), was discovered and characterized independently by two different groups employing two different assays. ASF was purified as an activity that could influence selection of alternative 5' splice sites in an SV40 early pre-mRNA (Ge and Manley 1990), suggesting a role for ASF in alternative splicing, and recapitulating in vitro a previously observed cell-specific pattern of alternative splicing (Fu and Manley 1987). In contrast, SF2 was identified initially as an activity required for splicing of a  $\beta$ -globin pre-mRNA in vitro (Krainer and Maniatis 1985) and was subsequently purified as a factor essential for splicing (Krainer et al. 1990a), but with activities in alternative splicing related to those of ASF (Krainer et al. 1990b). Isolation of cDNAs encoding the proteins confirmed that they are identical (Ge et al. 1991; Krainer et al. 1991). These early studies established an important point that remains central to our understanding of SR protein function: The proteins can function in vitro both as essential splicing factors, required for splicing of all pre-mRNAs tested to date, and as modulators of alternative splicing.

The sequence of ASF/SF2 immediately suggested that the protein might function in vivo in splicing control and, furthermore, could be a member of an evolutionarily conserved family of splicing factors. ASF/SF2 was found to share two significant features in common with genetically defined regulators of splicing in *Drosophila*. First, the protein contains an amino-terminal RNA recognition motif, or RNP-type RNA-binding domain (RBD). Although such domains had been observed in splicing factors, they were also known to be present in a wide variety of RNA-binding proteins not involved in splicing (for review, see Birney et al. 1993; Burd and Dreyfuss 1994). A carboxy-terminal region enriched in repeating arginine-serine dipeptides (the RS domain), however, was more strongly suggestive of a role in splicing. (For historical reasons, we refer to RS domains and SR proteins, even though both RS and SR refer to the same arginine-serine dipeptides.) Similar motifs had

**Figure 1.** Schematic representation of the domain organization of human SR proteins. All SR proteins contain an amino-terminal RBD (red) and a carboxy-terminal RS domain (green). In five of the known SR proteins the RS domain is immediately preceded by an additional degenerate RBD (blue). The RBDs of individual SR proteins display significant sequence similarities to the corresponding domains in other SR proteins but not to each other. The highest degree of sequence divergence is found in the portions of the molecules (orange) separating the first and second RBD, or the RBD and the RS domain when only one RBD is present. ASF/SF2 (Ge et al. 1991; Krainer et al. 1991) contains a stretch of nine consecutive glycine residues (G) in this region, whereas the corresponding part of SRp40 (Screaton et al. 1995) is composed of 30% arginine residues. The predominant residues present within these regions in other SR proteins are glycine (G), arginine (R), proline (P), and/or hydroxylated amino acids. In



addition, 9G8 contains a putative zinc knuckle of the CCHC family (Z), previously found in other nucleic acid binding proteins (Cavaloc et al. 1994). Note that SC35 (Fu and Maniatis 1992) was cloned as PR264 in human and chicken (Vellard et al. 1992). Human and chicken PR264 are 98% identical. SRp20 (Zahler et al. 1992) was originally cloned in mouse as X16 (Ayane et al. 1991), to which it is identical, and SRp40 as HRS in rat (Diamond et al. 1993). SRp55 and SRp75 were cloned, respectively, by Screaton et al. (1995) and Zahler et al. (1993b). Among *Drosophila* SR proteins, RBP1 (Kim et al. 1992) is the apparent homolog of SRp20, although it displays a similar degree of identity (~65%) to 9G8, whereas B52 (Champlin et al. 1991), which is likely *Drosophila* SRp55 (Roth et al. 1991), shares ~65% sequence identity with human SRp55.

been observed previously in only a few proteins, all known to be involved in splicing, with the closest match being to the *Drosophila* splicing regulators Tra (Boggs et al. 1987), Tra2 (Amrein et al. 1988; Goralski et al. 1989), and su ( $w^a$ ) (Chou et al. 1987). A considerable number of proteins containing RS domains or related motifs has since been identified, and the presence of an RS-like region continues to be diagnostic of a protein involved in splicing.

SC35 was the next SR protein to be identified. It was detected first with a monoclonal antibody raised against purified spliceosomes and was shown to be necessary for splicing and spliceosome assembly in vitro (Fu and Maniatis 1990). Isolation of cDNAs encoding SC35 revealed a primary structure very similar to that of ASF/SF2: an amino-terminal RBD and a carboxy-terminal RS domain (Fu and Maniatis 1992a). The two proteins were also shown to function interchangeably in in vitro assays: Each by itself could activate splicing of test pre-mRNAs in depleted extracts, and each could induce the same switch in selection of alternative splice sites in model pre-mRNAs when added to nuclear extracts (Fu et al. 1992). These findings contributed to the early view that SR proteins might be functionally redundant.

