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Signals and their transduction pathways regulating alternative splicing: a new dimension of the human genome

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The human genome project demonstrated that alternative splicing of genes is more the rule than the exception. Missplicing events are an important cause and indication of human disease. Changing alternative splicing patterns in response to an external stimulus seems to be a physiological process performed by many cells. Organisms regulate alternative splice site selection by changing the concentration and activity of splicing regulatory proteins. This is achieved by *de novo* protein synthesis, by regulation of the intracellular localization and by phosphorylation.

Most eukaryotic genes contain intervening sequences (introns) that interrupt the exonic sequences, which are joined in the pre-mRNA splicing process. A large number of exons are used alternatively; for example, the cell either includes an exon in the mature mRNA or omits this sequence as an intron. The sequencing of mammalian genomes demonstrated the importance of alternative splicing. In humans, a detailed analysis was performed for chromosome 22 and 19 (1). Of the 245 genes present on chromosome 22, 59% are alternatively spliced, and the 544 genes of chromosome 19 result in 1859 different messages. The comparison of expressed sequence tags (ESTs) with the human genome sequence indicates that 47% of human genes might be alternatively spliced (2). Since alternative splicing events usually result in multiple protein isoforms from a gene, this mechanism contributes to the surprising finding that only 23 000–40 000 genes (1,3) create a larger proteome, estimated to be >90 000 proteins (4).

The importance of alternative splicing is further illustrated by the increasing number of human diseases that have been attributed to missplicing events. The usage of alternative exons can be controlled by cells, for example in a developmental, tissue-specific or pathology-specific stage. As shown in this review, cells use known phosphorylation pathways, as well as relocalization and synthesis of splicing factors, to change their alternative splicing patterns. The emerging picture of signal transduction pathways regulating pre-mRNA alternative splicing will contribute to the development of therapies against diseases caused by missplicing.

SELECTION OF SPLICE SITES

The joining of exons is performed by the spliceosome, a macromolecular complex comprising five small ribonucleoprotein particles (snRNPs) and a total of at least 45 proteins (5). The catalytic events of this process have been determined in great detail, and have recently been reviewed (6,7). However, how the weakly conserved splice sites are recognized and often alternatively controlled remains unclear (8,9). The borders of an exon are defined by the 5′ and 3′ splice sites as well as the branch-point. Owing to the degenerate nature of these *cis* elements, additional elements known as silencers or enhancers are needed to define an exon (10). Proteins binding to these sequence elements can be subdivided into two major groups: heterogeneous ribonucleoproteins (hnRNPs) (11) and SR/SR-like proteins (12). Usually these proteins contain RNA-binding motifs and protein : protein interaction domains. Although the binding of each individual protein to *cis* elements on the RNA is weak, a high specificity is achieved through interactions of multiple RNA elements with proteins that can interact with other RNA-binding proteins. As a result, exons are recognized with a remarkable fidelity through combinatorial control of different proteins (9). Since the concentrations of these proteins differ between tissues (13) or cell types (14–16), exons can be recognized alternatively in a tissue-specific manner, indicating a cellular code for splice site selection. In a given tissue, the activity of splicing regulatory proteins can change during development (17,18), which explains the frequently observed change (19) of splicing patterns during development. The combination of the regulatory proteins also seems to vary between individuals of the same species, which explains the different alternative splicing of the same allele. This results in a different penetrance of a disease-causing gene (20,21) and indicates that alternative splice site selection could be a potential genetic modifier of human disease (22).

Although splicing can be biochemically separated from other gene expression events, it is clear that pre-mRNA splicing is

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tightly coupled to transcription (23), polyadenylation (24), editing (25), RNA surveillance (11) and transport (26). Most of these processes share common proteins, and in an intact cell mRNA is most likely created by 'gene expression' (27) or 'RNA factories' (28). As a result of this interdependence, splice site selection can be influenced *in vivo* by indirect effects, for example promoter activity (29).

SPLICING AND DISEASE

Changes in splice site selection are increasingly recognized as the cause or the consequence of human pathologies (reviewed in 30–32). Observed changes in splice site usage can result from mutations in regulatory elements or variations in the composition, concentration or localization of regulatory proteins. Mutations in splice sites account for at least 15% of point mutations causing disease in humans (33,34). Mutations in exonic elements can alter gene expression by changing splice site usage. Several of these mutations are translationally silent, which clearly demonstrates aberrant pre-mRNA processing as the most likely cause of disease (35). The nuclear concentration of regulatory proteins is changed in several diseases. For example, in mammary gland tumorigenesis (36) and ovarian cancer (37), the amount and ratios of SR proteins are altered. In brain ischemia, which is caused by stroke, SR proteins are translocated from the nucleus to the cytoplasma (38).

