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GENE REGULATION '97
The Regulation of Splice-Site Selection, and Its Role in Human Disease

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The pre-mRNA splicing machinery recognizes exons and joins them together with remarkable precision to form mRNAs with intact translational reading frames. Splicing requires canonical sequences at the intron/exon border, and mutation of these sequences may cause abnormal splicing patterns that affect gene expression and cause disease. Recent studies indicate that distinct sequence elements that are distant from the splice sites are also needed for normal splicing. These elements can affect splice-site recognition during constitutive splicing and also play important roles in directing alternative splicing, a common phenomenon in which multiple mRNAs, encoding functionally distinct proteins, are generated by use of different combinations of splice junctions, according to developmentally regulated or tissue-specific programs. A number of auxiliary splicing elements required for cell-specific modulation of alternative splicing have been found within introns that flank alternative exons. A second set of splicing elements, exonic splicing enhancers (ESEs), are found within both coding and noncoding exons. These enhancers direct the specific recognition of splice sites during constitutive and alternative splicing. The prevalence of alternative splicing as a mechanism for regulation of gene expression makes it a likely target for alterations leading to human disease. Below we summarize what is known about various sequences that affect splice-site selection and illustrate how changes in alternative splicing may lead either to disease or, conversely, to an amelioration of the effects of certain genetic lesions.

Intronic Splicing Elements and Splicing Regulators

Although modulation of the nuclear concentrations of constitutive RNA processing factors causes some alternative splicing events (Takagaki et al. 1996), this mechanism is unlikely to account for all regulated splicing observed in vertebrates. There are many examples in which different regulatory programs run concurrently within the same cell, suggesting that different alternatively spliced pre-mRNAs are regulated by distinct programs that use different sets of *cis* elements and *trans*-acting factors. Strong evidence that cell-specific factors are responsible for alternative splicing comes from studies on intronic elements that mediate cell-specific splicing (Guo et al. 1991; Tacke and Goridis 1991; Black 1992; Gooding et al. 1994; Huh and Hynes 1994; Ryan and Cooper 1996). One model system in which such elements have been identified is the cardiac troponin T (cTNT) gene (Ryan and Cooper 1996). Figure 1*A* shows a diagram of cTNT exons 4–6, in which the alternative exon 5 is included in embryonic striated muscle and is skipped in the adult. Exon inclusion in embryonic muscle requires intronic elements, referred to as "muscle-specific splicing enhancers" (MSEs), located a short distance upstream and downstream of the exon (shown as small boxes in fig. 1*A*). Evidence from transient transfection into embryonic muscle and nonmuscle cell cultures suggests that these elements regulate splicing via regulatory factors specific to embryonic muscle that promote inclusion of the exon (Ryan and Cooper 1996).

Few potential regulators of vertebrate alternative splicing have been found, but recent studies have identified one candidate, called "SWAP," a homologue of a *Drosophila* splicing regulator, suppressor of *white apricot* (Zachar et al. 1987; Denhez and Lafyatis 1994; Spikes et al. 1994). In both humans and *Drosophila,* the SWAP splicing factor negatively regulates its own expression at the posttranscriptional level. SWAP inhibits its splicing of its own pre-mRNA, which, in its unspliced form, encodes a nonfunctional truncated protein. Recent evidence from transient-transfection experiments demonstrates that overexpressed SWAP protein in mammalian cells regulates splicing of several alternatively spliced pre-mRNAs (Sarkissian et al. 1996). Significantly, in transient-transfection experiments, overexpression of the SWAP protein affects splicing patterns differently than does overexpression of a general splicing