ASF/SF2 and SC35 were soon shown to be members of a larger, evolutionarily conserved family of proteins (the SR proteins; Zahler et al. 1992). To isolate SR proteins, these authors utilized a simple two-step purification protocol that results in nearly homogeneous preparations consisting of six major proteins ranging in apparent size

from 20 to 75 kD. Proteins of similar sizes could be isolated from several species, suggesting that the protein family is evolutionarily well conserved from *Drosophila* to humans (see Fig. 1, legend). Further, the proteins are all recognized by a previously described monoclonal antibody (mAb104; Roth et al. 1990) that targets a phosphorylated epitope residing within the RS domain. Partial amino acid sequence revealed that the proteins all showed sequence similarity, and two, of ~30 kD, were found to be identical to ASF/SF2 and SC35. The purified proteins also all have the ability to function as essential splicing factors. Indeed, a recombinant form of one from *Drosophila*, SRp55, was found to function interchangeably with human ASF/SF2 in in vitro assays (Mayeda et al. 1992), adding to the view that the function of these proteins, though conserved throughout metazoan species, might be redundant.

The partial amino acid sequence data of Zahler et al. (1992) along with cDNA sequences of the SR proteins provide a structural basis for the defining of an SR protein (see Fig. 1). All SR proteins contain an amino-terminal RBD and a carboxy-terminal RS domain. In addition, some, but not all, SR proteins contain a second, internal RBD that is significantly diverged from the RBD consensus. For example, ASF/SF2, SRp40, SRp55, and SRp75 contain two RBDs, whereas SC35 and SRp20 contain only one. [Zahler et al. (1992) named the individual SR proteins according to their apparent molecular weight. By this nomenclature, ASF/SF2 is SRp30a and SC35 is SRp30b.] The differences in the sizes of SR proteins are

therefore largely determined by the presence or absence of a second RBD and by the length of the RS domain. Subsequently, two additional human proteins (9G8 and SRp30c) that are members of the SR protein family have been identified (Cavaloc et al. 1994; Screaton et al. 1995). These proteins may not have been detected by Zahler et al. (1992) because of differences in purification properties and/or lower abundance. In addition, by this definition, the *Drosophila* regulator Tra2 might also be considered an SR protein, as it contains an amino-terminal RBD and carboxy-terminal RS domain. Tra2, however, also contains a second RS region amino-terminal to the RBD, and there is no evidence that it can function as an essential splicing factor in vitro, which is a common feature of all other SR proteins. Classification of Tra2 as an SR protein is therefore ambiguous and depends on the stringency of the definition of an SR protein. It is likely that the SR protein family will continue to grow during the coming years. As mentioned above, there is also a large number of proteins that contain RS-like regions, but which do not fit the definition of SR protein. These proteins may constitute an SR protein superfamily [for review, see Fu 1995], which will be discussed here only in the case of individual members whose properties shed light on SR protein function.

#### Function of SR proteins: Redundant or unique?

Initial studies, referred to above, were consistent with the notion that SR proteins might be functionally redundant: The proteins tested showed similar abilities to activate constitutive splicing and to switch selection of alternative splice sites. To appreciate these results (and their limitations), the assays employed need to be described briefly. For essential splicing factor activity, individual SR proteins are added to so-called S100 extracts, which are essentially postribosomal cytoplasmic supernatants, usually prepared from HeLa cells, that contain all factors necessary for splicing (resulting from nuclear leakage during cell lysis) except the SR proteins, which remain in the nucleus and/or precipitate during extract preparation. Splicing of several different pre-mRNAs can be activated by virtually any single SR protein, suggesting both that there is little or no substrate specificity and that SR protein function is redundant in this assay. To measure effects on alternative splicing, SR proteins are added to nuclear extracts (which already contain endogenous SR proteins), and their ability to influence selection of alternative competing splice sites is determined. Most frequently, this assay has employed pre-mRNA substrates containing multiple 5' splice sites and a single 3' splice site, and the result obtained in most early experiments was a switch in preference so that the 5' splice site farthest downstream was used most frequently. Again, there was no evidence of substrate specificity, and the SR proteins tested behaved indistinguishably.

Despite these initial experiments suggesting at least partial redundancy, more recent work indicates clear differences in the behavior of individual SR proteins. Some of these differences will become more apparent in subsequent sections dealing with specific properties of the

SR proteins. Several studies with assays similar to those described above began to provide evidence that individual SR proteins can behave differently. Comparison of the *Drosophila* SR protein RBP1 (the apparent homolog of human SRp20) and ASF/SF2 revealed distinct properties in vitro. First, RBP1 was found to activate splicing in S100 extracts of only one of two test pre-mRNAs activated by ASF/SF2. In addition, the effects of the two proteins on alternative splice-site selection in nuclear extracts, although identical with an SV40 pre-mRNA, were distinct with adenovirus E1A pre-mRNA (Kim et al. 1992). Differential effects on alternative splice-site selection with these same two viral pre-mRNAs were also observed with individual purified human SR proteins, with SRp40 and SRp55 favoring the use of upstream rather than downstream 5' splice sites (Zahler et al. 1993a). It has also been possible to observe effects of SR proteins on the appropriate reporter genes overexpressed in transient transfection assays (Cáceres et al. 1994), and, in such assays, differences in the behavior of individual SR proteins have also been observed (Wang and Manley 1995; Screaton et al. 1995).

Fu [1993] used a somewhat different assay, which not only revealed differences in the behavior of specific SR proteins, but also provided insights to how they might function. It was shown that preincubation of a labeled pre-mRNA with a specific purified SR protein could result in commitment of the RNA to splicing, with commitment defined as the ability of the RNA to be spliced following the addition of excess competitor RNA and nuclear extract. A  $\beta$ -globin pre-mRNA was committed by preincubation with SC35 but not ASF/SF2 or other SR proteins, whereas HIV tat pre-mRNA was committed specifically by ASF/SF2 and not SC35. These experiments not only provided evidence for specificity in the behavior of SR proteins, but also indicated that these proteins can function at the earliest step in spliceosome assembly, a finding consistent with previous work (Fu and Maniatis 1990; Krainer et al. 1990a). This early function likely involves, in part, sequence-specific binding by the SR protein to the pre-mRNA.

