Determinants of SR protein specificity

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The SR (Ser–Arg) proteins are a family of nuclear factors that play multiple important roles in splicing of mRNA precursors in metazoan organisms, functioning in both constitutive and regulated splicing. Certain of these functions are redundant, such that any single SR proteins will suffice, but other functions are unique and are specific to a given family member. A number of studies during the past year have investigated the basis for SR protein specificity.

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Abbreviations

dsx	doublesex
ESE	exonic splicing enhancer
PRE	purine-rich element
RBD	RNA-binding domain
RNP	ribonucleoprotein
SELEX	systematic evolution of ligands by exponential enrichment
SR protein	Ser–Arg protein
tet	tetracycline

Introduction

SR proteins (Ser–Arg proteins) constitute a family of pre-mRNA splicing factors that are highly conserved throughout the metazoa (for reviews, see [1,2]). These proteins — of which about ten are currently known — have multiple functions in splicing and indeed appear to participate in virtually every step of the reaction. Biochemical experiments have provided strong evidence that SR proteins play essential roles in general, or constitutive, splicing. But they seem to be equally important in splicing regulation being able to modulate selection of alternative splice sites in a concentration-dependent manner and to contribute to activation (and repression) of splicing through interaction with elements in the pre-mRNA known as splicing enhancers (or silencers). Given their crucial role in constitutive splicing, it is somewhat surprising that SR proteins appear not to be conserved in *Saccharomyces cerevisiae*. Their presence thus seems to be more closely correlated with the complex alternative splicing that is characteristic of metazoans.

The primary structure of SR proteins — which range in size from about 20–75 kDa — is simple, with each containing one or two amino-terminal ribonucleoprotein (RNP)-type RNA-binding domains (RBDs) and a carboxy-terminal RS domain, which consists largely of repeating arginine–serine dipeptides and probably functions in protein–protein interactions (e.g. [3,4]). Although early work suggested that SR proteins might be functionally redundant, a number of subsequent studies have suggested that each protein probably performs at least some nonredundant functions. Most convincingly, one SR protein, SRp55/B52, is essential for proper development in *Drosophila* (e.g. [5]) and another, ASF/SF2, is essential for viability of a chicken B-cell line [6]. Given the ability of SR proteins to display both redundant and unique behavior, an important question is what features of the proteins are responsible for specificity? For example, how critical is sequence-specific high-affinity RNA binding for function? Do RS domains, which vary among SR proteins but are highly conserved evolutionarily, perform unique or redundant functions? These questions have received considerable attention during the past year and are the focus of this brief review.

The contribution of RNA binding specificity and affinity to SR protein function

Initial evidence for divergent RNA binding specificities among SR proteins came from the observation that two SR proteins, ASF/SF2 and SC35, differed significantly in their abilities to commit specific pre-mRNAs to the splicing pathway [7]. Since then, efforts made to understand the RNA-binding properties of SR proteins have included extensive application of the SELEX protocol (systematic evolution of ligands by exponential enrichment), which allows the selection of high-affinity binding sites from pools of random sequence RNA [8]. SELEX data have been obtained for a number of SR proteins, including human ASF/SF2 [9], SC35 [9,10•], SRp40 [11], and 9G8 [10•], and *Drosophila* RBP1 [12] and B52/SRp55 [13]. Overall the results clearly established that SR proteins are sequence-specific RNA binding proteins with distinct RNA binding specificities. SR proteins with two RBDs apparently require both for specific RNA binding [9,13]. In general, selected sequences yielded short consensus binding sites of 6–10 nucleotides without evidence of secondary structure requirement, although the B52 recognition motif may involve a hairpin-loop structure [13].

The functional significance of sequences identified by SELEX has been an important issue. A considerable amount of data supports the idea that the RNA sequences that form high-affinity binding sites for individual SR proteins are sufficient to function as exonic splicing enhancers (ESEs) [14,15]. These ESEs can be activated through specific binding of their cognate ligands [9,10•,11,16] and, in several instances, additional evidence for the biological significance of these sequences has been obtained. SELEX data predicted the presence of several binding sites for the *Drosophila* SR protein RBP1 within the so-called doublesex (dsx) repeats, which are essential elements of the prototypical ESE, the *Drosophila* dsx enhancer [12]. Site-specific UV cross-linking subsequently

showed that RBP1 present in *Drosophila* Kc cell extracts binds strongly to the dsx repeats, although binding seems to require the presence of two other factors, Tra and Tra2, which are absolutely required for enhancer activity [17]. In another example, SELEX with ASF/SF2 provided two closely related consensus binding sites, one of which, RGAAGAAC — termed the ASF/SF2 octamer (see Table 1) — showed high sequence similarity to purine-rich motifs previously identified in several natural ESEs [9]. The observation that multiple copies of the ASF/SF2 octamer indeed constitute a powerful ESE and that a number of previously defined ESEs match this sequence suggests an important role for ASF/SF2 in the activation of natural purine-rich ESEs [9].

