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Review

Silencers regulate both constitutive and alternative splicing events in mammals

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Abstract. Constitutive and alternative splicing events are regulated, in higher eukaryotes, by the action of multiple weak *cis*-acting elements and *trans*-acting factors. In particular, several evidences have suggested that silencers might have a fundamental role in preventing pseudoexon inclusion in mature transcripts and in defining constitutive exons by suppressing nearby decoy splice sites. Moreover, silencer elements allow the recruitment of regulatory factors to alternatively spliced exons, therefore participating in the modulation of alternative splicing pathways. Here we focus on splicing repression mechanisms in mammals, with particular concern to both exonic and intronic silencer elements, secondary structure formation and role in human genetic disease.
Recently, in addition to the availability of a growing number of sequence elements deriving from the analysis of individual regulated exons, approaches have been developed that allowed the systematic identification of splicing silencers. These methods and are briefly described, as well as the motifs they retrieved, and summary of silenced exons is provided.

Key words. Splicing silencer; *trans*-acting factor; *cis*-acting elements; constitutive splicing; alternative splicing; secondary structure; human genetic disease.

Introduction

Most protein-encoding genes in metazoans are interrupted by introns; production of functional messenger RNAs (mRNAs) in these organisms is therefore critically dependent upon the accuracy of pre-mRNA splicing, a highly regulated process assuring that introns are removed and an ordered array of exons is maintained in mature transcripts. The excision process involves two transesterification reactions and takes place in a large macromolecular complex, the spliceosome, that is composed of over 100 proteins and at least five RNA molecules (reviewed in [1]). While there is considerable understanding of the splicing reaction itself, much less is known about how splice sites are appropriately paired. It is now established that, in mammals, exons (and not introns, as initially speculated) are recognized by the splicing machinery [2]; this notion is referred to as the 'exon definition model' and most probably holds true in the great majority of cases. As a consequence, our present understanding of splicing processes in mammals is not far from a finding-needles-in-a-haystack view [3]: the typical situation of most pre-mRNAs envisages relatively small exons embedded in large intronic sequences with short splicing signals to establish which is which.
The panorama is even more complicated when alternative splicing events are considered: about 46–60% of human genes encode alternatively spliced transcripts [4, 5], and this mechanism is thought to provide gene expression

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control and protein functional diversification [6]. Alternative splicing events can be expected to entail additional complexity if compared to constitutive ones since specification of time (developmental stage, cell cycle phase etc.) and space (cell type, organ, tissue) information is often required. Moreover, in many cases, space and time coincide for more than one alternative event, resulting in a combination of different transcripts within the same cell. The major question is where does all this information reside? What factors either *cis*- or *trans*-acting regulate both constitutive and alternative exon recognition?

It is now clear that the presence of well-defined *cis*-elements (fig. 1), namely the 5′ and 3′ splice sites, the branch point and the poly-pyrimidine tract, are necessary but not sufficient to define intron-exon boundaries [7]. The concerted recognition of multiple weak elements located within exons as well as in intronic regions is nowadays thought to provide the necessary splicing information [8, 9]. Sequences having a role in splicing regulation are generally referred to as either enhances or silencers, depending on the effect they exert. Enhancers were discovered prior to silencers, and have been a matter of intense investigation. In particular, the best-studied exonic splicing enhancers (ESEs) represent binding sites for SR proteins [10, 11], which are thought to have a role in the initial steps of spliceosome assembly [12, 13] and to promote splicing by recruiting spliceosomal components through protein-protein interactions [14, 15]. Splicing enhancers located within intronic regions (ISEs) have also been described, although these elements as well as the *trans* factors they bind to have not been characterized thoroughly. While extensive analysis of ESEs and ISEs has led to wide knowledge and also to the availability of web facilities to identify them [16] (ESEfinder, http://rulai.cshl.edu/tools/ESE), splicing silencers remain enshrouded in poor understanding and ambiguous attribution.

This review will focus on splicing repression mechanisms in general with particular concern to both exonic and intronic splicing silencer elements (ESS and ISS, respectively). In particular, only elements and regulatory mechanisms pertaining to mammalian splicing regulation are treated in detail, while viral and lower eukaryote systems are not thoroughly described but rather cited to support experimental data or model definition.

Silencers and constitutive splicing

Human introns are typically thousands of bases long, and the analysis of human genes [17, 18] revealed that sequences matching splice site consensuses (pseudosites) are highly abundant in intronic regions with pseudoexons (i.e. intronic sequences displaying good 3′ and 5′ splice sites), outnumbering real exons by an order of magnitude [17]. Most likely, the majority of these false exons are not recognized by the spliceosome due to the intrinsic weakness of their splicing determinants. Indeed, the detailed analysis of one of these pseudoexons indicated that it was affected by multiple splicing defects [17], and comparison of pseudoexons with real exon sequences revealed that the former have, in general, weaker splice sites and lower ESE densities [18]. Nonetheless, some recent observations [19, 20] have suggested that a subpopulation of pseudoexons might exist in the human genome that require only subtle changes to become splicing competent. In particular, Pagani et al. [19] were able to demonstrate that a pathological 4-bp deletion in the *ATM* gene intron 20 disrupted a novel silencer sequence that prevented pseudoexon inclusion. The novel element was found to be complementary to U1 small nuclear RNA (snRNA) and to interact with U1 small nuclear ribonucleoprotein (snRNP) particles. These latter factors have been shown to be involved in the initial steps of spliceosomal formation by recognizing the 5′ splice site through base pairing between U1 snRNA and the 5′ site exon-intron junction [21, 22]; the authors therefore speculated that this element might exert its silencing activity by interfering with the nearby pseudosite. Whatever the mechanism underlying splicing repression in *ATM* intron 20, the above observation raises the interesting possibility that at least a proportion of pseudoexons might be prevented from splicing by *cis*-acting negative regulatory elements rather than by multiple splicing defects. This hypothesis is also in line with a previous report indicating [23] that splicing inhibitory sequences are rather common in the human genome, and therefore suggesting that splice silencing might be one of the mechanisms that repress pseudoexon inclusion. Blanchette and Chabot [24] had hypothesized that hnRNPA1 might have a role in intron definition. These authors compared human complementary DNA (cDNA) 1 and intronic sequences and noted that there is

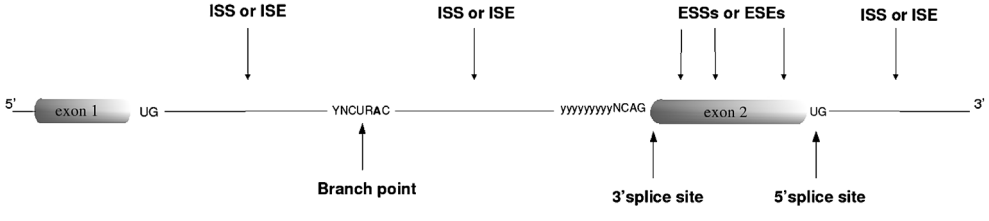

Figure 1. Schematic representation of cis-acting splicing regulatory elements.

a marked prevalence for hnRNPA1 binding sites in the latter, particularly in close proximity to 5′ and 3′ splice sites. The authors therefore envisaged a situation whereby interaction between intron-bound A1 molecules might cause looping out of intronic portions, thereby preventing inclusion of encompassing sequences and resulting in closer proximity of flanking exons. More recently, several reports have used different approaches to systematically identify silencer elements (see below), and in most cases the results are consistent with pseudoexons being enriched in ESS compared with real exons [18, 25, 26].

Intronic sequences that can repress splicing might therefore have a fundamental importance in the correct definition of real exons, and an exon prediction algorithm that integrates the contribution of putative ESS improves the identification of real exons versus pseudoexons [26]. In particular, it has recently been suggested that intronic silencers might play a relevant role in the definition of real splice sites. Indeed, Wang and co-workers [26] analyzed intronic regions located upstream of real 3′ splice sites and noted that silencers are more abundant in those sequences that contain decoy 3′ splice sites. The authors therefore proposed that while the presence of ESEs might help to identify the approximate location of real exons, silencer sequences might function to define splice sites by silencing nearby decoy sites.

Silencers and alternative splicing

Except for splice sites and branch points, most splicing regulatory elements were initially identified through the description and detailed analysis of alternative splicing events. Silencers were no exception to the rule, and most of our present knowledge comes from the study of viral transcripts: viruses that infect eukaryotic cells have evolved to exploit RNA splicing as a means to expand the coding capacity of their typically small genomes and as a mechanism of post-transcriptional gene regulation. The study of viral splicing regulation has therefore provided a great wealth of data concerning regulatory *cis*- and *trans*-acting factors; this knowledge has easily been extrapolated to cellular processes given that viruses rely on cellular pathways and components for their own splicing regulation.

Model organisms have also been instrumental in the identification of splicing regulatory elements and pathways. In particular, *Drosphila* has proved an extremely useful model to study alternative splicing events. Analysis of splicing regulation of the P-element transposase and of transcripts controlled by Sxl has provided relevant clues to the understanding of splicing silencing in mammals. It is now clear that whether an exon is constitutively or alternatively spliced often depends on both its splice site strength and on the presence of additional regulators. Alternatively spliced mammalian transcripts generally contain several *cis*-elements with positive or negative functions that often antagonize each other's action to achieve splicing regulation. Such observations have led to models of combinatorial control, whereby regulation of alternatively spliced exons is achieved through the net influence of several proteins that bind to different *cis*-elements flanking regulated splice sites (reviewed in [3]).

Cis-acting elements: few common features

Table 1 summarizes the location and function of identified ESS and ISS involved in the regulation of alternative splicing events. In several instances more than one element is employed to achieve splicing repression or to counteract the effect of splicing enhancers. Often, the precise function of these elements remains elusive, and features such as secondary structure formation (see below) or nearby sequence composition can deeply influence the action of these elements. Yet, in several cases these sequences have been shown to preserve their function when artificially located in a heterologous system, indicating that they represent autonomous regulators. Most of these sequences have been identified through the use of standard molecular biology techniques and validated using biochemical and cell transfection experiments. They therefore derive from the study of single (or a few) regulated splicing events. Conversely, recent work (see below) has provided lists of putative silencers and methods to systematically identify splicing repressors. These elements do not pertain to specific exons/transcripts and have been only partially validated experimentally. These sequences have therefore been grouped into a separate table (table 2) and might provide a useful resource to compare putative silencers that will be identified in specific studies on splicing regulation. Indeed, contrary to splicing enhancers, no dedicated algorithm has been made available for the prediction of silencers up to now, making hand inspection of candidate sequences the only way to detect significant homologies.

Trans-acting factors: ambiguity is a rule

Several protein factors have been reported in one or more specific case to bind either directly or indirectly to silencer elements. We describe below only the best-studied splicing factors involved in splice silencing.