#### RNA binding properties of SR proteins

The presence of RBDs in SR proteins naturally suggested that sequence-specific RNA binding would be important to their function. This was initially supported by studies of *Drosophila* Tra2, which binds with specificity to its target sequence, the *doublesex* (*dsx*) repeat element (Hedley and Maniatis 1991; Inoue et al. 1992). Evidence is now beginning to emerge reinforcing the idea that sequence-specific binding likely plays a significant general role in SR protein function.

The first demonstration of an interaction between a purified SR protein and specific RNA sequences was obtained with a recombinant derivative of ASF/SF2 lacking the RS domain (ASF $\Delta$ RS; Zuo and Manley 1994). The rationale for the deletion of this domain was that it is very basic (especially when the protein is isolated as the unphosphorylated bacterial form) and might enhance nonspecific interactions with RNA, perhaps obscuring

sequence-specific binding. A precedent for this behavior comes from the splicing factor U2AF, which has an RS-like domain that interferes with specific binding (to the polypyrimidine stretch of the 3' splice site; Zamore et al. 1992). Ultraviolet-cross-linking and gel mobility-shift assays showed that ASF/SF2 was able to recognize specifically two RNA fragments containing intact 5' splice sites, with mutations that disrupt the 5' splice site in each case reducing or eliminating binding. These results were satisfying because on the one hand they began to suggest how ASF/SF2 (and by extension other SR proteins) might function to activate splicing, or switch 5' splice sites, of many different pre-mRNAs, while on the other hand they offered the possibility of specificity if, for example, different SR proteins prefer distinct 5' splice site sequences. Although several subsequent studies, described below, support the view that ASF/SF2 function involves interactions at the 5' splice site, other studies have provided direct evidence that ASF/SF2 can function through sequences removed from 5' splice sites. While this does not argue against a role for 5' splice site binding, the functional significance of this interaction remains to be firmly established.

Do individual SR proteins have distinct RNA binding specificities? The first direct evidence that they do came from the analysis of proteins that bind to RNA sequences known as splicing enhancers. Although splicing enhancers and the role of SR proteins in their function are discussed below, here we mention several studies that provided evidence that SR proteins can bind to these elements with distinct specificities. Examination of the properties of a purine-rich bovine growth hormone gene exonic sequence that enhances splicing of an upstream intron showed that purified ASF/SF2 could both bind with specificity to this sequence and also enhance splicing when added to nuclear extract (Sun et al. 1993). In contrast, SC35 could neither bind the element nor activate splicing, providing evidence that the two proteins possess distinct RNA binding specificities. Several subsequent studies have extended this general finding to other enhancer elements and other SR proteins (e.g., Staknis and Reed 1994; Lynch and Maniatis 1995; Ramchatesingh et al. 1995). Together, these studies provide support for the idea that different SR proteins can recognize distinct, functionally important RNA sequences.

The above experiments did not identify the specific, minimal sequences that constitute the binding sites for individual SR proteins. To define such sequences, the SELEX protocol, which allows in vitro selection of high-affinity RNA binding sites (Tuerk and Gold 1990), was employed with several SR proteins. RS domain-deleted versions of ASF/SF2 and SC35 each selected purine-rich consensus sequences 8–10 bases in length (Tacke and Manley 1995). Importantly, however, the sequences selected by the two proteins were distinct, and the purified proteins bound preferentially to their own selected sequence. The full-length proteins bound RNA with the same specificity as the RS-deleted versions, providing evidence that the RS domain is not involved in determining RNA-binding specificity. One of the ASF/SF2-

selected motifs is virtually identical to a consensus sequence found in a number of purine-rich splicing enhancers (see below), and this sequence can in fact form an ASF/SF2-dependent splicing enhancer. Heinrichs and Baker (1995) carried out a similar SELEX analysis with *Drosophila* RBP1 and identified a distinct sequence not enriched in purines. Interestingly, this sequence was found in both the *dsx*-regulated intron and *dsx* repeat element, and evidence was presented that RBP1 participates in *dsx* regulation through interaction with these sequences (see below).

These findings have provided strong evidence that individual SR proteins can have distinct, functionally significant RNA binding specificities. Many questions remain, however. For example, are sequence-specific interactions the prime means by which SR proteins interact with RNA? Or might cooperative interactions with other SR proteins (Lynch and Maniatis 1995) or other splicing factors (e.g., U1 snRNP; Kohtz et al. 1994) be of equal or greater importance? The majority of SR proteins contain two RBDs, and in the case of ASF/SF2, both have weak binding affinity by themselves, but can function together both to increase affinity and to influence specificity (Cáceres and Krainer 1993; Zuo and Manley 1993; Tacke and Manley 1995). But why does ASF/SF2 contain two RBDs and SC35 only one? And how do the two RBDs actually interact to define binding sites?