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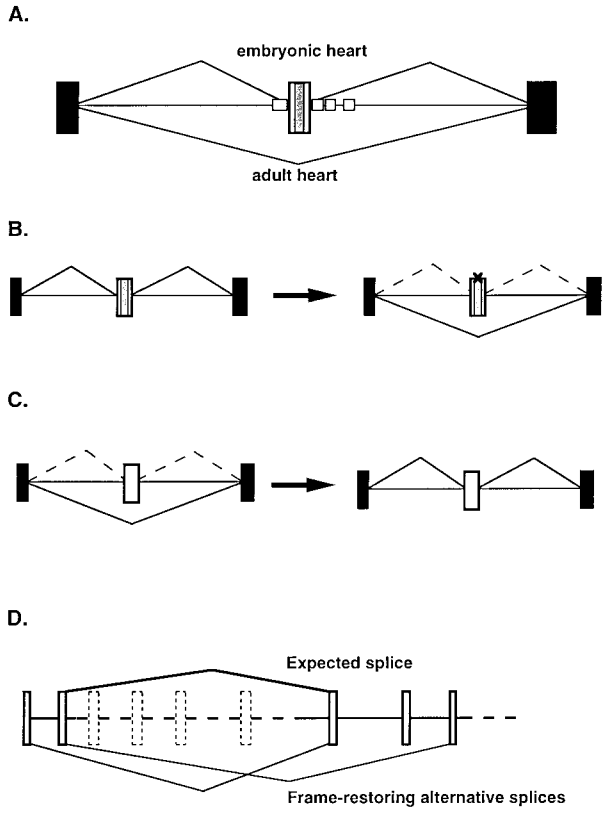

Figure 1 Alternative splicing in human disease. *A,* Alternative splicing cardiac troponin T pre-mRNA. Exons 4–6 of the 18 exon cardiac troponin T gene are shown diagramatically. Intronic elements (MSEs) required for inclusion of the alternative fifth exon in embryonic heart have been identified (*small unblackened boxes*). cTNT exon 5 contains an ESE which is represented as a gray-shaded box. *B,* ESE mutation in an exon-splicing enhancer, resulting in exon skipping. *C,* Alteration in splicing machinery: expression of distinct CD44 mRNAs, correlating with changes in the cellular splicing machinery. In cancer cells, the metastatic phenotype is associated with the expression of inappropriate CD44 protein isoforms. *D,* Restoration of reading frame (dystrophin), as a result of alternative splicing in BMD. The genomic deletion is indicated by dotted lines.

factor, indicating specificity in HsSWAP regulatory ability.

ESEs

Perhaps the best understood auxiliary splicing elements are ESEs, sequences that are internal to exons and that function to enhance the use of specific splice sites. The importance of exon sequences for splice-site recognition was first revealed in studies of artificial β-globin RNAs, in which the selection of either of two competing splice sites was influenced by adjacent exonic sequences (Reed and Maniatis 1986). Subsequent results from many experimental systems demonstrated that sequences internal to exons play a role in splice-site selection during both constitutive and alternative pre-mRNA splicing (see Coulter et al. 1997 and references within). The sequences of some of the best defined ESEs are summarized in table 1. These enhancers were identified as exonic sequences that, when altered or deleted, led to a change in the recognition of specific splice sites. Ultimate definition of an ESE is accomplished by a gain-of-function experiment in which the sequence enhances splicing of a heterologous, inefficiently spliced exon. The ESEs shown in table 1 are all functionally autonomous and, with only a few exceptions, share several common hallmarks described below.

The most obvious feature of ESEs is that most are purine rich. However, purine richness alone is not sufficient for splicing-enhancer activity. Experiments performed both in vitro and in vivo demonstrate that neither synthetic poly-G, poly-A, nor several other tested synthetic polypurine sequences act as splicing enhancers in exons known to require ESEs for correct splice-site recognition (Tanaka et al. 1994). Consistent with this, it has been observed that, in naturally occurring ESEs, some mutations that do not affect purine content nonetheless significantly impair the enhancer's function (Ramchatesingh et al. 1995). Moreover, when several cytosine residues were changed to thymidines in the purine-rich IgM ESE, the enhancer's activity was decreased (Tanaka et al. 1994). Thus, it is likely that specific sequences, which may include interspersed pyrimidines, are necessary for the function of purine-rich ESEs.