Heterogeneous nuclear ribonucleoproteins

hnRNPs have been reported to be involved in multiple aspects of messenger RNA biogenesis and metabolism [27]. They constitute a large group of RNA-binding pro-

teins that localize primarily in the nucleus, altough some of them engage in nucleo-cytoplasmic shuttling [28].

The most abundant hnRNPs belong to the A/B type. This family consists of a number of isoforms (A1/A1B, A2/B1, B2 and A3) showing extensive post-transcriptional and -translational modifications and sharing common RNA substrate specificities [29]. Members of the hnRNP A/B family have been identified as factors involved in the modulation of splice site selection. Using model substrates containing competing 5′ splice sites, hnRNP A1 was shown to induce a remarkable shift towards the use of distal 5′ splice sites [29]. This same protein has also been shown to affect 3′ splice site choice, as in the case of the K-SAM exon in fibroblast growth factor receptor-2 mRNA [30], finally resulting in exon skipping. In general, hnRNPA1 has been shown to negatively regulate several alternative exons (see table 1); application of the SELEX (systematic evoltion of ligands by exponential enrichment) approach has identified UAGGGA/U as the highest affinity 'winner' sequence for hnRNP A1 [31]. Yet, not all exons regulated by this protein have been reported to display winner binding sites, indicating that either hnRNP A1 binds other RNA sequences, maybe with lower efficiency, or that its effect is mediated by protein-protein interactions on the regulated pre-mRNA. Indeed, hnRNP A1 can interact with itself [32] and with other hnRNP proteins, as well as with U2 and U4 snRNPs [33]. It is therefore conceivable that either direct or indirect recruitment of hnRNP A1 to a regulated exon might result in the formation of a larger complex, which sterically affects spliceosome positioning or reduces its affinity for the splice sites.

hnRNP proteins belonging to the H group (hnRNPs F, H, H' and 2H9) are encoded, in humans, by different genes but share a similar structure [34] and a common core-binding site (GGGA) [35]. In particular, hnRNP H has been shown to act as both splicing repressor and activator. This factor is involved in promoting the inclusion of *c-src* exon N1 in neuronal cells [36]; conversely, hnRNP H acts as a powerful splicing repressor (table 1) in the rat β-tropomyosin (*β-TM*) gene by binding to the the UGUGGG motif and causing exon 7 skipping in nonmuscle cells [37]. The different action of hnRNP H in these two instances might be accounted for by the location of its binding sites: intronic in *c-src* and exonic in *β-TM*. Indeed, it has been shown [38] that the creation of an hnRNP H binding site within *CFTR* exon 9 results in diminished exon inclusion and that insertion of a UGUGGG motif in a constitutive exon determines partial exon skipping [18].

Probably, the best-studied hnRNP family member is hnRNP I, also known as polypyrimidine tract binding protein (PTB), which has been proposed to be a global repressor of weak or regulated exons [39]. PTB was initially identified as the factor binding the pyrimidine-rich region [40,41] and was consequently hypothesized to be a splicing positive regulator. PTB has strong RNA binding activity [42], and its preferred RNA binding site is UCUU flanked by pyrimidines [43]. Later studies revealed that PTB is not a positive regulator but rather a splicing repressor [39, 44] and assigned to U2AF the role of pyrimidine tract binding factor [45]. In particular, U2AF is dimeric, with the larger (65-kDa) and smaller (35-kDa) subunits interacting with the pyrimidine tract and the 3′ss, respectively [46, 47]. Early models of PTB repression suggested that it might compete with $U2AF^{65}$ for polypirimidine tract binding [48,49], in a similar fashion to the competition between $U2AF^{65}$ and *Drosophila* sex-lethal protein at the transformer-regulated 3′ splice site [50]. However, regulation by PTB often requires additional PTB-binding elements remote from the pyrimidine tract [43,51-56]. Multiple elements might therefore mediate cooperative binding of PTB [53] so as to interfere with recruitment of both $U2AF^{65}$ and other splicing factors. In the case of *c-src* exon N1, for example, cooperative binding to sites on both exon sides is required for repression [53]. It has therefore been suggested that PTB bound at flanking sites of regulated exons might interact across the exon sequence and result in strong splicing repression. Moreover, Wagner and Garcia Blanco [39] have suggested that PTB might act as a general repressor of weak exons, including pseudoexons, and therefore play a role in constitutive splicing decisions.

Finally, hnRNP G proteins have been shown, in humans, to be encoded by two genes: *RBMY* [57] is located on the Y chromosome and is expressed exclusively in germline cells in the testis [58]; *RBMX* is located on the X chromosome and is expressed in somatic cells [58]. The product of this latter gene has been implicated in splicing regulation of two mutually exclusive exons in the α-tropomyosin 3 gene (table 1). In particular, hnRNP G was shown to promote skipping of the skeletal muscle (SK)-specific exon and to enhance inclusion of the nonmuscle (NM) exon [59]. In this system, the effects exerted by hnRNP G are counteracted by hTra2β; indeed, hnRNP G binds to SK exon sequences, and hTra2β competes for binding to the same target; nonetheless, the precise sequence requirements for hnRNP G binding have not been determined. Interestingly, hnRNP G and hTra2β also play antagonistic roles (hnRNP G is a repressor) in the regulation of *MAPT* (microtubule-associated protein tau) exon 10 [60].

CELF/Bruno-like proteins

The CELF (CUG-BP and ETR3-like factors) proteins [61], also known as Bruno-like proteins, are a family of splicing regulators that includes six members (CUG-BP, ETR3 and CELFs 3–6) [61, 62]. Although the binding specificity of these proteins has not been studied systematically, recent reports [63, 64] have indicated that they have a preference

for uridine/purine repeat elements (UREs). CELFs are variably expressed, with CUG-BP, ETR3 and CELF4 being detectable in a variety of tissues, and CELF3 and CELF5 being specific to brain [61, 62, 64, 65]. CELF proteins are of particular interest, as their misregulation in myotonic dystrophy (DM) leads to the failure of properly regulating alternative splicing of various pre-mRNAs (see the genetic disease paragraph; reviewed in [66]). In some instances CELFs have been shown to counteract the effect of splicing repressors, therefore promoting exon inclusion; in other cases they have been reported to act as silencers or even as both repressors and activators on different exons in the same transcript. In particular, different family members have distinct effects on the inclusion of two mutually exclusive exons in the rat α-actinin transcript (table 1): both CUG-BP and ETR3 promote inclusion of the SK exon and skipping of the NM exon. In contrast, CELF4 acts as a repressor of both exons. Gromak and co-workers [67] demonstrated that activation of SK exon selection by CUG-BP is achieved by a derepression mechanism involving the displacement of PTB from the polypyrimidine tract. Indeed, the authors demonstrated that PTB and CUG-BP are in binding competition at adjacent sites at the 3′ end of the polypyrimidine tract.

SR proteins

SR proteins constitute a family of essential pre-mRNA splicing factors present in all metazoans [68]. Members of the SR protein family contain one or two N-terminal RNA binding domains, as well as a C-terminal arginine-serine (RS)-rich domain; this latter domain is thought to mediate protein-protein interactions with other RS domain-containing proteins [69]. In constitutive splicing, SR proteins have been proposed to promote both cross-intron and cross-exon interactions, and to participate in the recruitment of snRNPs into the spliceosome [69]. As far as alternative splicing is concerned, SR proteins have been shown to bind a number of ESEs, and function to stimulate the splicing of adjacent introns. Nonetheless, SR proteins have been described to act as splicing enhancers or suppressors depending on the location of their binding sites. In an adenoviral system [70], SR proteins are able to bind to an intronic element and suppress splicing of an alternative 3′ splice site, while the same SR protein-binding element was capable of splicing enhancer activity whem located within a viral exon.

Also, binding of SR proteins to a Rous Sarcoma virus ISE has been shown to determine nonproductive interactions of SF2/ASF and the snRNPs U1, U2 and U11 at this site, ultimately resulting in splicing repression [71]. Still, the question is not as simple as it might seem since the presence of binding sites for SR proteins in introns is not always associated with splicing inhibition. In fact, in the case of chicken β-tropomyosin, natural intron binding sites for SR proteins have been associated with enhancer activity [72]. Similarly, positioning the exonic splicing enhancer from the human fibronectin ED1 exon in the intron has shown no silencing activity [73]. Moreover, some reports have suggested that SR protein interaction with exonic sequences can result in splice silencing. In the case of CD45 [74, 75] inclusion of three alternative exons (A, B and C) is regulated upon T cell activation and depends on SR proteins; in particular, some of these proteins act as repressors, while others stimulate inclusion of the alternative exons (table 1). The *cis*-acting elements that mediate SR-protein binding and splicing regulation are presently unknown, although some evidence [76, 77] indicates that they might be located within exonic sequences (at least in the case of exons 4 and 6). Another instance of SR proteins acting as suppressors is the regulation of SRp20 alternative splicing with SF2/ASF and ASF3 repressing inclusion of exon 4 [78].

A different situation involving repressor activity of SR protein family members pertains to splicing regulation of hnRNP A1 exon 7B; modulation of exon 7B skipping entails several conserved elements (CEs) (table 1, fig. 2). One of these elements (CE9) has been shown to bind SRp30c and mediate repression of the downstream exon 8 3′ splice site [79]. Given the existence of competition between exon 7B and exon 8 3′ splice sites, the action of SRp30c might ultimately result in increased inclusion of the alternative exon. Nonetheless, given the redundancy of splicing regulators acting on exon 7B, the contribution of CE9 and SRp30c is difficult to establish. Although CE9 exerts splicing repression in a heterologous system, deletion of this element does not significantly affect the frequency of exon 7B inclusion. The authors therefore speculate that the contribution of CE9 to splicing control may become more important at higher concentrations of SRp30c.

Recently an SR-like protein, Tra2β, was described. Tra2β is very similar to *Drosophila* Transformer-2, which has an important role as a splicing activator in sex determination and sex-specific neural splicing [80], and as a repressor of its own splicing in the male germline [81]. The human Tra2β (hTra2β) has been more often reported to act as a splicing enhancer: it has been shown to activate exon 7 splicing in *SMN1* and *SMN2* (see below) and to interact with an ESE in *MAPT* exon 10 [82]. Recently, Stoilov et al. [83] demonstrated that hTra2β can promote inclusion of exon 2 in its own pre-mRNA and autoregulate its concentration. Yet some recent reports have indicated that this factor can also function as a splicing silencer; as reported above, in the case of human α-tropomyosin 3, hTra2β activates splicing of the SK exon (by counteracting hnRNP G) but inhibits inclusion of the NM exon [59]. Moreover, in the same tau pre-mRNA hTra2β has a role in suppressing exon 3 inclusion [84], and in the case of the EN exon of the clathrin light chain B, a similar

Table 1. Alternative exons under the regulation of splicing silence.