### Splicing enhancers and SR proteins

The *cis*-acting elements in pre-mRNA required for intron removal consist of sequences encompassing the 5' splice site, the 3' splice site, and the branch site. During the first decade of splicing research, studies centered almost exclusively on the defining and characterization of these sequences and the factors that interact with them. Although this area of research remains productive, in metazoan species, another type of sequence—the splicing enhancer—has emerged. Splicing enhancers most frequently lie within exons and facilitate splicing of the upstream intron. Enhancers are usually found associated with introns that are considered to be weak (typified by a 5' or 3' splice site that is a poor match to the consensus) and that are frequently subject to alternative splicing. An important conclusion is that SR proteins appear to play a role in the function of most of, if not all, such elements. In this section, we discuss splicing enhancers by focusing on SR protein involvement in their function.

The most thoroughly studied splicing enhancer is found in the *Drosophila dsx* gene situated about 300 bases downstream of the regulated intron. The element contains the so-called *dsx* repeats (six copies of a 13-base sequence), which are required for function and which appear to bind the regulators Tra and Tra2 (for review, see Rio 1993). In addition to these proteins, several studies have provided evidence that certain SR proteins associate with the enhancer and are required for function. Tian and Maniatis (1992, 1993) showed that enhancer-dependent splicing could occur in vitro in a HeLa cell nuclear extract supplemented with Tra and Tra2, and that several of the SR proteins, plus Tra and Tra2, could

be found in a complex bound to the enhancer. Furthermore, preincubation of the pre-mRNA with Tra and Tra2 plus specific SR proteins was sufficient to commit the RNA to splicing, establishing a functional role for SR proteins in enhancer-dependent splicing *in vitro*. Subsequent work showed that the enhancer can function in the absence of Tra and Tra2 when moved closer to the regulated intron (Tian and Maniatis 1994). Inspection of the sequence of the enhancer revealed a purine-rich element (PRE) embedded between the fifth and sixth repeats (Lynch and Maniatis 1995). Individually, the PRE and the repeats can function as short-range enhancers, but the two together are required for activity at a distance. Evidence was presented that, contrary to expectation, Tra2, as well as certain SR proteins, interacts preferentially with the PRE, whereas Tra and other SR proteins interact with the repeats. A likely scenario that emerges is that cooperative interactions between these proteins, and perhaps other factors, are required to assemble a stable and specific complex on the *dsx* enhancer.

Heinrichs and Baker (1995) also provided evidence for the involvement of an SR protein in *dsx* regulation. As mentioned above, they first determined an RBP1 consensus binding site by SELEX analysis. Several matches to this consensus were observed in the *dsx* repeat region, and mutations in these sites reduced splicing efficiency in transient transfection assays. Furthermore, overexpression of RBP1 enhanced female-specific splicing in transfected cells, supporting a role for RBP1 in the function of the *dsx* enhancer. These results and the *in vitro* studies described above together provide evidence that SR proteins are necessary for the activity of the *dsx* splicing enhancer. Although there are differences between the details of these experiments, it is noteworthy that two quite different approaches implicated SR proteins in the function of this element.

Splicing enhancers have now been described in a number of mammalian pre-mRNAs. Most frequently they have been found in exons downstream of introns subject to alternative splicing. Whether this frequency results because enhancers are preferentially associated with introns that may be subject to regulation or because such introns are simply studied most frequently remains to be determined. That splicing of certain introns could be affected by nearby exonic sequences has been appreciated for some time. Until recently, whether such sequences function by influencing RNA secondary structure or by binding *trans*-acting factors was not known. Watakabe et al. (1993) provided the first evidence that a splicing enhancer could bind a specific splicing factor. They found that an enhancer sequence in the last exon of mouse IgM heavy chain pre-mRNA specifically bound U1 snRNP. An RNA-RNA interaction with weak base pairing to sequences in the enhancer is likely involved. Although the functional significance of U1 snRNP binding to the enhancer is not known, this snRNP also associates with a splicing enhancer in avian retroviral RNA (Staknis and Reed 1994). Watakabe et al. (1993) also defined the sequences required for enhancer function and compared

them with sequences in the half-dozen or so elements previously believed to have enhancer-like activity. In each case, one or more short, purine-rich sequences were found, leading to the suggestion that at least one class of splicing enhancer element consists of such sequences. Indeed, subsequent studies have, with few exceptions, identified related purine-rich sequences as splicing enhancers (e.g., Yeakley et al. 1993; Dirksen et al. 1994; Tanaka et al. 1994; Humphrey et al. 1995).

Despite the fact that U1 snRNP was the first *trans*-acting factor identified to interact with a splicing enhancer, SR proteins have been found to associate specifically with many of these elements (e.g., Lavigne et al. 1993; Sun et al. 1993; Staknis and Reed 1994; Lynch and Maniatis 1995; Ramchatesingh et al. 1995). These results provide a strong indication that, as suggested by the experiments with the *Drosophila dsx* enhancer, SR proteins play an important role in the function of mammalian enhancers. The SELEX experiments of Tacke and Manley (1995) provided further support for this idea. Specifically, a purine-rich octamer that emerged as a preferred ASF/SF2-binding site also matched a consensus that could be drawn from a number of previously described splicing enhancers, similar to that noted by Watakabe et al. (1993). Three copies of the octamer formed a splicing enhancer that could activate splicing of a weak upstream intron in nuclear or S100 extracts. Splicing in S100 extracts was found to require specifically ASF/SF2 (SC35 would not suffice), but also at least one additional, unidentified nuclear factor. This finding contrasts with the requirements for enhancer-independent splicing of strong introns, which are relatively promiscuous in their requirement for SR proteins and do not require additional nuclear factors.