Since no consensus sequence that describes all known purine-rich ESEs has been recognized, these elements are difficult to identify through simple sequence comparisons. The lack of a clear consensus among purine-rich ESEs might be explained if the known enhancers derive from several functionally distinct classes of elements or if *trans*-acting factors that recognize these enhancers have flexible RNA-binding–site specificities. In support of the former possibility, recent studies suggest that there are qualitative differences in the way in which some purine-rich enhancers behave when they are inserted into the same alternatively spliced RNA (authors' unpublished data). Further evidence for the existence of multiple types of ESEs comes from recent molecular screens in which either an in vitro functional assay (Tian and Kole 1995) or in vivo (Coulter et al. 1997) functional assay is incorporated into a combinatorial selection strategy (see Antic and Keene 1997 [in this issue]), the "SELEX" method originally developed by Tuerk and Gold (1990). In the in vivo selection, enhancers were identified by inserting a large pool of random 13-nucleotide sequences into the middle exon of a three-exon construct and selecting for those that enhanced inclusion of the resident exon. This allowed the cellular splicing machinery to identify those sequences with splicing-enhancer activity, on the basis of function. The in vivo SELEX strategy identified two general classes of splicing en-

Table 1

ESE Sequences

Sequence[a]	Gene	Reference(s)
Purine-rich ESEs:		
GGAAGGACAGCAGAGACCAAGAG	Human IgM (exon M2)	Watakabe et al. (1993), Tanaka et al. (1994)
GAGAUGUGAUGAAGGAGAUGGGAGG	Human *HPRT* (exon 3)	Steingrimsdottir et al. (1992), Tanaka et al. (1994)
GAAGAAGAC	Human fibronectin (exon ED1)	Lavigueur et al. (1993), Caputi et al. (1994)
GAAGAAGAAG	Human calcitonin (exon 3)	Yeakley et al. (1996)
CUUCCGGAAG	Bovine growth hormone (exon 5)	Hampson et al. (1989), Dirksen et al. (1994)
GAGGAAGAGAAAAGGGCAGCAGAGGAGAGGCA	Chicken caldesmon (exon 5)	Humphrey et al. (1995)
AAGAGGAAGAAUGGCUUGAGGAAGACGACG	Chicken cTNT (exon 5)	Xu et al. (1993)
AAAGGACAAAGGACAAAA	*Drosophila doublesex* (exon 4)[b]	Lynch and Maniatis (1995)
ACEs:		
ACUUCAACAAGUU	Human calcitonin (exon 4)	van Oers et al. (1994)
CCACCAGAAGGUAUG	Chicken cTNT (exon 16)	Wang et al. (1995)
UCUUCAAUCAACA	*Drosophila doublesex* (exon 4)[b]	Ryner and Baker (1991), Inoue et al. (1992), Lynch and Maniatis (1995)

[a] With the exception of the ACE in human calcitonin, each of the sequences shown has been demonstrated to have autonomous enhancer activity in an exogenous exon. In their native RNA, many of these sequences are likely to function in combination with additional exonic enhancer elements that are not shown.

[b] For regulated splicing both the purine-rich and ACE enhancers of *doublesex* are required, but both sequences independently confer constitutive splicing-enhancer activity.

hancers: a purine-rich motif resembling previously identified ESEs and a novel A/C-rich enhancer called "ACE." ACE enhancer activity was confirmed by point mutants and by the ability of the selected elements to activate splicing of additional exons. Although not previously recognized as a class of splicing enhancers, functional ACE elements have been identified in several exons in which splicing has been studied extensively (van Oers et al. 1994; Wang et al. 1995; Coulter et al. 1997) (see table 1). Multiple purine-rich and non–purine-rich enhancers were also identified in a similar SELEX-based approach, by use of an in vitro assay to select sequences that improve splicing efficiency (Tian and Kole 1995). Interestingly, both the in vivo selection and the in vitro selection identified a complex group of enhancers with a number of sequences represented only once in the set of selected clones. This strongly suggests that a large number of sequences can function as ESEs and that use of auxiliary splicing enhancers within exons may be widespread within the genome.