Organism	Gene	Exon	Splice site choice	Effect	*Cis*-acting element		*Trans*-acting factor	Other	Ref.
					Sequence	Location			
Human	4.1R	16	cassette	exon skipping during early erythroid differentiation	ce: ggugaaaacattt atatcagacatagc aatttaatgttggag	exonic	hnRNP A/B	diminished expression of hnRNP A/B during erythropoiesis correlates with ex 16 inclusion	[106]
Rat	α-actinin	SM	mutually exclusive exons	exon skipping in nonmuscle cells	multiple ucuu motifs	intronic (upstream)	PTB, CELF4	PTB is displaced by CUG-BP/ETR-3	[63, 67]
		NM		exon skipping in muscle cells	multiple ug/cug repeats	intronic (flanking the branch point)	CUG-BP, ETR-3, CELF4, FOX-1, PTB		[63, 67]
Human	α-tropomyosin 3	SK	mutually exclusive exons	exon skipping in nonmuscle tissues	unknown	exonic	hnRNPG	hnRNP G and hTra2β have opposite effects on the splicing regulation of these two mutually exclusive exons	[59]
		NM		exon skipping in muscle	unknown	unknown	hTra2β		
Rat	α-tropomyosin	3	mutually exclusive exons	exon skipping in smooth muscle cells	three ucuu motifs	intronic (120–170 nt upstream)	PTB	PTB interacts with raver1, which functions as a co-suppressor	[43, 54, 55, 111, 165]
					three ugc motifs (ure): aucacgcug ccugcugcaccccac ccccuuccccuucc uucccccaccccg uacuccacugccaac uccc	intronic (50-70 nt upstream)	PTB		
					four ugc motifs (dre)+pyrimidine-rich tract (dy): cuggaugccgccucu gcugcugcgcacauu ucauuuauauucugu ccuuucccuuuuuc uccucuucuuuaccu ccucccuuuggguug	intronic (130–200 nt downstream)	PTB		
Human	amyloidβ precursor protein	8	cassette	neuron-specific	uuu	intronic (38 to 36 upstream)	unknown		[178]
Mouse	amyloidβ precursor protein	15	cassette	exon skipping prevalent in non-neural tissues	UCR:uucuuuu uuuucccagcugc uccuuuuucauga cuuguuuauugca aaugcuucccuug cuc	intronic (69-138 nt upstream)	PTB?		[177]
					DCR:gccucugc ucucagcacugug cgcuucagcaaac augccucucuggg	intronic (21-67 nt upstream)	PTB?		
Human	ATP synthase γ-subunit	9	cassette	exon skipping in muscle cells	aguucca	intronic (within the PPT)	PTB		[167]
				exon skipping in skeletal and cardiac muscle cells	four gcaug motifs	intronic (upstream of the branch point)	Fox-1		[59]

Table 1. (continued)

Organism	Gene	Exon	Splice site choice	Effect	*Cis*-acting element		*Trans*-acting factor	Other	Ref.
					Sequence	Location			
Rat	β-tropo-myosin	7	mutually exclusive exons	exon skipping in nonmuscle cells	uguggg	exonic	hnRNP H		[37, 160]
					IRE:aaccccaccc ccucaccccgucg ucgcgccaccccac ugucucaccucacu gugcccucacgcuc cauccugccacacg ccccugcag	intronic (1-88 nt upstream)	PTB	a protein complex (including FUSE and sam 68) interacts with PTB and assembles on the IRE	
Mouse	BK channel α-subunit	STRE X	cassette	exon skipping in pituitary cells, Ca++ mediated	CARRE: ccuu gccauuaa ccgc gcucuuccucuc cucccauccacca caugguuauag	intronic (1-53 nt upstream)			[176]
Human	c-H-ras	IDX	cassette	alternative splicing in different cell lines and human tissues	gggacggcaggg cagugagggagg cgagggccgggg ucugggcucacg ccc	intronic (11–61 nt downstream)	hnRNP A1/ p68	SC35 and SRp40 can antagonize the inhibitory action of hnRNP A1	[95]
Mouse	c-src	N1	cassette	exon skipping in nonneural cells	four cucucu motifs	intronic (2 upstream and 2 downstream)	PTB		[52, 53]
Human	cardiac troponin T	5	cassette	exon skipping in adult cardiac muscle	cgcugcggc	intronic (18–26 nt upstream)	MBNL	MBNL action is counter-acted by CUG-BP binding to a sequence downstream of exon 5; this splicing event is deregulated in DM1	[134]
					ugucucgcu	intronic (36–44 nt upstream)	MBNL		
Mouse	caspase-2	9	cassette	exon skipping in tissues other than brain and skeletal muscle, associated with apoptosis	in50up:gcuuau uaacugcaaugua cuuuuuauuguu uuucauuccagu uaagg	intronic (140-188 nt downstream)	U2 snRNP (decoy 3′ Ss)		[173, 174]
					in50dn:auuucu gcuugacucuuc caaaucuuccuuc ucuuacccuugc auuuucucucugu	intronic (189-244 nt downstream)	PTB		
Human	CD44	v5	cassette	exon inclusion during embryonic development and lymphocyte activation	multiple elements within the exon	exonic	hnRNP A1	deregulated during tumor progression	[169, 170]
Human	CD45	4,5,6 (or A, B, C)	cassette	exon skipping in T cells and activated T cells	unknown	exonic?	SF2/ASF, SC35, SRp30c, SRp40, and SRp75, SWAP	Srp55, SRp20 and 9G8 promote exon inclusion	[74, 75]
		4	cassette	exon skipping in T cells and activated T cells	not exactly determined	exonic	SWAP/ unidentified protein complex		[153]
Rat	clathrin light chain B	EN	cassette	exon skipping in nonneural tissues	cucuuucucuuu aacccugugccug ccugucuugcug ucugucuucccc caccuaacuccuu cuccuaacgguu uuccucaag	intronic (1-85 nt upstream)	PTB/nPTB	CUG-BP might counteract the effect of PTB	[115]

Table 1. (continued)

Organism	Gene	Exon	Splice site choice	Effect	*Cis*-acting element		*Trans*-acting factor	Other	Ref.
					Sequence	Location			
Human	cystic fibrosis trans-membrane conductance regulator	9	cassette	variable degree of exon skipping	(TG)m(T)n polymorphic element	intronic (upstream of the AACAG of the 3′ ss)	TDP-43		[136, 137, 139, 142]
					uuaauuucaaga	exonic	SF2/ASF, Srp55, Srp75		
					pce:ttcttttgttctt cactattaagaactt aatttggtgtccatg tctctttttttttctagt ttgtag	intronic (4-71 nt upstream)	PTB	PTB is counteracted by TIA-1	
					ISS: unpublished	intronic (117–264 nt downstream of the 5′ss)	SF2/ASF, Srp55, Srp75		
Human	DNA ligase III	β	alternative terminal exon	exon selection only in testes	ccaagucaaaa uuuac	exonic	37-kDa protein		[171]
Human	fibroblast growth factor receptor 1	α	cassette	exon skipping in glyoblastoma	ISS1: ccgcuuaaugcug cuacagcugcuuc cuaacuuugccuc uuucuuccu	intronic (241 nt upstream)	PTB	ISS1 and ISS2 are functionally redundant	[162]
					ISS2:aucugccc ccacucugcuuca gaaacugcugccc acuaacauugcuc ccugccugccgcg ugg	intronic (97 nt downstream)	unknown		[161]
Human	Fibroblast growth factor receptor 2	BEK (IIIb)	mutually exclusive exons	exon skipping in epithelial cells	ucuuuagguucc cuuucaauucu	exonic	PTB		[163]
		K-SAM (IIIc)		exon skipping in non-epithelial cells	uagg	exonic	hnRNP A1		[30, 164]
Rat	fibroblast growth factor receptor 2	IIIb	mutually exclusive exons	regulated alter-native exon inclusion in different cell types	UISS1:cucauu gugaucuccuccc ucccacagcucuu uaggugua	intronic (103–143 nt upstream)	PTB		[98, 99]
					UISS2: uggug ggaccauaggcag cac	intronic (49–70 nt upstream)	unknown		
					DISS1: contains 4 ucuu motifs, sequence not published	not published	PTB?		
					DISS2: contains 3 ucuu motifs, sequence not published	not published	PTB?		
		IIIc			ISAR:caaacaaa uucaaagagaacg gacucugugggcu gauuuuuccaugu guucaauc	intronic (227-281 nt upstream)	unknown	this sequence forms a secondary structure with an upstream r egulatory region; the stem formation promotes exon IIIc skipping and enhances exon IIIb inclusion	[101]
					gcaug	intronic (221-226 nt upstream)	unknown		[103]

Table 1. (continued)

Organism	Gene	Exon	Splice site choice	Effect	*Cis*-acting element		*Trans*-acting factor	Other	Ref.
					Sequence	Location			
					ISE/ISS-3: ugu ggugaugggccug cagaggugagcug gccggugucucuc agugucucuuggu ugugggcuuugug gacgggcug	intronic (upstream)	unknown	this element promotes inclusion of exon IIIb	[100]
Human	fibronectin	EDA	mutually exclusive exons	exon skipping in hepatocytesvariable inclusion in other tissues	caaggcc	exonic	unknown	the silencer element function depends exclusively on the complete EDA exonic context; possible formation of a secondary structure	[92, 104]
		IIICS	complex alternative region	diverse alternative events varying with tissue and developmental stage	unknown	unknown	SWAP		[87]
Rat	GABAA receptor γ2 subunit	24-nt exon	cassette	exon skipping in nonneural cells	uguuucucuuucu cuccuuuccuuuu ccuucuucuuauu	intronic (51–89 nt upstream)	PTB/nPTB		[115, 168]
					gcaauucucuuu ucuguc	intronic (13-31 nt upstream)			
Mouse	hnRNP A1	7B	cassette	autoregulated	CE1: uagagu	intronic (145-148 nt upstream)	hnRNP A1	possible interaction between hnRNP A1 molecules bound to CE1a and CE4 to increase the frequency of exon skipping	[94, 24, 124]
					CE4: agcuagauuagc cuuuuagagcuu	intronic (35-58 nt downstream)	hnRNP A1/ unknown factor		
					CE610:ccugcuc ugcugugcuaccu ccuccuggcuuua agcuggggccgcc uccccaaaauaag uaggugaaugag ugguua	intronic (128-204 nt downstream)	n.a.	possible duplex formation between CE61O and the 5′ss of exon 7B promoting exon skipping	
		8 (3′ ss)	see text		CE9:cuggauua uuaacugaaugcc ucacucagagaau gaa	intronic (370-418 nt downstream)	SPp30c	This element suppresses the use of exon 8 3′ss and therefore is thought to promote the use of exon 7B 3′ss	[79]
Mouse	IgM	M2	cassette	regulated during B cell maturation	cuaguaaacuua uucuuacgucuu uccuguguugcc cuccagcuuuuau cucugagauggu cuucuuucuag	exonic	PTB		[172]
Human	insulin receptor	11	cassette	exon skipping in tissues that do not respond to insulin	uuacucggacac auguggccuccaa gugucagagccc aguggu	intronic (52–95 nt upstream)	CUG-BP	CUG-BP is counteracted by MBNL proteins; alternative splicing regulation is altered in myotonic dystrophy	[131]
Mouse	myelin-associated glyco-protein	12	cassette	increased exon skipping in quaking mice	QUASE:cgggg gccucagugugc cagccuccgggcc cuagcugggcuu cgggguuggug	intronic (8 to 61 nt downstream)	QKI-5		[180]