SR proteins can also function negatively to repress utilization of specific splice sites. A purine-rich sequence located just upstream of a regulated 3' splice site in the adenovirus late transcript was found to inhibit use of that site when excess SR proteins were added to nuclear extracts (Kanopka et al. 1996). Inhibition appears to result from interference with recruitment of U2 snRNP to the nearby branch site, likely by a steric hindrance mechanism. Interestingly, the purine-rich sequence responsible for inhibition was found to function as a splicing enhancer when placed in the downstream exon, and, conversely, an active splicing enhancer, consisting of ASF/SF2 consensus binding sites, was found to block splicing when substituted for the natural, upstream sequence. These findings indicate not only that SR proteins can function negatively (see also Zuo and Manley 1993), but also that the position of the purine-rich binding site can determine whether the element acts positively or negatively. SR proteins have also been found to bind specifically to a well-characterized negative regulatory element in Rous sarcoma virus RNA (McNally and McNally 1996), although the mechanism of splicing inhibition in this case is not yet established.

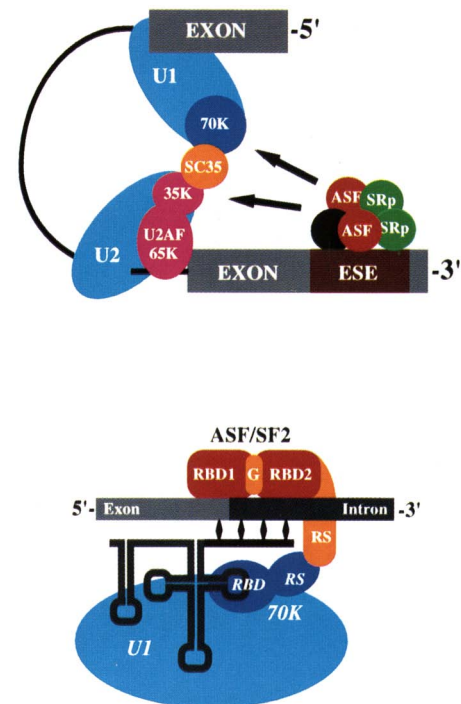
The studies described above indicate that splicing enhancers are likely to play important roles in controlling RNA splicing. As with transcriptional enhancers, it

seems likely that they will be found to consist of different sequence motifs, and to function via interactions with a variety of different factors. It is noteworthy, however, that splicing enhancers are usually situated in exons and their sequences must therefore be constrained by the requirements of the specific open reading frame. It is conceivable that this may have influenced the nature of the RNA–protein interactions that have evolved. Perhaps formation of stable complexes via cooperative interactions between factors with somewhat limited sequence specificity is most compatible with an RNA sequence that must perform a second, unrelated function. In any event, it now seems clear that complexes containing SR proteins and other nuclear factors assemble on exonic RNA sequences that are frequently rich in purines. How do such complexes actually function to activate splicing? Again analogous to the way in which transcriptional activators are thought to work, the answer appears to involve protein–protein interactions, as described in the next section.

### Protein–protein interactions: The role of the RS domain

Once bound to RNA, how do SR proteins exert their function? Accumulating evidence supports the view that specific protein–protein interactions among RS-domain proteins play key roles in early steps of spliceosome assembly (see Fig. 2). Wu and Maniatis (1993) used Far-Western, coimmunoprecipitation, and yeast two-hybrid analysis to provide evidence for several specific protein–protein interactions. Specifically, ASF/SF2 and SC35 were found to interact with each other, as well as with the *Drosophila* Tra and Tra2 proteins (also see Amreïn et al. 1994). Importantly, interactions between the SR proteins and two other splicing factors, the U1 snRNP-specific protein 70K (70K) and the essential splicing factor U2AF, were detected. U2AF facilitates binding of U2 snRNP to the branch site (Zamore and Green 1989) and consists of two subunits, a large [U2AF<sup>65</sup>] RNA-binding subunit that recognizes the polypyrimidine tract at the 3' splice site and a small [U2AF<sup>35</sup>] subunit (Zhang et al. 1992). It is noteworthy that all three of these polypeptides contain regions resembling RS domains. Two-hybrid analyses provided evidence that the SR proteins could interact with 70K and U2AF<sup>35</sup> simultaneously (Wu and Maniatis 1993).