Alternative Splicing, ESE Function, and SR Proteins

A key step in the understanding of splicing-enhancer function was the realization that purine-rich ESEs are recognized by members of the SR family of splicing factors, a group of structurally related and highly conserved RNA-binding proteins (Roth et al. 1990; Fu 1995). SR proteins play essential roles in the early steps of splice-site recognition and interact directly with snRNP-associated proteins to facilitate assembly of the prespliceosome complex on the pre-mRNA (Fu 1995; Manley and Tacke 1996). SR proteins bound to ESEs are thought to recruit key splicing factors to a localized region near the affected splice site, enhancing its recognition (Berget 1995; Reed 1996).

SR proteins affect alternative-splice-site selection in vitro. Interestingly, individual members of the SR family proteins differ qualitatively in which splice sites they select (Zahler et al. 1993). This raises the possibility that tissue-specific expression of SR proteins may drive variations in splicing patterns. For instance, the expression levels of specific members of the SR protein family during T-cell activation correlate with the level of inclusion of a CD44 variable exon, implying a possible role for SR proteins in formation of specific CD44 splice variants (Screaton et al. 1995).

The interaction between SR proteins and ESEs may be an important target for regulation by other tissue-specific factors. By far the best-described example of this comes from studies on the pre-mRNA that encodes the *Drosophila* sex-determination factor, *doublesex* (McKeown 1992). In this case, the assembly of a functional SR protein complex on the repeated ESEs in the *doublesex* pre-mRNA depends on the presence of two regulatory proteins, transformer and transformer-2, which bind SR proteins and associate with ESEs (Tian and Maniatis 1993; Amrein et al. 1994; Heinrichs and Baker 1995; Lynch and Maniatis 1995, 1996). Because transformer protein is expressed only in female flies (Boggs et al. 1987), formation of the ESE/SR complex is sex specific.

Although vertebrate ESEs are typically found in alternatively spliced pre-mRNAs, it remains uncertain whether all of these sequences function in cell-type–specific splicing. In some cases it appears that ESEs serve only as general splicing elements that improve the utilization of suboptimal splice sites that would otherwise be ignored by the general splicing machinery; for instance, the alternatively spliced cTNT exon 5 contains an ESE that improves the efficiency of exon inclusion (see fig. 1*A*), but cell-type–specific regulation is conferred by the intronic MSE elements (Xu et al. 1993; Ryan and Cooper 1996). It is important to note that cell-specific differences in exon inclusion are observed even in the absence of the cTNT ESE, indicating that it is not required for regulation. Nonetheless, given the frequency at which ESEs are found in alternatively spliced pre-mRNAs, it is difficult to imagine that cell-type–specific regulation does not involve ESEs.

A Role for ESEs in Human Genetic Disease

Fifteen percent of all point mutants that result in human genetic disease create an RNA splicing defect (Krawczak et al. 1992). A significant fraction of these affect exon sequences that are distinct from the consensus splicing signals near the intron/exon borders. Disease-causing point mutations that are likely to disrupt ESEs have been identified in a variety of genes (see table 2). Of the nine mutations shown in table 2, all but one has been demonstrated to disrupt splicing in the affected gene (fig. 1*B*). Four of the mutations are within purine-rich regions, two are within AC-rich regions, and three are in sequences that do not resemble ACE or purine-rich ESEs. Demonstration that the exon mutations listed in table 2 disrupt splicing enhancers will require evidence that the affected sequences function autonomously and that enhancer function is lost in the mutant. A splicing enhancer has been most convincingly identified in the case of the hypoxanthine phosphoribosyltransferase (HPRT) gene, *HPRT*. *HPRT* mRNA splicing was found to cause HPRT deficiency in a significant fraction of lymphocyte colonies selected from normal human subjects. In one study (Steingrimsdottir et al. 1992), 3 of 34 *HPRT* splicing mutations were located internal to the exonic portion of splice sites and did not introduce cryptic splice sites or stop codons (for a review of the effects of stop codons on RNA processing and stability, see Maquat 1995). At least one of these mutants affects a sequence that functions as an autonomous ESE when inserted into another gene (Tanaka et al. 1994).