Table 1. (continued)

Organism	Gene	Exon	Splice site choice	Effect	*Cis*-acting element		*Trans*-acting factor	Other	Ref.
					Sequence	Location			
Mouse	Neural cell adhesion molecule	MSD b	cassette	exon inclusion only during myogenesis	unknown	intronic	unknown		[175]
Rat	NMDA receptor 1	5	cassette	exon skipping in hindbrain	gcuuuagcgccg ucauuuucaacc guuuauaaucuu cuucugugucug cauauuuucucu gugcacauuauu caucag	intronic (1-78 nt upstream)	PTB, nPTB, NAPOR	CUG-BP might counteract the effect of PTB	[107]
		21	cassette	exon skipping in forebrain	not fully characterized	first 705 nt of intron 21	unknown	NAPOR binds the sequence and possibly displaces a *trans*-acting repressor	[115]
Human	PTB	11	cassette	exon skipping	two ucuu motifs	exonic	PTB	exon skipping is responsible for degradation through NMDA; raver1 acts as a co-suppressor and CELF4 as an activator	[109]
Human	sarco(endo) plasmic reticulum Ca2+ ATPase 2	25	cassette	exon skipping in neural cells	unknown	exonic	unknown		[166]
Human	SRp20	4	cassette	exon skipping upon serum stimulation of starved cells	unknown	unknown	SF2/ASF	SRp20 acts as an enhancer of exon 4 inclusion in its own mRNA	[126]
Mouse	stem cell factor	6	cassette	exon skipping during the early stages of testicular development	unknown	exonic	unknown complex	acidic pH favors exon skipping	[179]
Human	survival of motor neuron 2	7	cassette	exon skipping in spinal cord	uagaca	exonic	hnRNPA1	the low exon 7 inclusion levels render SMN2 incapable of compensating for loss of SMN1 in patients suffering from SMA	[145, 181]
					guaaaaugucuug ugaaacaaaauac uuuuuaacaucca uauaaa	intronic (68–112 nt upstream)	33-kDa protein		
Human	tau (microtubule associated protein tau)	3	cassette	exon skipping in fetal brain; variable degree of exon skipping in adult central nervous system	uuag	exonic	Nova1, SWAP, KSRP, hTra2β		[84]
		10	cassette		ucaaaggauaau aucaaa	exonic	unknown	hTra2β, CELF3 and CELF4 promote exon inclusion; alteration of this splicing event by pathogenetic mutations causes FTDP-17	[158, 159]
					ISS: ucacacgu	intronic (11-18 nt downstream)	unknown		

n.a., not applicable; ss, splice site.

silencing effect has been shown when hTra2β concentrations are increased [85]. Although in these latter instances the *cis*-acting element(s) mediating the hTra2β effect remain to be clarified, Stoilov et al. [83] searched for common motifs in exons that are regulated by this factor and identified a putative hTra2β consensus: GGARGARR (R = A or G).

Miscellaneous

SWAP

This factor was initially identified in *Drosophila,* where it regulates splicing of the *white-apricot* allele of the *white* gene (SWAP, suppressor-of-white-apricot); human and murine homologs were then identified and reported to harbor COOH-terminal RS domains [86]. SWAP regulates splicing of intron 2 in its own pre-mRNA by promoting the use of a downstream 3′ splice site [87]; moreover, this same factor can determine the exclusion of the entire IIICS region from the fibronectin mRNA when cotransfected with a minigene vector, and acts as a silencer on CD45 exon 4 [87].

Fox-1

This factor (feminizing on *X*) was initially identified in *Caenorhabditis elegans,* where it was suggested to regulate sex-specific splicing of *xol-1* pre-mRNA [88]; fox-1 homolog were then identified in zebrafish, mouse and man [89]. In mammals Fox-1 is expressed in heart, skeletal muscle and brain, and it localizes to the nucleus. Jin et al. [89] demonstrated that Fox-1 can act as both splicing repressor and activator and suggested that it might represent a muscle-specific splicing regulator. Indeed, as summarized in table 1, Fox-1 has a role in inducing muscle-specific exon 9 skipping in ATP synthase γ-subunit pre-mRNA and participates in repressing inclusion of rat α-actinin NM exon [89]. Meanwhile, the same factor promotes inclusion of the SM exon (skeletal muscle) in the same α-actinin pre-mRNA, possibly through antagonizing the repressive effect of PTB [89]. Fox-1 has been reported to exert these effects by binding to the GCAUG pentanucleotide; this observation raises the interesting hypothesis that Fox-1 might have a role in neuron-specific splicing. In fact, the motif UGCAUG has been found to be overrepresented in flanking introns of brain-specific exons [90].

Pre-mRNA secondary structures and splicing repression

Recent studies have proposed that secondary structure formation on pre-mRNAs has the potential to act as a modifier on splicing regulation. In principle, secondary structures might act as *cis* elements, and their action can be exerted in multiple ways. First, RNA duplexes can be recognized, usually in a sequence-independent fashion, by double-stranded RNA binding proteins [91]. Second,

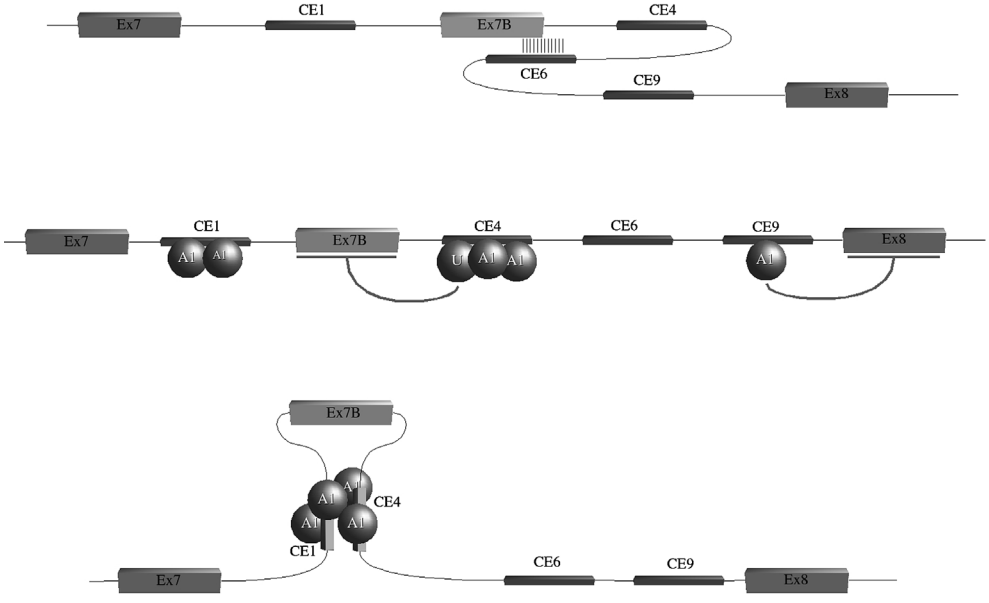

Figure 2. Regulation of hnRNP 1 alternative splicing. Conserved elements (CE) involved in exon 7b splicing control are represented. Duplex formation with CE6 is thought to sequester the exon 7b 5′ splice site. Although selection of the exon 8 splice site is repressed by SRp30c bound to CE9, two different mechanisms repress usage of the exon 7B 3′splice site: an unknown (U) trans-acting factor binds CE4 and represses the exon 7b 3′splice site. Moreover, multiple hnRNPA1 molecules bind both CE1 and CE4, and their interaction causes looping out of the pre-mRNA region containing exon 7B and brings in closer proximity the 5′ splice site of exon 7 and the 3′ splice site of exon 8.

RNA duplexes can either occlude or favorably display other *cis*-acting elements [92, 93]. Finally, RNA duplexes can approximate otherwise distant *cis* elements, bringing them into closer proximity and allowing interaction. Although to our knowledge no specific instance of splicing regulation by double-stranded RNA binding proteins has been described, both secondary structure sequestration of *cis*-acting elements and approximation of distant sequences have been shown to result in splicing silencing. In particular, secondary structure formation has been proposed to regulate inclusion of exon 7B in hnRNP A1 mRNA; Blanchette and Chabot [94] have suggested that formation of a duplex structure between the 5′ splice site of exon 7B and a conserved element (CE6) located in the downstream intron might have a role in splicing repression (fig. 2) and act in concert with other regulatory elements. Similarly, secondary structure formation has also been hypothesized to have a role in the regulation *c-H-ras* splicing [95]. In this transcript, inclusion of the alternative IDX exon is responsible for the production of a shorter protein due to the presence, within IDX, of an in-frame termination codon. An intronic ISS has been identified immediately downstream of IDX (table 1), and a protein complex containing hnRNP A1, hnRNP H, FUS/TSL and p68 helicase has been shown assemble on this element [95]. Among the complex components, hnRNP A1 and p68 have been assessed to act as splicing negative regulators. Yet Guil and co-workers [95] noted that the *ras*-ISS is only active in the presence of the IDX exon; detailed sequence analysis of ISS and IDX predicted the formation of a long stem-loop structure that might sequester the exon sequence, including the 5′ splice site. Similar structures can be predicted to occur on rat, hamster and mouse c-ras pre mRNAs, although in this latter case, the structure stability is questionable. The authors were able to demonstrate that the ISS/IDX RNA sequence could compete efficiently with inhibitory factors in in vitro assays, while IDX or ISS sequences alone could not, therefore substantiating the hypothesis that secondary structure formation might have a role in regulation. Of extreme interest, in our opinion, is the fact that an RNA helicase, p68, has been found to participate in complex formation and demonstrated to have inhibitory effects. The presence of an RNA helicase might indeed act as a stabilizer or destabilizer of a long secondary structure forming on *ras* pre-mRNA and might favor inhibitory structure formation and IDX sequestration. Alternatively, p68 might play an important role in modifying and modulating interaction between the pre-mRNA and its *trans*-acting factors, as recently proposed [96]. Indeed, another RNA helicase, p72, was previously shown to have a role in the positive regulation of CD44 exon v4, and in this case the formation of a secondary structure has been hypothesized, as well [97].