The above results suggest two roles for SR proteins in spliceosome assembly (see Fig. 2, top). First, SR proteins (specifically ASF/SF2 and/or SC35), perhaps when bound to a splicing enhancer, may aid in the recruitment and binding/stabilization of U2AF to the polypyrimidine tract and subsequent recognition of the branch site by U2 snRNP (see also Lavigne et al. 1993; Wang et al. 1995). This may be particularly important in the case of weak and/or regulated 3' splice sites, such as in the *dsx*-regulated intron. It has recently been shown that SR proteins bound specifically to a downstream enhancer can facilitate binding of U2AF to the *dsx* polypyrimidine tract and that this interaction requires U2AF<sup>35</sup> (Zuo and Maniatis 1996). Second, SC35 (and perhaps other SR proteins)



**Figure 2.** SR proteins are involved in various steps of spliceosome assembly. (Top) ASF/SF2 and SC35 connect 5' and 3' splice site complexes through a network of protein–protein interactions (see text for detail). Activation of splicing of weak introns by exonic splicing enhancer (ESE) elements requires sequence-specific binding of SR proteins (SRp) such as ASF/SF2 (ASF), and other nuclear factors, such as, for example, Tra2 (black circle), to the ESE. SR proteins help to connect the enhancer complex to the 5' and/or 3' splice site via protein–protein interactions with U1 70K and/or U2AF<sup>35</sup> (arrows), thereby facilitating or stabilizing binding of U1 and/or U2 snRNPs. SC35, and perhaps other SR proteins, may also function to bridge the U1 and U2 snRNP complexes. (Bottom) SR proteins such as ASF/SF2 recruit U1 snRNP to and/or stabilize its interaction with the 5' splice site. ASF/SF2 binds at or near the 5' splice site via its RBDs, while its RS domain interacts with the RS region in the U1 70K protein. 70K is in turn bound via its own RBD to the first stem–loop of U1 snRNA, and the 5' end of U1 snRNA base pairs with the 5' splice site. Note that the mechanism by which the two RBDs of ASF/SF2 cooperate in RNA recognition is not known, nor is it clear that the RS regions of the two proteins, although necessary for association, interact directly.

teins) may help bring together the 5' and 3' splice sites by bridging U1 snRNP at the 5' splice site (via 70K) and U2 snRNP at the 3' splice site (via U2AF). This is consistent with previous work indicating that SC35 is required for interaction of U1 and U2 snRNP at the 3' splice site (Fu and Maniatis 1992b).

Additional evidence indicating that specific protein interactions are important for SR protein function was provided by Kohtz et al. (1994). These authors used *in vitro* assays to provide evidence for the direct interaction between ASF/SF2 (and other SR proteins) and U1 70K. They showed further that purified U1 snRNP and ASF/SF2 could cooperate to bind RNA containing an intact 5'

splice site. Analysis of U1 snRNPs lacking specific proteins provided evidence that 70K was necessary for cooperative binding. Furthermore, the ASF/SF2 RS domain was required for both cooperation with U1 snRNP and binding to 70K. These data support the idea that an important function of ASF/SF2 (and possibly other SR proteins) may be to aid in recruitment/stabilization of U1 snRNP to the 5' splice site (Fig. 2, bottom). This is consistent with other studies indicating that SR proteins can function in the recruitment of U1 snRNPs to 5' splice sites, a process that likely plays a role in SR protein-mediated selection of alternative 5' splice sites (Eperon et al. 1993; Zahler and Roth 1995). It is also possible, however, that the cooperative binding of U1 snRNP and ASF/SF2 may be important to recruit SR proteins to the 5' splice site rather than the other way around (Jamison et al. 1995). Consistent with this idea, recent studies have suggested that, in several cases, splicing can proceed *in vitro* in the absence of U1 snRNP if the concentration of SR proteins is increased (Crispino et al. 1994; Tam and Steitz 1994). This may reflect recruitment of U6 snRNP to the 5' splice site, as evidence has been presented that base-pairing between this sequence and U6 snRNA can be rate-limiting for splicing in the absence of U1 snRNP (Crispino and Sharp 1995), and experiments suggesting interactions involving SR proteins and U6 snRNP have been described (Tam and Steitz 1995; Roscigno and Garcia-Blanco 1995).

The emerging picture of SR protein function indicates that protein-protein interactions play an important role and that RS domains are necessary for association. These interactions aid in the recruitment and stabilization of snRNPs to the pre-mRNA, and there are likely multiple distinct points during the splicing cycle at which SR proteins intervene. But there are a number of important questions outstanding: For example, are the RS domains of different SR proteins functionally equivalent, or do they engage in specific, distinct interactions? Are the highly charged RS domains sufficient for interaction (i.e., do RS domains interact directly) or are other parts of the proteins required? Finally, how does phosphorylation influence RS domain interactions? Although nothing is really known about this specific question, it is clear that RS domains are extensively phosphorylated, and the next section summarizes what is known about SR protein kinases.

### Phosphorylation of SR proteins

SR proteins are phosphoproteins, and several studies have indicated that the majority of the modifications occur on serines in the RS domain. For example, a frequently used antibody that reacts with all SR proteins (mAb104; Roth et al. 1990) recognizes a phosphoepitope within the RS domain. Mapping of tryptic phosphopeptides produced from ASF/SF2 indicated multiple phosphorylations in the RS domain (Colwill et al. 1996). Three important questions regarding SR protein phosphorylation stand out. What kinases are responsible? What are the functional consequences of phosphorylation? And can phosphorylation be a regulatory mecha-

nism? Very little is known about the latter two questions, but information regarding the possible identity of the kinases involved has recently emerged.

The first activity capable of phosphorylating SR proteins was found associated with immunoaffinity-purified U1 snRNPs (Woppmann et al. 1993). The kinase responsible for this activity, which remains unidentified, was found to phosphorylate serines both in the RS domain of ASF/SF2 as well as in the related RS region of U1 70K. Although the functional significance of this phosphorylation is unknown, evidence has been presented that at least partial dephosphorylation of 70K is required for splicing catalysis, although not for spliceosome assembly (Tazi et al. 1993).