More recently, however, binding assays with biotinylated RNA revealed that, in addition to ASF/SF2, one or both human homologs of *Drosophila* Tra2 bind the ASF/SF2 enhancer in HeLa nuclear extracts [18•]. Both human Tra2 proteins were shown to bind oligo(GAA) with high affinity and to specifically activate the ASF/SF2 enhancer *in vitro* [18•], supporting the notion that ASF/SF2 and Tra2 proteins have overlapping RNA binding specificities. Given the documented properties of Tra2 as a splicing regulator in *Drosophila* sex determination [19] (for review, see [20]) and the additional finding that Tra2, unlike ASF/SF2, is not an essential splicing factor [18•], targeting of purine-rich ESEs by Tra2 may be a relevant mechanism for the regulation of splicing in mammals. How and why ASF/SF2 and Tra2 recognize such similar sequences remains to be determined. Interestingly, the second ASF/SF2 consensus binding site — the decamer AGGACGAAGC — is strikingly similar to a purine-rich element (PRE) in the dsx enhancer (see Table 1). Consistent with this, ASF/SF2 binds specifically to the PRE within the dsx enhancer [17] and the PRE can serve as an ASF/SF2-specific ESE [16].

Although most natural ESEs identified to date seem to be purine-rich, a variety of non-purine-rich ESEs have been identified by functional SELEX, that is iterative selection from randomized sequences employing either *in vitro* [21] or *in vivo* [22] splicing assays for selection rather than binding. Using a nuclear-extract-based *in vitro* selection procedure, Schaal and Maniatis [23] identified both purine- and pyrimidine-rich ESEs. The purine-rich sequences bore some resemblance to the ASF/SF2 binding sites described above, and the two pyrimidine-rich sequences could be activated specifically by SC35 in an SR-protein-dependent splicing assay. A screen for ESEs within the pre-mRNA identified two related SC35-specific ESEs in the first and second exon respectively [24•]. Site-specific UV cross-linking indicated that SC35 binds to the heptamer UGCUGUU in human β-globin exon 2 and a highly similar heptamer (UGCCGUU) was detected in exon 1. Strikingly, two different groups used conventional SELEX to determine virtually identical pyrimidine-rich consensus sequences that bear significant similarity to the SC35 heptamer (Table 1), although the functional significance of these sequences was not determined [9,10•]. Taken together, these results suggest that both conventional and functional SELEX are suitable approaches to identify related motifs that can function as SR protein-specific ESEs; moreover, the presence of ESE-like elements in the β-globin pre-mRNA, which was not previously believed to require such sequences for splicing, indicates that these elements may be more widespread than anticipated.

Table 1

SR protein biding sites.

Protein	RNA Sequence	Context
Tra2β	AAGAAGAA	SELEX [18•]
ASF/SF2	RGAAGAAC	SELEX (octamer) [9]
	AGGACAGAGC*	SELEX (decamer [9]
	$AA(AGGACAA)_2AA$	dsx PRE [16]
	SRSASGA	Functional SELEX [26]
SC35	GUUCGAGUW*	SELEX [9]
	UGUUCSAGWU*	SELEX [10•]
	UGCNGYY	Functional SELEX [23]
	UGCUGUU	β-globulin [24•]
9GB	AGACKACGAY	SELEX [10•]
	GGACGACGA	Functional SELEX [23]
SRp20	CCUCGUCC	Functional SELEX [23]
	GCUCCUCUUCC	Calcitonin/CGRP [25]
	YWCUUCAU	SELEX (mutant 9G8) [10•]

Comparison of high-affinity binding sites for individual SR proteins and Tra2β with sites defined by function. Except for those sequences marked by asterisks, evidence for their function in splicing enhancement has been provided (see indicated references for details). Symbols for alternative bases: K = G/U, R = A/G, S = C/G, W = A/U, Y = C/U, N = A/C/G/U.

Extending these conclusions, conventional SELEX [10•] and functional SELEX [23] have also led to the identification of splicing enhancer sequences specific for 9G8 (Table 1), with GAC repeats suggested as a consensus motif by both studies. In addition to the RBD, a CCHC (in the single letter code for amino acids) Zn knuckle — which is characteristic of 9G8 — proved to be a determinant of RNA-binding specificity of 9G8. Mutation of the gene encoding 9G8 to change the first two cysteine residues to glycines altered the outcome of the SELEX procedure, generating mostly pyrimidine-rich sequences [10•]. Moreover, *in vitro* binding and splicing activation studies indicate that SRp20 can activate ESEs that carry the consensus binding site YWCUUCAU (where Y = C/U and W = A/U) for mutant 9G8 [10•]. This is likely to reflect the high sequence similarity between the RBDs of 9G8 and SRp20, which does not contain a Zn knuckle. Pyrimidine-rich sequences closely matching this consensus motif have also been identified as natural targets of SRp20, including an intronic polyadenylation enhancer in the calcitonin/CGRP gene [25] (Table 1).