Another well-documented example of secondary structure having a role in splicing regulation involves alternative splicing of fibroblast growth factor receptor (*FGFR*) genes. In the rat *FGFR2* pre-mRNA inclusion of one out of the two mutually exclusive exons IIIb and IIIc (BEK and K-SAM, respectively, in the human gene) determines the production of FGFR2-IIIb or -IIIc. Different *cis*-acting elements have been reported to regulate alternative exon inclusion; two ISSs (UISS, upstream ISS) repress splicing of exon IIIb (see table 1 and fig. 3) by recruiting PTB and possibly other *trans*-acting suppressors [98]. Two other ISSs (DISS, downstream ISS) are located downstream of the exon, and one of them also binds PTB [99]. A further element, located within the intron separating exons IIIb and IIIc, was recently identified and termed ISE/ISS-3 [100]. This sequence both promotes splicing of exon IIIb and represses IIIc. The mechanism responsible for ISE/ISS-3 splicing silencer activity has not been determined, yet the intrinsically weak branch point of exon IIIc is required for silencer activity, suggesting that factors binding to this element might interfere with branch point recognition. Another *cis*-acting regulatory sequence, the ISAR (intronic splicing activator and repressor) is located between the DISSs and ISE/ISS-3; similarly to this latter element, ISAR plays a dual role in splicing regulation, promoting exon IIIb inclusion and exon IIIc skipping [101]. Muh et al. [102] have demonstrated that ISAR forms an RNA stem structure with a conserved element (ISE-2) which is located 611 nt upstream of ISAR. Indeed, these authors indicated that the ISAR-ISE-2 system can be replaced without loss of splicing regulation by two completely unrelated sequences provided that the

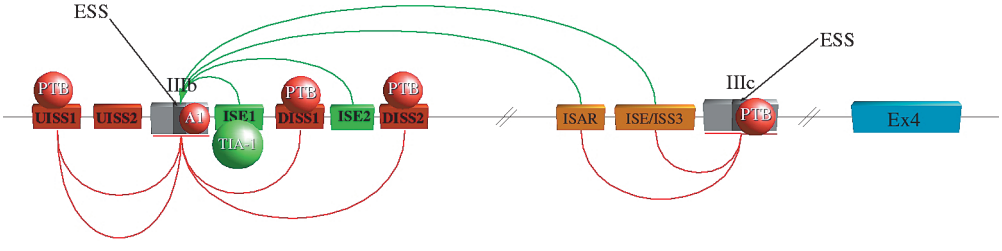

Figure 3. Schematic representation of *cis*-acting elements and *trans*-acting factors that regulate FGFR-2 pre-mRNA splicing. Exons IIIb and IIIc are mutually exclusive; details are described in the text. UISS, upstream ISS; DISS, downstream ISS.

secondary structure formation is preserved. Accordingly, ISAR and ISE-2 are highly conserved from human to frog, and they are predicted to form the same secondary structure in all organisms. Moreover, since alternative splicing of exons that represent homologs of IIIb and IIIc has been observed for FGFR1 and FGFR3, Muh et al. [102] analyzed intron sequences from the paralogous region of FGFR1 genes and verified that ISAR-ISE-2 elements are conserved in sequence and predicted structure. These authors therefore speculated that stem-loop structures in FGFR1 and FGFR2 pre-mRNAs migh be recognized by cell-type-specific *trans*-acting factors that control exon choice. Yet, in a more recent study, Baraniak et al. [103] indicated that the main function of stem formation is bringing into close proximity elements that are located in ISE2-ISAR flanking regions; in particular these authors identified a GCAUG sequence element downstream of the ISAR core which is required for activation of exon IIIb and repression of exon IIIc; the juxtaposition of this sequence element, as well as of ISE/ISS-3, to the DISSs, might therefore be required for efficient suppression of exon IIIc and enhancement of IIIb inclusion.

Another example of secondary structure formation having a role in splicing silencing involves fibronectin EDA exon. In particular, the first 118 nt of this exon were shown to harbor a splicing silencer [104]. This region has the potential to form three stem loops, and the region comprising the first stem loop was found to be completely conserved among human, dog and rat sequences, while chicken and frog fibronectin sequences contain several substitutions that do not compromise stem-loop formation [104]. Indeed, mutation analysis indicated that substitutions that abolish the secondary structure result in partial loss of ESS function that can be restored by second site mutations which regenerate the structure. The function of the two distal stem-loop structures, if any, is at present unknown, and mutations partially disrupting the second stem had extremely slight effects on ESS function. The mechanism responsible for splice silencing in the presence of secondary structure formation is still unknown, but the authors speculate that the stem loops might recruit *trans*-acting factors that, in turn, modulate the efficiency with which the EDA 3′ splice site is recognized.

It is worth noting that a recent study has indicated that secondary structure formation might have a role in the regulation of exon skipping in a number of transcripts. In fact, Miriami [105] and co-workers have applied an interesting approach (see below) to identify sequence elements associated with alternatively spliced exons and retrieved two motifs that seem to be able to promote the formation of secondary structure with regulatory potential. Such motifs are present in one or more copies in both flanking introns of 50 out of 54 alternatively skipped exons. The authors noted that while one motif was highly enriched in pyrimidines (mainly C), the other one was extremely rich in G residues (table 2); moreover, the relative order of the two elements was always conserved in the corresponding flanking introns (up and downstream of the regulated exon, fig. 4); these features are consistent with the motifs forming secondary structures through base pairing. The authors therefore proposed a model whereby stem-loop structure formation between elements located in the flanking introns would cause the alternative exon to loop out and the splice sites of nearby introns to be closer, eventually resulting in exon skipping; conversely, the formation of intra-intronic stem loops would determine close proximity of the alternative exon splice sites to those of its flanking exons, probably resulting in exon inclusion. Although this model has not been experimentally validated, we consider it is intriguing and conceivable, especially in the light of the above-reported instances of secondary structure regulation of alternative splicing events. In the case such a model were verified, it is quite evident that any factor that is able to promote inter-rather than intra-intronic base pairing or vice versa would shift the balance towards exon skipping or inclusion; therefore, the two identified elements cannot, on this basis be regarded as pure silencers but rather as general regulators.

Different mechanisms allow regulation of splicing silencing

Although in recent years considerable knowledge has been accumulating on *cis*-acting elements as well as on the *trans*-acting factors that bind them, much less clear are the mechanisms that exploit identified elements/factors to achieve regulation of splicing pathways. As is evident from data in table 1, the action of most silencers is exerted in a tissue/cell-type-specific manner or depending on developmental stage. Other splicing events are regulated in response to different kinds of stimuli (differentiation, lymphocyte activation) or even result in the tissue-specific effect of a disease-causing allele. The molecular pathways that underlie such complex events are only beginning to be elucidated.

Although some tissue-specific splicing factors have been identified, our current knowledge of splicing modulation in mammals supports a view whereby alternative splicing is regulated by specific combinations of widespread factors and not by simple expression of dedicated activators and repressors. Indeed, the best-studied spicing regulators, namely SR and hnRNP proteins, are expressed in a wide range of tissues, cell types and developmental stages. Still, in many instances it is probably the relative abundance of inhibitory proteins that can modulate splicing patterns; in the case of 4.1R, alternative splicing of exon 16 is controlled by an ESS interacting with

Table 2. Silencer elements identified through systematic approaches.

Consensus/motif	Comments	Experimental validation	Identification method	Ref.
UCCNNCUCCCUCUCCCU CCC (A/G)GG(G/C)UGGAGGGN AGGGGGG	possible formation of secondary structures	no	search for common motifs in intronic sequences of 54 alternatively spliced human exons	[105]
(U/G)G(U/A)GGGG	resembles hnRNP H binding site consensus	yes (motif insertion in a) minigene reporter construct, transfection in COS-7 cells and RT-PCR	In silico comparison of 6-mers frequency in internal pseudoexons versus real exons; clustering and alignment of hexamers to identify motifs	[18]
CUAGAGGU UCUCCCAA		yes (unable to induce skipping of a constitutive exon)		
U(U/A)UUAU UA(A/G)NUAU AAAUG(G/C)U (A/G)U(A/U)(A/C)UGU U(G/C)UU(A/U)GAU UUA(A/U)(A/U)A(U/A) UAAAAUG AUGU(A/U)AU AA(C/G)CUU(A/U)		yes (insertion of at least one sequence per cluster in a minigene reporter construct; transfection in 293 human cells and RT-PCR)	in silico comparison of 8-mer frequency in internal noncoding exons versus pseudoexons and intronless gene 5′UTRs, in this study 974 putative splicing silencers were identified	[25]
UU(C/U)GUUCCUU GUA(A/G)GUUU(A/G/U)		yes (insertion of more than one decamer per cluster in a reporter construct; transfection in 293 and/or HeLa cells; GFP assay and/ or RT-PCR)	in vivo reporter screening of a random 10-mer library; clustering and alignment of decamers to identify motifs	[26]
(U/G)NGG(G/U)GGGU	resembles hnRNP H binding site consensus			
UAGUUUA U(U/G)(U/G)UUAGNUA				
GGUUU(A/G)GGGUA GUA(G/A)GU(U/A)(U/A)(G/A)(G/U)	resemble hnRNP A1 binding site consensus			

RT-PCR, reverse-phase polymerase chain reaction.

hnRNP A/B; Hou and co-workers [106] have demonstrated that exon 16 is progressively included in the transcript upon erythroblast differentiation and that exon inclusion parallels a decline of hnRNP A/B expression levels. Possibly, in many instances it is the variation in activator/repressor ratio that shifts the balance towards exon skipping or inclusion. Many exons have in fact been shown to be regulated by distinct factors with antagonistic activity. Although several reports have indicated that SR proteins act antagonistically with PTB or hnRNP A1, the most common situation probably envisages substrate-specific combinatorial interactions among splicing factors rather than fixed repressor/activator partner pairs.

For example, distinct SR proteins can counteract each other, exerting opposite effects on the inclusion of three alternative exons in CD45 mRNA (table 1); accordingly, changes in SR protein expression are observed upon T cell activation, i.e. when a shift in CD45 splicing pattern is observed [74].

Antagonistic effects can also be exerted by *trans*-acting factors that act alternatively as both repressors and activators on different target RNAs. This is the situation of GUG-BP and MBNL proteins in the regulation of cardiac troponin T and insulin receptor pre-mRNAs (table 1); alteration in the relative availability of these proteic factors results in altered splicing patterns and DM (see below).