Proximal spinal muscular atrophy (SMA) provides a good example that illustrates changes of alternative splicing patterns in a human disease. SMA is a neurodegenerative disorder with progressive paralysis caused by the loss of α-motor neurons in the spinal cord. It is the second most common autosomal recessive disorder and the most frequent genetic cause of infantile death (39). The gene responsible for the disease is *SMN1* (survival of motor neurons), and the disease is caused by loss of (96.4%), or mutations in (3.6%), the *SMN1* gene (40). *SMN2* is a nearly identical copy of the *SMN1* gene, but cannot compensate for the absence of *SMN1* because it is processed differently. Owing to a single nucleotide difference in exon 7, this exon is skipped in *SMN2*. Therefore the proteins generated by the two genes differ in their C terminus, which is most likely crucial for the function. The exon enhancer containing the single nucleotide difference has been characterized (41,42) and found to be of the purine-rich GAR type. Systematic searches for *trans*-acting factors identified SRp30c and transformer2β, members of the SR-(related) family of proteins (43,44). In co-transfection experiments, an increase in the concentration of both factors result in stimulation of exon 7 usage. In a mouse model, administration of high doses of sodium butyrate increases both SR-protein levels and exon 7 usage and enhances survival of the animals (45). In a cell culture system, aclarubicin, an anticancer drug used in chemotherapy, was also shown to increase exon 7 usage (46). To date, the link between the two substances and a change in splice site selection remains unclear. The SMN model illustrates the following general points: (i) diseases caused by splicing defects are common; (ii) a point mutation is sufficient to cause a phenotype; (iii) alternative exon usage is regulated by several factors *in vivo*; and, importantly, (iv) alternative exon usage can be changed by proper cell treatment or stimulation.

SIGNALS THAT CHANGE THE SELECTION OF SPLICE SITES

Changes in splice site selection not only occur as a result of a disease, but are a normal adaptation of a cell. Studies in recent years showed that numerous stimuli, such as growth factors, cytokines, hormones and depolarization, result in a change in splice site selection. Most of the systems investigated play a role in the immune or nervous system, which reflects the high abundance of alternative splicing in these organs (2,47,48). For some systems, parts of the signal transduction pathways have been worked out, but for most of the signals these pathways remain to be determined. The paradigms applied can be roughly subgrouped into three categories: stimulation of a receptor, cellular stress and neuronal activity (Table 1). Several studies performed in intact animals with physiological stimulation protocols resulted in an alteration of splice site selection. For example, when mice are forced to swim, this stressful experience is enough to change the alternative splicing pattern of the acetylcholine esterase pre-mRNA (49). Another example is the expression of a Praja1 splice variant after pavlovian fear memory training (50). Nutritional status, especially the amount of polyunsaturated fatty acids, regulates the alternative splice variants of glucose-6-phosphate dehydrogenase (51,52). These examples show that changes in alternative splicing patterns are part of a physiological adaptation. The changes seen in models of pathology, such as stroke (38) and cocaine (53) and ethanol (54) abuse, are most likely a more pronounced manifestation of the adaptations that occur under normal physiological conditions.

Currently, three major mechanisms are known that change alternative splice site usage in response to a stimulus: synthesis, phosphorylation, and a change in localization of splicing regulatory proteins. Treatment of T cells with phorbol ester stimulates the Ras-pathway by activating PKC, which changes the splicing pattern of CD45. This change in splice site selection is blocked when protein synthesis is inhibited (55). In other systems, a change in splice site selection does not require protein synthesis and is mediated solely by phosphorylation. For example, changes in splicing patterns of CD44 (56) and a plasma membrane Ca^{2+}-ATPase (57) occur without protein synthesis. Expression of SR protein kinases or their inhibition can change splice site selection *in vivo*, suggesting that phosphorylation of SR proteins is a common regulatory principle (58,59). The application of several models of cellular stress results in a change of the intracellular localization of splicing regulatory proteins. A subset of SR proteins shuttles between the nucleus and the cytosol (60). When cells are stressed by osmotic shock or ultraviolet irradiation, some SR proteins and hnRNP A leave the nucleus and accumulate in the cytosol. As a result, splice site selection in a reporter gene is changed (61). When cells are subjected to heat shock, the SR proteins SRp30c and SF2/ASF accumulate in stress-induced nuclear bodies in the nucleus. Again this change is accompanied by a change in splice site selection (62).