The first SR protein kinase to be purified and sequenced was SRPK1 (SR protein kinase 1; Gui et al. 1994a,b). Purified SRPK1 was shown to phosphorylate several SR proteins and its activity to be specific for serines in the RS domain. SRPK1 activity, and the levels of phosphorylated SR proteins, were both found to be highest in M phase cells, raising the possibility that the phosphorylation is cell-cycle regulated. Elevated concentrations of SRPK1 were also found to inhibit splicing *in vitro*. It is conceivable that excess kinase could have prevented dephosphorylation of SRPK1 targets, presumably SR proteins. As mentioned above in the context of 70K, and consistent with experiments showing that phosphatase inhibitors can block splicing catalysis *in vitro* (Mermoud et al. 1992), dephosphorylation of certain proteins appears to be required during splicing. Addition of protein phosphatase to nuclear extracts, however, also inhibits splicing, but at a very early step in spliceosome assembly (Mermoud et al. 1994). As addition of purified SR proteins could rescue the inhibition, it may be that a cycle of SR protein phosphorylation-dephosphorylation must occur during the splicing reaction.

Another kinase implicated in SR protein phosphorylation is Clk/Sty. This kinase, discovered by two groups independently, is a prototypical dual-specificity kinase, capable of phosphorylating tyrosines as well as serines and threonines (Ben-David et al. 1991; Howell et al. 1991). Clk/Sty was first implicated in SR protein phosphorylation from the results of yeast two-hybrid interaction assays that selected cDNAs encoding several of the SR proteins (Colwill et al. 1996). In fact, Clk/Sty contains an amino-terminal RS region enriched in arginine and serine residues, including multiple RS dipeptides. Consistent with the view that RS domains provide protein-protein interaction surfaces, the RS region of Clk/Sty and the RS domain of ASF/SF2 are both essential for interaction in the two-hybrid assay. Recombinant Clk/Sty efficiently phosphorylates serines in the RS domain of ASF/SF2, and the pattern of phosphorylation closely resembles that detected *in vivo*. The kinase itself is extensively autophosphorylated, on tyrosine and threonine as well as serine. The function of this autophosphorylation is unknown. Both Clk/Sty (Colwill et al. 1996) and SRPK1 (Gui et al. 1994a) are able to cause the redistribution of splicing factors within the nucleus when overexpressed. This ability must result from hy-

perphosphorylation of target proteins (i.e., SR proteins) because a catalytically inactive derivative of Clk/Sty, although localizing with SR proteins, does not cause their relocalization. The molecular interactions responsible for these effects remain to be elucidated.

Clk/Sty is the prototype of a family of related kinases, the Clk kinases. cDNAs encoding two similar but clearly distinct Clk kinases have been isolated from human cDNA libraries (Hanes et al. 1994). Although currently entirely speculative, the existence of multiple Clk isoforms raises the fascinating possibility that different cells may possess distinct combinations of SR proteins and Clk kinases, which could dictate cell-specific patterns of alternative splicing. Consistent with this possibility, cell-type and/or tissue-specific differences in the patterns of expression of SR proteins or mRNAs (Ayane et al. 1991; Diamond et al. 1993; Zahler et al. 1993a; Sreaton et al. 1995) and Clk mRNAs (Hanes et al. 1994) have been reported. In any event, further studies on SR protein kinases promise to yield important insights into both the mechanism and the regulation of pre-mRNA splicing.

### SR proteins and splicing in vivo

With the exception of the honorary SR protein Tra2, almost everything we know about SR protein function derives from in vitro analyses. As has been detailed above, SR proteins were discovered by in vitro assays and have been characterized largely by biochemical experiments. What is known about the proteins in vivo, for the most part, suggests that they function in vivo as they do in vitro. For example, a number of splicing factors are known to have distinct subnuclear localizations, and SR proteins share this distribution (for review, see Spector 1993). The results of transient cotransfection assays, in which specific SR proteins are overexpressed together with an alternatively spliced reporter transcript, have been largely consistent with expectations from in vitro experiments (Cáceres et al. 1994; Wang and Manley 1995; Sreaton et al. 1995), although one pre-mRNA (the SV40 early transcript) did not behave as predicted (accumulation of mRNA was strongly inhibited; Wang and Manley 1995).

As mentioned at the beginning of this review, true alternative splicing appears not to occur in yeast, and, for perhaps related reasons, authentic SR proteins have also not been described in *S. cerevisiae*. Thus it has not been possible to apply the powerful genetic-biochemical approach available in this organism to SR proteins. The only relevant genetic experiments to date have been with the *Drosophila* SR protein B52. B52 was first identified as a chromatin-associated antigen thought to play some role in transcription or chromatin structure (Champlin et al. 1991). Subsequent studies, however, showed the protein to be homologous to SRp55 (Roth et al. 1991) and to function in in vitro splicing assays (Mayeda et al. 1992). A B52 null allele results in lethality during development, establishing that B52 must provide at least one nonredundant function necessary for proper development (Ring and Lis 1994). Analysis of a number

of specific transcripts in homozygous mutant larvae, however, did not reveal any defects, leaving uncertain a role for B52 in specific splicing events in vivo. Peng and Mount (1995), in a screen for novel modifiers of the well-studied *white apricot* allele, identified a dominant allele of B52, called B52<sup>ED</sup>, that modified expression from several different mutant genes in a manner consistent with a role in pre-mRNA splicing. Interestingly, the mutation in B52<sup>ED</sup> resulted in the change of a single residue in the amino-terminal RBD, raising the possibility that altered RNA-binding properties of B52<sup>ED</sup> may be responsible for the mutant phenotypes observed. Nonetheless, considerably more work will be required to document the roles played by SR proteins in vivo.