Given these observations, it is conceivable that even slight tissue- or developmental stage-specific variations in expression levels of distinct splicing factors might

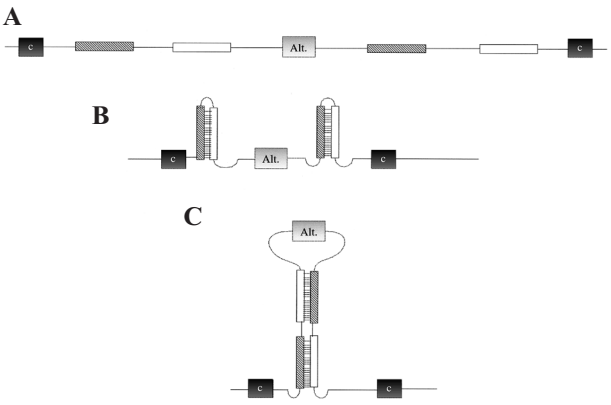

Figure 4. Schematic representation of the model proposed by Miriami and co-workers [105]. The relative order of the two elements is always conserved in the corresponding flanking introns (*A*). The formation of intra-intronic stem loops would determine close proximity of the alternative exon splice sites to those of its flanking exons, probably resulting in alternative exon inclusion (*B*). Conversely, stem loop structure formation between motifs located in the flanking introns would cause the alternative exon to loop out and the splice sites of nearby introns to be closer, eventually leading to exon skipping (*C*). Alt, alternatively spliced exon; c, constitutive exons.

well allow regulation of specific mRNA substrates. One extremely interesting observation refers to splicing regulation of NMDA receptor 1 mRNA (*NMDAR1*). Zhang and co-workers [107] demonstrated that regulated skipping of exons 5 and 21 in the *NMDAR1* transcript is achieved through regional expression in the rat brain of NAPOR (neuroblastoma apoptosis-related RNA-binding protein), a CELF-related splicing factor. In the adult rat brain NAPOR is expressed in a forebrain-enriched, hindbrain-deficient fashion, and this expression pattern coincides with the splicing pattern of exons 21 and 5 in the NMDAR1. In particular, in the forebrain, high NAPOR levels contribute to the repressive effect on exon 5 together with PTB/nPTB (table 1), while stimulating inclusion of exon 21. It is at present unknown how NAPOR exerts its effect on both exons, but a region in intron 21 was identified [107] that acts as a silencer element and that is also able to bind NAPOR; this factor might therefore enhance exon 21 inclusion through competitive binding with an unidentified splicing repressor. Conversely, in the hindbrain, NAPOR expression is low, resulting in diminished repression of exon 5 and decreased activation of exon 21. In this brain region, therefore, the major transcript contains exon 5 but lacks exon 21.

In addition to variation in expression levels, different mechanisms allow regulation of silencer activity. In the case of PTB, for example, alternative splicing of PTB messenger itself has been shown to have an impact on target pre-mRNAs. Three major PTB forms are generated by alternative splicing of exon 9: PTB2 and PTB4 differ from PTB1 by the insertion of 19 or 26 amino acids, respectively, which derive from the optional inclusion of the exon using one of two alternative 3′ splice sites [40, 108]. Wollerton and co-workers [109] have demonstrated that the three PTB isoforms have distinct activities in regulating α-tropomyosin (α-*TM*) but not α-actinin alternative splicing; they showed that, while PTB1 expression either had no effect or relieved α-*TM* exon 3 skipping, PTB4 acted as a repressor on the same exon and PTB2 had an effect intermediate between that of PTB1 and PTB4. Conversely, no difference was observed when the alternative SK/NM exons of α-actinin were coexpressed with PTB1, 2 or 4. The authors also suggested that PTB1-induced increase in exon 3 inclusion might be due to competition for binding to regulatory elements and that PTB1 overexpression might result in displacement of the stronger repressor PTB4. If this were the case, the PTB1/PTB4 ratio might have a role in the regulation of a subset of splicing events. Other authors [98] have analyzed the activity of the different PTB forms on splicing regulation of FGF-R2 exon IIIb and found no difference, indicating that alternatively spliced PTB forms might have distinct functions depending on the context or on the presence of other factors. Indeed, a PTB-interacting protein, raver-1 [110], has been shown to function as a co-suppressor in the regulation of α-*TM* exon 3 [111]. Raver1 is a shuttling protein which redistributes from nucleus to cytoplasm during differentiation of myogenic cells [110]. Variation in raver 1 localization has therefore been hypothesized to play a role in regulating alternative splicing events [111]. Yet the ability of this protein to interact with different PTB forms is presently unknown.

Moreover, in addition to hnRNP I (PTB), which is ubiquitously expressed, three tissue-specific paralogs have been identified which might play a role in modulation of tissue-specific regulation of PTB targets. ROD1 and smPTB are mainly expressed in hematopoietic and smooth muscle cells, respectively [112, 113], but only a few functional data are available. While the relevance of these two factors in tissue-specific splicing modulation awaits further investigation, some evidence suggests that the PTB neural paralog might play a pivotal role in making splicing decisions in the mammalian central nervous system. nPTB/brPTB [114] is expressed predominantly in neuronal cells, and it has been shown to exert a weaker repressive effect compared with PTB on *c-src* N1 exon, contributing to the neuronal selection of N1 [114]. Moreover, the two paralogous genes show complementary expression patterns. PTB is expressed at low levels in adult human and mouse brain, and in the cerebellum its expression decreases with increasing postnatal age [115]; conversely, nPTB is mainly expressed during postnatal growth [116]. This expression shift, together with the weaker repressive effect exerted by nPTB, might help explain how PTB repression is specifically relieved in neural cells for a number of regulated transcripts (*c-src*, $GABA_A$ receptor $\gamma 2$ subunit, *NMDAR1,* clathrin light chain B).

As reported above, splicing patterns can also be modulated in response to specific stimuli, meaning that mechanisms must exist within the cell that couple splicing regulation to signal transduction. This topic has been addressed in a recent review [117]; we will therefore only mention a few findings that specifically refer to silencing factors. In particular, we consider extremely interesting some novel reports indicating that modulating the subcellular distribution of splicing factors might affect splicing regulation by altering the ratios of enhancers/silencers in the nucleus.

HnRNP A1 was observed to accumulate in the cytoplasm as a consequence of either osmotic stress or ultraviolet (UV)-C irradiation; conversely, these stimuli did not change the localization of splicing factor SF2/ASF [118]; in the case of hnRNP A1, osmotic stress also induced its phosphorylation. Whether phosphorylation is the direct cause of hnRNP A1 accumulation is unknown, but Van der Houven van Oordt and co-workers [118] were able to demonstrate that activation of the $MKK_{3/6}$-p38 stress-response pathway is required and sufficient for hnRNP A1 cytoplasmic translocation. Most important for the purpose of this review, the same authors showed that osmotic stress or irradiation changed the splicing pattern of a reporter adenovirus E1A gene in the direction that would be expected following a decrease in the hnRNP A1/SF2ASF ratio.

Similar data have been reported for PTB, Xie et al. [119] showed that Ser-16 is a target of protein kinase A (PKA) phosphorylation in vitro and in vivo and that phosphorylated PTB accumulates in the cytoplasm. PKA transduces the signals of many extracellular factors, including hormones and neurotransmitters; therefore the stimuli that regulate PTB localization can be expected to be manifold, and pathway dissection requires further study.

In the case of both PTB and hnRNP A1, the authors did not test whether protein activity was also modulated as a consequence of phosphorylation. But it is conceivable that a shift in protein localization alone might be sufficient to modulate splicing regulation and repression.

Silencing of silencers and splicing factors

Transcripts encoding splicing factors and regulators undergo splicing regulation themselves. In many instances the significance of alternatively spliced forms is far from clear, while in other cases some data are beginning to elucidate possible regulatory significance. Moreover, recent reports have indicated that several genes encoding RNA binding proteins with roles in splice site selection autoregulate their expression, among them hnRNPA1 [120], Srp20 [78], SC35 [121], 9G8 [122], hTra2β [83] and SWAP [87]. Here we review some aspects of autoregulation events that exploit the use of splicing silencers.

hnRNP A1

hnRNP A1 pre-mRNA contains an alternative exon (7B) which is either included to produce hnRNA A1B or excluded to yield hnRNA A1 [123]. In HeLa cells the abundance of A1B mRNA is approximately 5% that of A1, and the different activities and specificities of the two hnRNP forms are presently unknown. At least four distinct *cis*-acting elements have been identified that contribute to regulated exclusion of exon 7B (see fig. 2). Chabot and co-workers have indicated that the frequency of exon inclusion is primarily determined by competition between the 3′ splice sites of exons 7B and 8. This is probably due to the formation of a duplex structure between the 5′ splice site of exon 7B and CE6 [94] (see fig. 2). Although, as reported above, selection of the exon 8 splice site is repressed by SRp30c bound to a *cis*-acting conserved element (CE9) [79], at least two different mechanisms seem to repress usage of the exon 7B 3′ splice site. Indeed, the composite CE4 element displays binding sites for both hnRNPA1 itself and for another unknown splicing regulator [24]. This latter element acts to suppress utilization of the exon 7B 3′ splice site. The second silencing mechanism involves hnRNPA1: the protein can bind to both CE4 and to CE1 (which is located upstream of the alternative exon). The interaction of hnRNPA1 molecules bound to CE4 and CE1 is thought [24] to cause the looping out of the pre-mRNA region containing exon 7B and to bring in closer proximity the 5′ splice site of exon 7 and the 3′ splice site of exon 8 (see fig. 2). This mechanism is probably enforced by the action of multiple hnRNP A1 binding sites encompassing both the CE1 and CE4 regions [124]. The authors suggest that multiple binding sites might act cooperatively and improve the efficiency of hnRNP A1 molecules bound to these elements so as to stabilize loop formation.

PTB

In addition to alternative splicing of the exon 9 region, another PTB alternative transcript has more recently been identified [125]. In particular, PTBtr (PTB truncated) derives from skipping of exon 11, which results in protein truncation. Wollerton and co-workers [125] were able to demonstrate that the PTB messenger lacking exon 11 is removed by nonsense-mediated decay (NMD). PTB itself was found to repress exon 11 inclusion through binding to exonic sequences: the alternative exon contains a stretch of 15 pyrimidines with two optimal UCUU binding sites. These data indicate that PTB regulates its abundance through a negative feedback loop. Moreover, in the same system (HeLa cells), the PTB-interacting protein raver1 (see above) was found to increase exon 11 skipping, while CELF4 expression led to a towfold increase in exon 11 inclusion. This latter finding is particularly remarkable given that CELF proteins are known to counteract the

action of PTB in some alternative splicing events, and therefore CELF4 might have a role in maintaining adequate levels of an antagonistic regulator.