Regulation of PKC isoforms by insulin

The regulation of protein kinase C (PKC) βII alternative splicing provides a good example how a hormonal signal changes splice site selection (Fig. 1A). Application of insulin

Table 1. Stimuli that change alternative splicing patterns

Stimulus	Signal transduction	Alternatively spliced transcript	Refs
Stimulation of a receptor			
Phorbol ester; concanavalin A; TCR stimulation	PKC, Ras	CD44, exon v5	(56,66)
phytohemagglutinin	PKC, Ras	CD45, exons 4–6	(55)
Phorbol ester, phytohemagglutinin	PKC, Ras	CD45, exon 4	(55,75)
Phorbol ester; PDGF, EGF	?	PTP-1B	(76)
Insulin	PI-3K	PKCβ, exon βII	(63,65,77)
Insulin	?	Insulin receptor, exon 11	(78)
FGF-1, FGF-2	?	FGFR-2 and -3, exon III	(79)
TNF-α, IFN-γ	?	CD44, exons v6 and v9	(80)
IFN-γ, IL-1β, TNF-α, LPS	?	Nitric oxide synthase (iNOS)	(81)
IL-1β, PDGF TGF-β	?	Fibronectin, exon IIICS	(82)
TGF-β	?	Fibronectin, exon EIIIA	(83,84)
Dexamethasone	?	Insulin receptor	(85)
Hypophysectomy, ACTH	?	Slo	(86)
NGF, dexamethasone	?	NF1	(87)
NGF	Ras	Agrin	(88)
Neuronal activity			
Pilocarpine-induced neuronal activity	?	Htra2β, exon 3; clathrin light chain B, exon EN	(68)
Induction of long-term potentiation	?	Syntaxin 1, 3	(89) (90)
Hippocampal kindling	?	Glutamate receptor A and B, exon FLIP, FLOP	(91)
Rise in intracellular Ca	?	Ca^{2+}-ATPase	(57)
Depolarization		BK potassium channel, STREX	(69)
Dopamine, cocaine	?	Ania-6	(53)
Ethanol	?	$GABA_A$ receptor	(54)
Neuronal stimulation	?	Homer-1	(92)
Pavlovian fear memory	?	Prajala	(50)
Cellular stress			
pH	?	ATP synthase γ subunit, exon 9	(93)
pH	?	Tenascin-C	(94)
Osmotic shock UVC irradiation	MKK3/6-p38	Adenovirus E1A (reporter)	(61)
Forced swim stress		Acetylcholinesterase	(49,95)
Ischemia	Localization	ICH-1	(38)
Heat shock	Localization	Adenovirus E1A (reporter)	(62)
Cold shock	?	Neurofibromatosis type 1, exon 4a	(96)
Heat shock	?	Dopa decarboxylase	(97)
Heat shock	?	Testosterone repressed message 2	(98)
Heat shock	?	Heat-shock protein 47	(99)
Cadmium	?	Bronze2 (Zea mays)	(100)
Miscellaneous			
Nutritional status, polyunsaturated fatty acids	?	Glucose-6-phosphate dehydrogenase	(51,52)
Concanavalin A	?	Qa-2 exon 5	(101)

Signals known to influence alternative splicing patterns are shown in the first column. Signal transduction pathways implicated in the signaling are shown in the second column. The third column indicates the alternatively spliced transcript.
ACTH, adrenocorticotrophic hormone; EGF, epidermal growth factor; FGF, fibroblast growth factor; FGFR, FGF receptor; IL, interleukin; INF, interferon; LPS, lipopolysaccharide; MKK, mitogen activated protein kinase kinase; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PI-3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PTP, phosphotyrosine phosphatase; STREX, stress axis exon; TCR, T-cell receptor; TNF, tumor necrosis factor.

to myotubes results in the incorporation of the PKCβII exon within 30 minutes of stimulation (63). Activation of the insulin receptor by insulin recruits a p85/p110 phosphatidylinositol 3-kinase (PI-3K) to the activated insulin receptor complex. As a result, phosphatidylinositol 3,4,5-trisphosphate is formed. This substance activates several protein serine–threonine kinases. One or several of these kinases phosphorylate predominantly the SR protein SRp40. SRp40 acts on a purine-rich RNA element that was first identified in the intron adjacent to regulated fibronectin EIIIB exon (64). In the PKCβ pre-mRNA, this element is located in the intron downstream of the βII exon, which parallels the situation in fibronectin. Similar to the fibronectin EIIIB system, an increase in SRp40 concentration results in inclusion of the downstream exon (65). However, the exact link between an increase in SRp40 activity and its phosphorylation remains to be determined. One possibility is that specifically SRp40 is recruited from speckles, intranuclear storage sites of numerous factors involved in pre-mRNA processing.