Pertubations in cellular splicing factors may affect either the efficiency of splicing or the regulation of alternative splicing and therefore may also be associated with disease states; for instance, changes in the efficiency of splicing have been associated with malignant transformation and metastasis (Mochizuki et al. 1992; Oyama et al. 1993; Salmi et al. 1993). The best example of this is the cell-surface–adhesion molecule CD44 (reviewed in Cooper and Dougherty 1995). Extensive alternative splicing of the CD44 pre-mRNA, involving $\geqslant$12 variable exons, produces a variety of protein isoforms according to complex tissue-specific and developmental regulatory patterns. Although the specific functions of the isoforms are unknown, variable exons encode parts of a multifunctional extracellular domain that bind components of the extracellular matrix—in particular, hyaluronan and proteoglycans (Aruffo et al. 1990). A number of studies suggest that reexpression of CD44 variable exons determines the metastatic potential of some cancer-cell lines. Expression levels and RNA splicing patterns of CD44 correlate with the expression of certain SR proteins and differ between malignant cells and matched normal cells (Tanabe et al. 1993; Screaton et al. 1995). More important, overexpression of specific CD44 splice variants is sufficient to establish metastatic potential in nonmetastasizing cell lines (Gunthert et al. 1991; Dougherty et al. 1992). Altering expression of metastasis-associated isoforms by antibodies or antisense reduces metastatic behavior of cell lines (Seiter et al. 1993; Merzak et al. 1994).

The correlation between metastasis and patterns of CD44 splicing suggests that metastatic transformation can result from changes in components of the splicing machinery that affect cell-specific or general exon recognition (fig. 1*C*). If so, the *cis*-acting ESEs and the *trans*-acting SR proteins may provide targets for novel therapies that will ameliorate genetic diseases by altering patterns of pre-mRNA splicing (for an example, see Sierakowska et al. 1996) Interestingly, in the case of some muscular dystrophies, naturally occurring alternative splicing may provide a mechanism by which severe mutations are tolerated. Approximately 60% of the cases of Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are caused by intragenic deletions within the 2.4-Mb dystrophin gene. In the more severe form, DMD, genomic deletions put the mRNA out of frame, whereas in the milder form, BMD, the remaining exons encode in-frame proteins that contain internal deletions (Malhotra et al. 1988; Koenig et al. 1989). Presumably, the shortened in-frame proteins retain residual function, which results in a less severe phenotype. However, rare exceptions to this "reading frame theory" were found, for both DMD and BMD; for example, cases that presented clinically as BMD contained out-of-frame genomic deletions. Analysis of dystrophin mRNAs by reverse-transcriptase–PCR (RT-PCR) in several of these BMD patients demonstrated a low level of in-frame mRNAs produced by alternative splicing of one or more exons (see fig. 1*D*) and suggest a potential mech-