Srp20

This factor has also been shown to autoregulate its own splicing. Two forms of SRp20 have been identified, one that lacks exon 4 and one that includes it in the transcript. Skipping of exon 4 determines the translation of an RS domain-lacking protein. SRp20 itself was found to act as a positive regulator on exon 4 inclusion, while SF2/ASF and ASF3 functioned as suppressors [78]. Jumaa and co-workers also reported that the exon 4-containing mRNA is the major product in starved murine lymphoma cells but progressively decreases upon serum stimulation [126]. The significance of such a change in splicing pattern is at present unknown as well as the different activity of the two SRp20 isoforms on the regulation of other splicing pathways.

Systematic identification of ESS/ISS

As reported above, in recent years some groups have focused their attention on splicing silencers and, in particular, on the systematic identification of sequences that might be able to exert this function in human genes. In the majority of cases, identification of silencers has integrated computational (in silico) and experimental wet (in vivo, in vitro) techniques.

Systematic computational approaches have generally been based on the construction of two or more databases of sequences which are expected to carry different splicing determinants; computational analysis led to the identification of discriminating features between groups, and these features were then used to obtain motif definitions. Different approaches have been characterized by the choice of sequence groups; in particular, alternatively spliced exons and pseudoexons have been analyzed against real constitutive exons to find putative splicing silencers [18, 25, 90,105, 127]. Although powerful, these approaches need to consider that, when searching differences in base composition between pseudo- and real exons, particular care must be used since pseudoexons differ from real ones in that they are noncoding sequences and lack relevant sequence information (for nuclear transport, mRNA stability etc.) other than splicing regulation. Also, given that alternative splicing is regulated by both silencing and enhancing elements, motifs found using alternative and constitutive exon comparison require further investigation in order to assess their behavior as silencers.

Differences between sequence groups have been computationally detected using diverse approaches. The most widely used [18, 25, 90, 105] imply evaluating k-mer frequency in the selected sequence databases and the application of different statistics to identify over- or under represented k-mers. Various algorithms have been applied to define consensuses by clustering retrieved k-mers and aligning their instances to obtain matrices reporting nucleotide frequencies at each position.

Brudno and co-workers [90] compared sequences flanking brain-specific alternatively spliced exons with a control set of sequences obtained from constitutive exons. They used different length word counts and resampling statistics to identify words enriched in alternative exon flanks. As reported above, retrieved sequences could not, without experimental analysis, be classified as either silencers or enhancers; nonetheless, these authors noted that brain-specific exons displayed increased frequencies of motifs corresponding to putative PTB binding sites in their upstream intronic sequences, suggesting that PTB (or nPTB) might have a role in the regulation of a wide range of neuron-specific alternative splicing events.

A similar approach in sequence selection has been applied by Miriami and colleagues [105]; they used two different algorithms (MEME, Gibbs sampling) to identify overrepresented motifs in unaligned sequences from exon-body and flanking introns of non-tissue-specific alternatively spliced exons. Two distinct motifs were identified (table 2) and searched for in control groups. The authors showed that the identified motifs were scarcely represented in constitutive exons (negative control) and enriched in another different set of alternative exons. These motifs have been proposed to regulate alternative splicing events through the formation of secondary structures (see above).

Finally, Zhang and Chasin [25], as well as our group [18], followed a similar computational approach to the one previously described by Fairbrother et al. [128] and used pseudoexons to identify putative splicing silencers as well as enhancers. In particular, Zhang and colleagues compared 8-mer frequencies (allowing a single mismatch) between a set of real noncoding constitutive exons and, independently, a set of pseudoexons and a set of intronless gene 5′ untranslated regions (UTRs). We performed 6-mer frequency comparison between a set of selected pseudoexons and, also independently, a group of real exons and one of pseudoexon flanks. In both cases z-scores were used to identify over- or underrepresented elements; these latter were then aligned and clustered to obtain motif consensuses. Experimental analysis of retrieved motifs was performed by both groups, and the summarized results of these studies are reported in table 2 together with motif consensuses.

In addition to the ones described above, other methods have been employed [127] that are based on a wider and more complex range of sequence features that are extracted from the compared sequence sets. These features are then

used to train a computational classifier for automatic discrimination between sequences belonging to each specific group (namely pseudoexons and real ones). Features giving the major contribution to sequence discrimination can be further analyzed to establish their role as splicing silencers and to obtain motif consensuses. Given the flexibility of these methods in selection of sequence features, they are particularly suited for complex sequence information extraction, and their classifier nature makes the results obtained readily available to be incorporated in gene-finding algorithms.

A completely different approach to systematically identifying silencer sequences has been applied by Wang and co-workers [26]. In an exhaustive and innovative study, these authors used a three-exon *GFP* (green fluorescent protein)-based reporter construct to screen a library of random decamers so as to identify sequences that could induce skipping when inserted in the central *DHFR* exon 2. This procedure allowed identification of 141 ESS decamers that were then clustered on the basis of sequence similarity and multiply aligned using CLUSTALW. Seven ESS motif were finally retrieved through this approach (table 2), and individual decamers from each cluster were subjected to experimental verification. Frequency distribution analysis of these motifs or of related hexamers (i.e. hexamers that are overrepresented in the set of decamer ESS) indicated that they are more abundant in pseudo- rather than in real exons and in alternative exons than in constitutive ones. These authors speculated that these sequences might have a relevant role in splice site definition by suppressing decoy splice sites located in close proximity to real sites.

Silencers and human genetic disease

We report here below the known examples of silencer mutations that cause human genetic disease. These instances show that single nucleotide variations can have a profound effect on splicing regulation and, as a consequence, disease manifestation or outcome. In general, mutations that alter or create splicing silencers can be expected to be underestimated in the statistics of disease-causing mutations for at least two reasons: first, the lack of reliable prediction algorithms makes it difficult to determine whether an identified substitution is likely to disrupt or create a *cis*-acting silencer, so that transcript analysis (which is only feasible in a minority of cases) is required to ascertain the effect on splicing; second, intronic regions, where ISS might reside, are rarely subjected to mutation screening. Therefore, in the absence of transcript analysis data, most mutations involving silencers can be expected to remain undetected or, if they are located within coding sequences, to be classified as missense or nonsense or neutral substitutions.

Myotonic dystrophy (DM, OMIM: #160900)

DM is a complex multisystemic disorder and represents the most common inherited disease of skeletal muscle in adults. The genetic basis for DM1 is a dominantly inherited unstable expansion of CTG triplet repeats in the 3′-untranslated region of the DM protein kinase (*DMPK*) gene [129]. Our current understanding of DM1 pathogenesis envisages a toxic gain of function by the mutant *DMPK* RNA resulting in disregulation of RNA-binding proteins and a *trans*-dominant effect on pre-mRNA alternative splicing [130]. Indeed, CUG-expanded transcripts accumulate within the nucleus in ribonuclear foci and compromise the regulation of alternative splicing for a subset of muscle transcripts, including the insulin receptor (*IR*) [131], the muscle-specific chloride-channel (*CIC-1*) and the cardiac troponin T (*cTNT*) genes [130, 132].

In healthy subjects, the mature IR exists as two isoforms: the A isoform lacks exon 11 and is expressed ubiquitously, while the B isoform contains exon 11 and is expressed predominantly in liver, muscle, adipocytes and kidney. Savkur et al. [131] have shown that DM1 patients express unusually high levels of IR-A in skeletal muscle tissue, which leads to reduced responsiveness to the metabolic effects of insulin. These same authors were able to demonstrate that a previously identified [133] se-

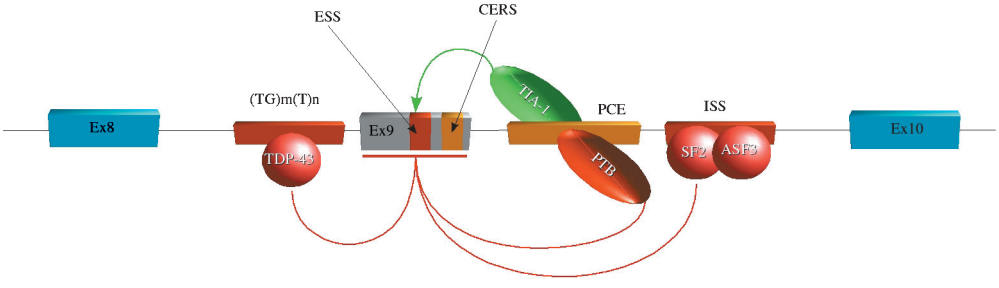

Figure 5. Schematic representation of *cis*-acting elements and *trans*-acting factors that regulate *CFTR* exon 9 splicing (see text for details). Positive and negative regulatory elements are in green and red, respectively. Elements that act as both activators and repressors are in orange. TIA-1 and PTB compete for binding to the PCE.

quence located within intron 10 (table 1) is responsible for exon 11 splicing deregulation in DM1. In particular, this sequence probably acts as a CUG-BP-dependent splicing silencer since it binds to CUG-BP in vitro and is necessary for switch to the IR-A isoform (table 1). Conversely, inclusion of exon 11 is specifically stimulated by three MBNL (muscleblind) family members [134].

cTNT pre-mRNA splicing is also deregulated in DM patients; in particular, exon 5 is included at unusually high levels in the cardiac myocites, possibly contributing to reduced myocardial function and conduction abnormalities [130]. CUG-BP positively regulates inclusion of exon 5 through binding to a pre-mRNA region downstream of the regulated exon. Yet, more recently, Ho and co-workers [134] were able to demonstrate that cTNT exon 5 is also subjected to negative regulation by MBNL1, 2 and 3; in particular, these authors identified two MBNL binding sites immediately upstream of exon 5 that are required for splice silencing. Therefore, CUG-BP and MBNL proteins exert opposite effects on at least two disregulated transcripts, and the altered splicing patterns in DM patients predict a reduced and increased activity of MBNL and CUG-BP, respectively. Indeed this is the case: MD1 patients display increased levels of CUG-BP in Western blot analysis, while MBNL proteins are thought to be sequestered in nuclear foci [135].