Regulation of CD44 by the Ras/Raf/MEK/ERK pathway

The change of CD44 isoforms in response to T-cell activation is another example of how phosphorylation cascades can regulate

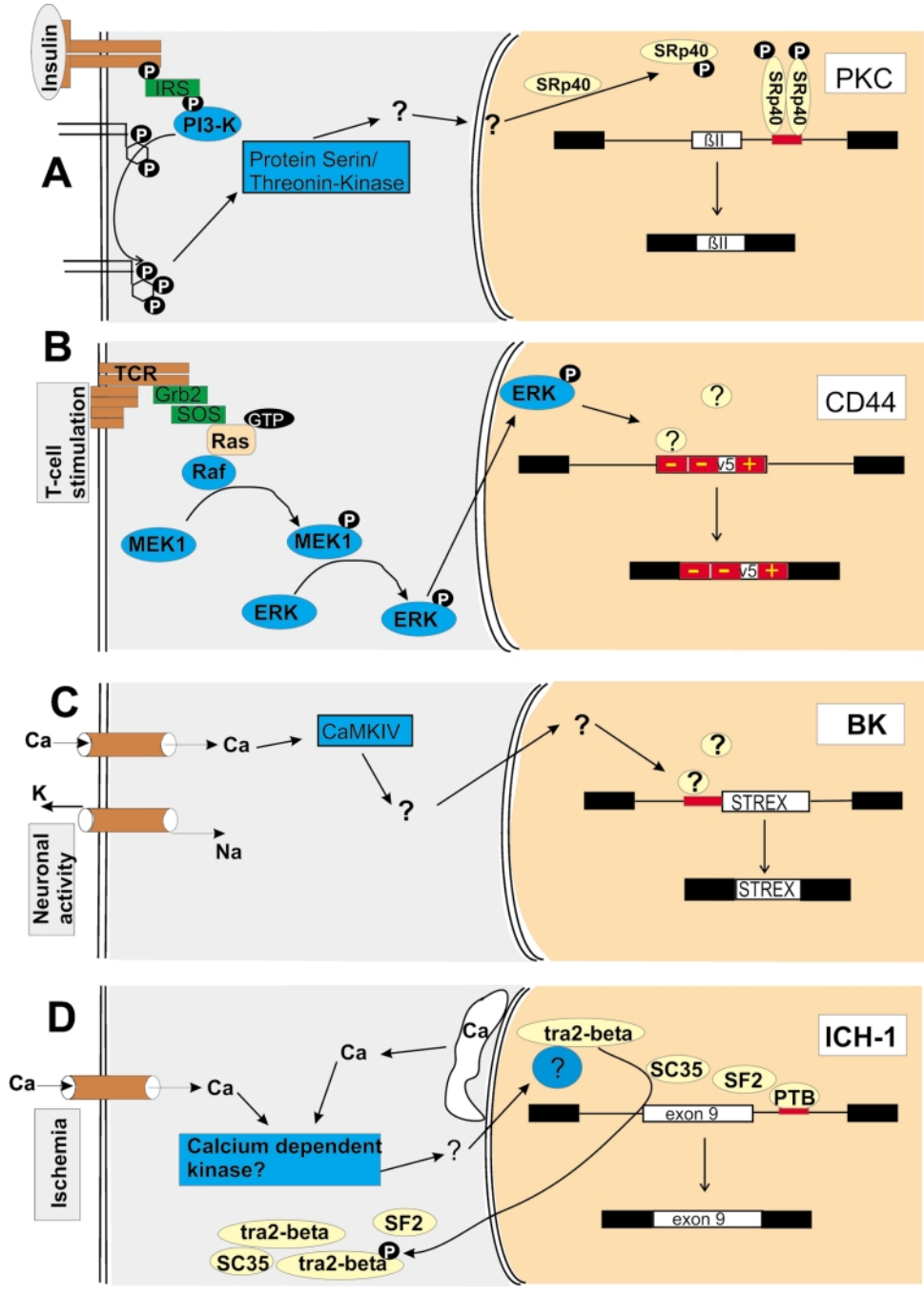

Figure 1. Examples of signal transduction pathways that regulate alternative splice site selection. The stimuli are shown in gray boxes on the left. Receptors or ion channels are shown in brown. The cytosol is shown in gray and the nucleus in sand color. Kinases are shown in dark blue and splicing regulatory proteins in yellow. Adapter molecules are shown in green. Constitutive exons are shown as black boxes and introns as lines. The alternative exons are shown as white boxes. Regulatory RNA elements are shown in red. The name of the gene is shown in a white box. For simplicity, only one of the alternative mRNA isoforms is shown. (**A**) Regulation of PKC splicing by insulin. Binding of insulin activates PI3-K via binding to the insulin receptor substrate (IRS). PI3-K forms phosphatidylinositol 3,4,5-trisphosphate, which activates an unknown protein serine–threonine kinase that ultimately phosphorylates SRp40, which acts on an intronic element that leads to exon βII inclusion. (**B**) Regulation of CD44 after T-cell stimulation. Stimulation of the T-cell receptor activates Ras via Grb2 (growth hormone-binding protein 2) and Sos ('son of severless') binding. Ras activates Raf, which phosphorylates MEK1, which phosphorylates ERK. Phosphorylated ERK translocates into the nucleus and releases repressors on two silencing elements of the alternative exon v5. This exon is subsequently included. (**C**) Regulation of the BK STREX exon by neuronal activity. Neuronal activity opens voltage-gated calcium channels. The rise in intracellular calcium activates CaMKIV, which phosphorylates unknown proteins that bind to an intronic element at the 3′ splice site of the STREX exon. De-repression of this element results in an inclusion of the STREX exon. (**D**) Regulation of the *ICH-1* gene by ischemia. Ischemia evokes a rise in the intracellular calcium levels by opening plasma membrane calcium channels and releasing calcium stored in the endoplasmatic reticulum (white structure). The rise in calcium concentration probably activates a calcium-dependent kinase, which ultimately leads to a translocation of SR proteins from the nucleus to the cytosol. The alternative exon of the *ICH-1* gene is repressed by the SR proteins SC35 and SF2, possibly by indirect interaction with a pyrimidine-rich repressing element downstream of the alternative exon. The decrease in nuclear SR protein concentration results in inclusion of the exon.