Liu *et al.* [26•] employed functional SELEX based on complementation of SR-protein-depleted extracts with specific

recombinant SR proteins to identify splicing enhancers specific for ASF/SF2, SRp40 and SRp55. The motifs obtained differed significantly from the consensus binding sites previously determined by conventional SELEX [9,11,13]. A difficulty in evaluating the general significance of these motifs stems from the observation that the effectiveness of individual sequences depended at least in part on the context of the entire selected sequence: the individual motifs were found to have reduced activity when transferred from the selected sequence to a sequence from the initial random pool. The authors suggested that the selected sequences might contain more degenerate, medium or low-affinity binding sites, although this raises the question of how the high degree of SR protein specificity observed in the activation assay was achieved. In addition, this context effect seems to somewhat diminish the value of these motifs in predicting the presence of specific ESEs within genes. In fact, when the authors used statistical scoring methods to determine the presence of their deduced consensus sites in four previously characterized natural enhancers, the highest scores frequently mapped outside the enhancer sequences. It appears that the results of functional SELEX may depend, at least in part, on experimental design. The particular sequence context and the stringency of the selection procedure — only three rounds of selection were used in the experiments of Liu *et al.* [26•] — are likely to be important variables that can influence the strength and accordingly the sequence of the selected elements. Nonetheless, this study provides evidence that at least in certain contexts degenerate, low-affinity sites can function as SR-protein-specific ESEs.

In summary, conventional and functional SELEX approaches have contributed greatly to our current understanding of the specificity of SR-protein–RNA interactions and their significance for splicing activation. A conclusion from these studies is that both specific, high-affinity sequences and degenerate, lower-affinity sites can function as SR-protein-specific ESEs. It seems likely that nature employs sequences with a continuum of affinities as ESEs. Important questions will be to understand how these sequences are integrated with other splicing signals in the pre-mRNA and how they contribute to splice site recognition on the one hand and the control of alternative splicing on the other.

RS domains: specific or redundant?

The development of both *in vitro* and *in vivo* assays that reveal nonredundant functions for individual SR proteins allowed the design of experiments to determine whether or not RS domains contribute to SR-protein specificity. Although the experimental approaches varied considerably, all used chimeric proteins with heterologous RS domains. One of the first efforts employed a 'commitment' assay, in which pre-incubation of different pre-mRNAs with specific individual SR proteins allows subsequent splicing in a manner dependent on the identity of the SR protein [7]. Chandler *et al.* [27] showed that, for two SR proteins (ASF/SF2 and SC35) with two different pre-mRNAs, the RBD was sufficient to determine specificity and that the identity of the RS domain was not important. In contrast, using a different assay Gravely *et al.* [28•] provided evidence that the activity of an RS domain correlated directly with the total number of arginine and serine residues (or RS dipeptides) contained in it. Although the effects were relatively small (2–4 fold), the pattern held with only minor exceptions for RS domains from six different SR proteins. In this assay, RS domains were fused to the bacteriophage MS2 RBD and the purified fusion proteins used to activate splicing of substrates containing a single MS2 binding site situated downstream of an intron with a weak 3′ splice site. The apparent discrepancy between these two studies could reflect the very different assays employed. If so, this would suggest that RS domains might show at least quantitative differences in enhancer-dependent splicing; however, it is also possible that the absence of an authentic SR protein RBD in the MS2–RS fusions exaggerated the importance of RS domain identity. For example, the RS domain of ASF/SF2 is necessary but not sufficient for its well-characterized interaction with the U1 snRNP-specific 70 kDa protein [29] and, perhaps reflecting this, the MS2–RS fusion proteins cannot function in the absence of authentic SR proteins [30].