Cystic fibrosis (CF, OMIM: #219700)

Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) are responsible for CF, the most common autosomal recessive disorder in caucasians. *CFTR* mutations can also be associated with nonclassical forms of CF characterized by tissue-specific disease manifestation such as congenital bilateral absence of vas deference (CBAVD) and idiopathic pancreatitis. In CFTR pre-mRNA, exon 9 is alternatively spliced; exon 9-minus transcripts yield a prematurely truncated polypeptide; and variable levels of exon skipping have been correlated with variable phenotypic outcomes. Splicing regulation of this exon has been studied in detail, revealing unexpected complexity in terms of both *cis*-acting elements and *trans*-acting factors (fig. 5). A polymorphic locus (TG)mT(n) immediately upstream of the 3′ exon 9 splice site can modulate exon 9 inclusion frequency: a high number of TG repeats and a low number of T tracts have been shown to induce increased levels of exon skipping [136]. In particular, an abbreviated tract of five thymidines (5T) is found in approximately 10% of individuals in the general population, and when present in *trans* with a severe *CFTR* mutation, the 5T allele can result in male infertility, nonclassic cystic fibrosis or a normal phenotype. Groman and co-workers [136] analyzed a population of subjects carrying a severe *CFTR* mutation on one *CFTR* gene and a 5T allele on the other, and demonstrated that individuals with the 5T tract adjacent to either 12 or 13 TG repeats were substantially more likely to exhibit an abnormal phenotype than those with 5T adjacent to 11 TG repeats. The effect on exon 9 skipping is mediated by binding of TDP-43 to the (TG)m repeat [137]; it is not clear how elongation of the T tract can partially relieve this inhibition, but it has been speculated that the effect of T tract length might simply reflect increased distance of pre-mRNA-bound TDP-43 from the 3′ splice site; TDP-43 was originally described to bind a poly-pyrimidine-rich region of the HIV-1 *trans*-acting responsive (TAR) DNA element and function as a transcriptional inhibitor [138]; up to now, no clear cellular function has been ascribed to it, so Buratti and co-workers speculated that this protein might suppress exon 9 splicing through steric hindrance of $U2AF^{65}$ binding to the 3′ splice site. Interestingly, TDP-43 has been found to be predominantly expressed in tissues that are particularly affected by CF, namely pancreas, lung and genital tract [137]. In addition to the TG(m)T(n) locus, both an ISS and an ESS have been identified that exert their effect by binding to SR proteins, although the molecular mechanisms of splicing inhibition through SF2/ASF binding is not well understood [139]. Moreover, a stretch of 15 nt within exon 9 have been shown to act as a CERES (composite regulatory elements of splicing) in that silencing and enhancing functions are juxtaposed in a relatively short region [140]. Two other such elements were described [141] in CFTR exon 12: these elements were hypothesized to exert their effect in a context-dependent manner so as to determine exon-specific combinations of *trans*-acting factors and/or RNA structures that correctly define the entire target exons. Accordingly, CERES cannot act autonomously in a heterologous system, and therefore these sequences have not been included in table 1. Finally, a polypyrimidine-rich controlling element (PCE) is located immediately downstream of the exon 9 5′ splice site and participates in splicing regulation [142]. In particular, TIA-1 binding to the PCE probably recruits U1 snRNP to the weak 5′-splice site and increases exon inclusion. Conversely, PTB, which has a negative role on exon 9 splicing, competes with TIA-1 for binding to this sequence, suggesting that PTB might induce exon skipping through antagonizing the binding of TIA-1. Yet a minigene carrying mutations in the PCE still responds to PTB overexpression, indicating that additional mechanisms must be involved. Indeed, Zuccato and co-workers mention that PTB is able to bind exon 9 sequences, possibly multimerizing with PCE- bound molecules and forming a repressive complex.

Spinal muscular atrophy (SMA, OMIM: #253300)

SMA is a common human genetic disease that is the leading cause of hereditary infant mortality. In 96% of

patients, the pathology is caused by homozygous loss of the telomeric copy of the survival motor neuron gene (SMN1). The human *SMN1* gene is located within a duplicated chromosomal region which contains a second, centromeric *SMN* gene (*SMN2*). While *SMN1* primarily expresses a full-length transcript, the centromeric *SMN* gene predominantly yields a truncated isoform [143] that lacks exon 7; a single C-to-T nucleotide difference at position +6 in exon 7 differentiates *SMN2* from *SMN1* and is responsible for preferential exon 7 exclusion [143]. Previous [144] studies on the mechanisms leading to exon 7 skipping indicated that the transition was responsible for an ASF/SF2-dependent ESE disruption. But, more recent work [145, 146] has demonstrated that the C-to-T variation results in the creation of an ESS: the sequence CAGACA in *SMN1* is changed to UAGACA in *SMN2,* and this latter motif displays splice silencing and hnRNPA1-binding activities. A second element with splice-silencing activity has been identified in *SMN* intron 6 (table 1); this element probably acts in synergy with the ESS to determine exon skipping in *SMN2*. Indeed, an ESE is located within exon 7 and is functional in both *SMN1* and *SMN2*. This sequence is bound by a complex containing hTra2ß, hnRNP G and probably other SR proteins [147]; therefore, inhibition of exon 7 splicing in *SMN2* results from competition between positive and negative regulatory factors that bind distinct sequences within the same exon.

Ataxia-telangiecstasia (AT, OMIM: #208900)

Recessive mutations in the *ATM* gene are responsible for ataxia telangiecstasia, a pathology characterized by cerebellar degeneration, immunodeficiency, radiosensitivity and cancer predisposition. The majority of *ATM* mutations are single base pair substitution or microinsertions and deletions that result in protein truncation or aberrant splicing patterns. Yet, as reported above, Pagani et al. [19] have demonstrated that disruption of a silencer element in intron 20 results in the inclusion of a 65-nt cryptic exon. The mutation responsible for silencer disruption is a 4-nt deletion and results in 100% cryptic exon inclusion from the mutated allele.

Multiple sclerosis (OMIM: #126200)

CD45 is a transmembrane glycoprotein possessing tyrosine phosphatase activity, which is expressed on all nucleated hematopoietic cells. In both human and mouse [148, 149], CD45 deficiency leads to severe combined immunodeficiency, while mutations that cause CD45 hyperactivity determine lymphoproliferation and autoimmune disease phenotypes in mice [150]. As reported above, exons 4, 5 and 6 of CD45 are alternatively spliced, and it has been speculated that alternative exon usage might alter the dimerization ability of CD45 molecules, which, in turn, might downregulate phosphatase activity [150]. In particular, exon 4 (or A) is the most tightly regulated of the CD45 variable exons (reviewed in [151]); this exon is included at high levels in naive T cells, while activated and memory T cell mainly express exon 4-minus CD45 forms. A C77G polymorphism within exon 4 was previously shown to result in increased exon inclusion and to correlate with the development of multiple sclerosis [152]. More recently, Lynch and co-workers [153] were able to demonstrate that the C77G polymorphism is responsible for disruption of a splicing silencer element resulting in improved exon 4 inclusion and increasing the risk of disease. These authors identified a region of exon 4 that functions as an ESS. The ESS exerts its effect by blocking recognition of the 5′ splice site and can repress exon inclusion when inserted in a heterologous context. The authors also demonstrated that the ESS binds to a protein complex that probably mediates repression, although they did not analyze its single proteic components. In addition to the ESS, additional splicing regulatory elements within exon 4 modulate its inclusion. In particular, two enhancer sequences as well as a second, weaker silencing element at nucleotides 169–188 were identified.

More recently, another polymorphism in CD45 was described [154] that alters the relative proportion of alternatively spliced forms. This variation is located in exon 6 (A138G) and is responsible for increased expression of CD45 transcripts lacking exons 4, 5 and 6. The mechanism which is responsible for such alteration remains unknown as well as its effects (if any) on phenotype and disease.

Frontotemporal dementia with parkinsonism associated with chromosome 17 (FTDP-17, OMIM: #600274)

Tau is a microtubule-associated protein involved in neuronal morphogenesis, axon polarity and axonal transport. During central nervous system development the tau transcript undergoes regulated alternative splicing [155], and in adults exons 2, 3 and 10 are alternatively spliced, yielding six different isoforms. Exon 10 encodes one of four microtubule-binding motifs, and mutations that increase inclusion of this exon cause FTDP-17 [156, 157]. Several *cis*-acting splicing regulators have been identified within exon 10 and the downstream intron sequence. In particular, intronic FTDP-17 mutations that increase exon inclusion are clustered in an intron, 10 ISS [158, 159]. This sequence has been shown to function in a position-independent manner and to be flanked by a modulator [159]. This latter element, the ISM (intronic splicing modulator) counteracts the effects of the ISS but does not function as an ISE per se, being able only to mitigate ISS-dependent splicing inhibition. Although the *trans*-acting factors binding to the ISS/ISM sequences are at present unknown, it has

been hypothesized [159] that factors associating with the ISM might sterically hinder the stability or association of ISS-specific inhibitory factors, thus allowing indirect enhancement of exon 10 inclusion. Exon 10 also displays an ESS (table 1): this sequence is responsible for FTPD-17 when mutated and is embedded within ESE sequences. Therefore, the current model for tau exon 10 regulation proposes that multiple *cis*-acting sequences (including weak 5′ and 3′ splice sites) act in concert to provide the correct balance between exon definition and exon skipping; any alteration in this balance is probably pathogenetic, as evidenced by the description of FTDP-17 mutations affecting these sequences.

Concluding remarks

The observations reported above clearly indicate that our knowledge on the *cis*- and *trans*-acting factors that repress splice site selection and inhibit exon inclusion in pre-mRNAs is far from exhaustive. The combinatorial associations of factors, the integration of multiple synergistic or antagonizing signals in the modulation of one same splicing event, as well as the dual role of many regulatory proteins and their ability to act as both repressors or activators depending on the system and substrate they interact with, make deep understanding of splicing regulation a tantalizing and difficult task. In particular, information about mechanisms linking signal transduction to splicing regulation is missing in many instances. Still, possibly, other features of splicing regulation (including silencing) are far more obscure than these ones. Transcription and splicing are spatially and temporally linked within the cell [182], with splicing often being co-transcriptional (reviewed in [183]). It was recently demonstrated that alterations in promoter elements that influence the elongation rate of the polymerase have an impact on alternative splicing decisions, and insertion within regulated genes of RNA polymerase II pause sites can alter alternative exon inclusion frequencies [184, 185]. In particular, Robson-Dixon and Garcia-Blanco [185] demonstrated that the insertion of pause sites in different positions with respect to known ISSs of the *FGFR-2* mRNA had different effects on the silencing of exon IIIb. Interestingly, these authors also indicated that the cell-type-specific regulation of *FGFR-2* required co-transcriptional splicing and did not occur when minigenes were transcribed in vitro and transfected. Such a situation is not difficult to envisage: different *cis*-acting elements required for silencing or splicing of an exon gradually become available as the polymerase proceeds along its template; the order and kinetic of synthesis of each *cis*-element can therefore be expected to reflect the binding order and kinetics of positive rather than negative regulators, perhaps depending on the their relative availability. Moreover, elongation rate might well have an effect on pre-mRNA secondary structure formation, possibly favoring specific structures rather than others with equal or even more favorable thermodynamic stability. Finally, proteins associated with the transcription complex may recruit splicing factors or influence their binding to substrate RNAs. Such events and interactions might not be difficult to conceive, but are extremely difficult to analyze in detail and almost impossible to predict.

Considerable effort will be required before a comprehensive description of splicing regulatory mechanisms is achieved; such knowledge will probably provide deeper understanding of fundamental biological processes as well as human phenotypic variability, genetic disease, predisposition to pathology and modulation of clinical outcomes.

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