### Summary and perspectives

Substantial evidence has accumulated over the last five years indicating that, at least in vitro, SR proteins are both essential for constitutive splicing and also able to participate in regulated splicing, by modulating selection of alternative competing splice sites as well as by functioning in the activation of splicing enhancer elements. We have also learned that SR proteins constitute a family of highly conserved proteins found throughout metazoa, as well as in plants (Lazar et al. 1995). Their activity appears to involve specific protein-RNA and protein-protein interactions that facilitate assembly of splicing complexes at specific sites. Phosphorylation, particularly of serines in the RS domain, likely plays an important role in their function and/or regulation. Therefore, we know a good deal about the SR proteins in general, but many questions remain unanswered. Indeed, in some cases, the questions that need to be asked are just becoming clear.

One important issue concerns the actual number of distinct SR proteins. The answer to this question will depend in part on how SR proteins are defined. The simplest definition might be any protein containing one or two amino-terminal RBDs and a carboxy-terminal RS domain. As illustrated in Figure 1, there are now eight known proteins that meet these criteria. Will this be the bulk of the SR proteins, or will there be tens or even hundreds more? The latter view might hold if there are SR proteins of lower abundance, and perhaps greater sequence specificity, than the currently known family members. These proteins might be analogous to Tra2 in *Drosophila*. If they do exist, however, a central, basic question arises: How is specificity achieved in splicing control? Is it driven by high-affinity, high-specificity interactions, analogous to the way transcriptional regulation appears most frequently to occur? Or could it be that cooperative interactions involving lower-affinity and/or lower-specificity RNA binding give rise to gene-specific regulation? If the latter is the case, cell-specific changes in the concentration of one or more of the abundant SR proteins, perhaps coupled with changes in phosphorylation, may suffice. For that matter, when we speak of changes in cell- or tissue-specific splicing of a particular transcript, how limited is the change? Is the splicing pattern of one or a very small number of tran-



scripts altered, or might there be a "wave" that changes the pattern of a significantly larger number of transcripts? Finally, again by analogy with transcription factors, which can have different types of DNA-binding domains (e.g., homeo domains, zinc fingers) as well as different types of activation, or effector, domains (e.g., acidic, glutamine rich), will there be different classes of alternative splicing factors? Supporting this possibility, certain hnRNP proteins, which contain RBDs but lack RS domains, have been shown to modulate alternative splicing in much the same way that SR proteins do (e.g., Cáceres et al. 1994).

Are there fundamental differences in the ways SR proteins function as essential splicing factors for introns with strong splicing signals, as concentration-dependent effectors of alternative splice-site selection, and as activators of splicing enhancers? One view is that all these activities center around the ability of SR proteins to recruit and/or stabilize snRNP binding to the pre-mRNA, functioning as a "glue" to hold the spliceosome together until catalysis causes the complex to dissolve. Perhaps with strong splice sites, cooperative interactions between for example U1 snRNP and SR proteins allow recognition of low-affinity SR-binding sites (possibly including the 5' splice site itself). It is noteworthy that analogous interactions have been described in transcriptional regulation. For example, the basal factor TFIID has been reported to stabilize DNA binding of the activator protein p53 (Chen et al. 1993). For weaker splicing signals, stronger SR protein-binding sites, which take the form of splicing enhancers, are necessary. In the case of competition between splice sites, some combination of splice-site strength, enhancer strength, and perhaps relative proximity between these elements, determines which sites are used. A related issue concerns the possible redundancy of SR proteins in vivo. One possibility is that they are redundant for some functions and unique for others, which would be consistent with in vitro data. For example, perhaps any SR protein can function in conjunction with strong splice sites, where the importance of sequence-specific RNA binding might be reduced. This view assumes that RS domains are able to function interchangeably, an important issue that needs to be addressed.

How are SR proteins regulated? As noted above, significant differences in expression of different SR proteins have been observed in various cell types or tissues. But the functional consequences of these changes remain to be determined. In addition, cDNAs corresponding to alternatively spliced forms of SR protein mRNAs have been described (e.g., Ge et al. 1991; Screaton et al. 1995), but their significance is unknown. Lastly, phosphorylation offers a clear possibility for regulation of SR protein activity. But little is known about how (indeed if) such regulation occurs. Assuming though that it does, it will eventually be important to understand the signaling pathways that activate the kinases involved.

SR proteins play critical roles in pre-mRNA splicing, apparently functioning at multiple points to facilitate the splicing reaction. They also likely play critical roles

in splicing regulation. Although recent studies have begun to provide considerable insights into mechanism, the above discussion makes clear that a good deal remains to be learned. Work in the future should answer many of the outstanding questions regarding the biochemistry of the proteins. A perhaps more long-term, but equally if not more important, goal will be to uncover the functions and regulation of SR proteins in vivo.

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