Table 2

Exonic Mutations That Cause Disease and Affect Splicing

Natural Sequence[a]	Change		Gene Affected	Disease	Reference
	Nucleotide	Amino Acid			
ATGAGAGTGATTCGCGTGGGTACCCGCAAGAG	C→G	Silent	Porphobilinogen deaminase	Intermittent porphyria	Llewellyn et al. (1996)
ATTGGAGACACGGTGAG	G→A	Silent	Integrin GPIIIa	Glanzmann thrombocytopenia	Jin et al. (1996)
CCTTATGAACGACTGGAGTG	C→T	Silent	Fumarylacetoacetate hydrolase	Hereditary tyrosinemia I	van Amstel et al. (1996)
CCTGTAAGTATAATGGAAAAGATGAGGTCTGCCTGACTTT	A→G	Silent	Pyruvate dehydrogenase E1a	Leigh encephalomyelopathy	de Meirleir et al. (1994)
GCAATGGTGGGAGATGGAATCAATGACTCCCAGCTCTGGC	G→A	Gly→Arg	*MNK*	Menkes disease	Das et al. (1994)
CAGGAGGGGGAGCGAGACTTC	C→T	Gln→stop	Adenosine deaminase	Adenosine deaminase deficiency[b]	Santisteban et al. (1995)
agCTACCACAGCCCCTAAACCCGCAACAG|TTGTTACRGGTT[c]	G vs. A	Silent	Episialin	Allelic difference	Ligtenberg et al. (1990)
agTCTCTGCCCACAGTGATACCACTGCAG|A[c]	C→T	Thr→Ile	Arylsulfatase A	Metchromatic leukodystrophy	Hasegawa et al. (1994)
agCTTGCGCCGGGACATAGAAG	C→T	Leu→Pro	β-Hexosaminidase β-subunit	Sandhoff disease	Wakamatsu et al. (1992)

[a] Lowercase letters denote intronic sequence; uppercase letters denote exonic sequence; and the mutated is underlined.

[b] The case of adenosine deaminase deficiency was reported to be due to a mutation in the putative splicing enhancer; however, since this mutation also introduces a stop codon, the actual mechanism of exon skipping remains to be determined.

[c] The vertical bar represents an alternative downstream 3′ splice site; when R = G, the upstream 3′ splice site was used; and when R = A, the downstream 3′ splice site was used.

anism for partial rescue of protein function. In one study, mRNA was analyzed from BMD patients with an out-of-frame deletion of exons 3–7. The expected RNA in which exons 2 and 8 were joined was most abundant. However, low levels of two in-frame RNAs were also detected in which either exons 2 and 10 or exons 1 and 8 were joined (Chelly et al. 1990). These in-frame alternative splice products were detected in normal muscle, but at one-tenth the level in the patient's muscle. The mechanism for the higher level of aberrantly spliced product in the BMD patient is unknown. In normal cells, the dystrophin pre-mRNA undergoes extensive alternative splicing that is regulated according to cell type and developmental stage. It is possible that the natural propensity of the huge dystrophin pre-mRNA to undergo alternative splicing allows expression of low levels of in-frame mRNA. Similarly, alternative splicing may explain the 1%–10% of "revertant fibers" found in muscles of 50% of DMD patients with out-of-frame deletions (Sherratt et al. 1993). These fibers contain dystrophin epitopes that are located downstream of the predicted termination codon, so that expression of frame-restored mRNAs must occur (Hoffman et al. 1990; Klein et al. 1992). Although most evidence suggests that revertant fibers are due to a somatic mutation, evidence from RT-PCR analysis supports the possibility that revertant fibers contain low levels of frame-restoring alternative splicing (Sherratt et al. 1993). It will be of interest to determine whether these fibers contain somatic mutations in the dystrophin gene that result in altered splicing.

The observation that mutations within exons can affect splicing may provide a molecular explanation for how some disease-causing mutations interfere with gene function; for example, previously unexplained "silent" third-codon-position mutations might alter ESE sequences and thus affect splicing. Many missense mutations in exons have been assumed to cause disease via disrupted protein function, but, as can be seen in table 2, mutations that alter protein-coding regions may also affect splicing. Prior to the discovery of splicing enhancers, elements required for splice-site selection were assumed to occur in introns, and it seemed unlikely that coding exons would contain such "overlapping" information for RNA processing (Padgett et al. 1986). With the regulatory role of exons in splicing now established, and with some of the molecular events in alternative splicing becoming clear, mutations in either ESEs or genes for splicing factors are emerging as potential causes of human genetic disease

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