RS domains have for some time been implicated in subcellular protein targeting [31,32]. SR proteins accumulate predominantly in nuclear structures referred to as speckles. Although it is beyond the scope of this review to discuss the details of SR protein nuclear localization, speckles seem likely to function as storage and/or recycling sites for these and other splicing factors (reviewed in [33,34]). Using transient cotransfection assays, Cáceres *et al.* [35] provided evidence that RS domains can have different capacities to direct SR protein localization to speckles. Specifically, they showed that, whereas the RS domains of both SRp20 and ASF/SF2 were sufficient to target a heterologous, unrelated protein to the nucleus, only the SRp20 RS domain could direct the fusion protein to the speckles. This finding supports the view that RS domains can behave distinctively, although its physiological importance is unclear: ASF/SF2 contains other motifs that are capable of directing the protein to speckles. The same authors [36] also provided evidence that some, but not all, SR proteins can 'shuttle' between the nucleus and cytoplasm (e.g. [37]). Again, using chimeric proteins and transient transfection assays, they showed that RS domains could play a dominant role in this process. For example, the RS domain of ASF/SF2, a shuttling protein, could convert SRp40, a nonshuttling protein, into a shuttler, whereas an ASF/SF2 derivative containing the SRp40 RS domain was unable to shuttle. Based on these results, the authors proposed that individual RS domains have distinct properties in directing different patterns of subcellular localization. This is a plausible and interesting possibility but it will be important in the future to confirm

these findings under more physiological conditions and to show that such differences are functionally significant.

An important question in considering possible RS domain functional redundancy is whether different RS domains can substitute for one another under physiological conditions *in vivo*. This issue has been investigated in two quite different systems. In one study, Wang *et al.* [38•] used a chicken B-cell line (DT40-ASF) in which the only source of ASF/SF2 is from a tetracycline (tet)-repressible promoter. DT40-ASF cells die in the presence of tet and thus a test for function is whether a given protein, produced following stable transformation, can rescue the tet-induced lethality. Although ASF/SF2 derivatives lacking, or with deletions in, the RS domain were previously shown to be nonfunctional in this assay [6], cells expressing ASF/SF2 chimeric proteins containing RS domains from any of several different SR proteins were fully viable in the presence of tet. Either of the two RS domains from human Tra2 also appeared to be fully functional, extending the apparent RS domain redundancy beyond classic SR proteins. An RS-like domain from the splicing factor U2AF65 was inactive, however, and the purified ASF/SF2–U2AF65 RS-domain chimera was also completely nonfunctional in *in vitro* splicing assays. This seemed entirely reasonable at the time, as previous studies had noted significant functional differences in the ASF/SF2 and U2AF65 RS domains. For example, the former has been implicated in protein–protein interactions ([39] and references therein), the latter in protein–RNA interactions [40]. Additionally, in contrast to the requirement of the ASF/SF2 RS domain for viability of chicken DT40 cells, the U2AF RS domain is dispensable in *Drosophila* [41•]. Overall, however, these studies indicate that RS domains are in general functionally redundant for whatever nonredundant ASF/SF2 functions are required for viability of DT40-ASF cells.

A different picture emerged when the ability of different RS domains to substitute for one of the two RS domains of Tra2 in *Drosophila* was examined [42•]. In this case, different RS domains varied considerably in their ability to restore Tra2 function. The authors tested RS domains from two SR proteins, from Tra and from the *Drosophila* homologue of U2AF65, dU2AF50, in assays measuring Tra2 function in somatic sex determination and in the male germline. Remarkably, the two SR protein RS domains displayed the weakest function, whereas the dU2AF50 RS domain functioned nearly as effectively as the Tra2 RS domain it replaced. These results indicate that in the context of *Drosophila* Tra2, RS domains can differ considerably in activity.

There are a number of possible explanations, which are not mutually exclusive, for the differences in RS domain behavior in the two assay systems just described. One reflects the fact that Tra2 is not a classic SR protein. Its structural organization is distinct from that of SR proteins, it does not function in constitutive splicing *in vitro* and it is not essential for *Drosophila* viability ([18•] and references therein). Nonetheless, it is important to recall that either of the RS domains from human Tra2 will functionally substitute for the ASF/SF2 RS domain in DT40 cells [38•], indicating that, at least in vertebrates, these domains are not completely incompatible. A second possibility is that there are species-specific differences in RS domain compatibility between insects and vertebrates, such that RS domains have assumed a more generic function in vertebrates. Finally, RS domains may be largely redundant in the functions measured in the simpler cell-viability assay but less so in the context of the intact organism. This view is attractive because it is consistent with the very high degree of sequence conservation between individual SR proteins (and Tra2) throughout evolution, which is highly suggestive of specific functions. Why the U2AF RS-like domain behaved so differently in the two systems is completely unclear, however, and illustrates that significantly more work is required to understand the intricacies of RS domain function.

Studies conducted to date have given mixed results regarding the specificity of RS domain function. It seems likely that, just as with SR proteins themselves, RS domains have both redundant and nonredundant functions. Future studies should help to delineate the specific interactions involved, to define their structural basis and mechanistic consequences and, ultimately, to elucidate their contribution to splicing control.

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