splice site selection (Fig. 1B). CD44 is a transmembrane protein that can form multiple isoforms due to alternative splicing of internal cassette exons. The expression of different CD44 isoforms is characteristic of malignant tumors. Systematic overexpression and inhibition of components of the mitogen-activated protein kinase (MAPK; also known as extracellular signal-regulated kinase, ERK) pathway in T cells, showed that activation of the T-cell receptor switches on Ras, which activates the serine–threonine kinase Raf. Raf acts on a MAPK/ERK kinase (MEK; also known as MAPK kinase, MKK)—in the T-cell model most likely MEK1. Finally, MEK1 activates ERK by phosphorylation. Activated ERK translocates into the nucleus and acts on a factor that silences the 5′ and middle part of the alternative exon v5. As a result, exon v5 inclusion is stimulated after T-cell receptor activation (66). How ERK influences CD44 exon v5 splicing is still unclear. However, co-transfection and binding studies implicate hnRNP A as part of the regulated exonic enhancer (67). The regulation of exon v5 does not require protein synthesis, demonstrating that signal-induced phosphorylation of regulatory splice factors is the regulatory principle. Interestingly, the Raf/MEK/ERK pathway is activated by a mutant constitutive active form of Ras, which is found in many tumor cells. Therefore, activation of this pathway could account for the changes in alternative splicing seen in cancer.

Change of alternative splicing as a response to neuronal activity

A change of alternative splice site selection after neuronal stimulation has been observed in several systems. In most of these cases, the subsequent change of protein isoforms is part of an adaptation to neuronal activity, and will likely be used to memorize the stimulus (49,50,53,68,69). The analysis of the STREX (stress axis) exon of the large-conductance Ca^{2+} and voltage-activated potassium (BK) channel revealed the existence of a Ca^{2+}/calmodulin-dependent protein kinase (CaMK) response element (CaRRE) (Fig. 1C). This pyrimidine-rich element is located in the 3′ splice site of the regulated STREX exon and is responsible for STREX repression after neuronal stimulation. The element is specific for CaMKIV, since it does not respond to CaMKI or II. It can be transferred to other ion channels, where it mediates CaMKIV-specific exon regulation. It is therefore possible that this element orchestrates a coordinated response to the rise in intracellular calcium that follows neuronal activity (69). However, the factors binding to this element and their interaction with CaMK remain to be determined.

Change of intracellular localization of splicing factors after stress

When cells are stressed by pH change, osmotic or temperature shock, they often respond with a change in splice site selection. Several studies showed a remarkable specific response of cellular splice site selection to these unspecific stimuli (Table 1). The lack of oxygen in an ischemic event is another cellular stress that evokes a response in affected neurons (Fig. 1D). When the brain area that lacked circulation and oxygen for several minutes was examined, it became apparent that several, but not all, splicing regulatory proteins translocate from the nucleus to the cytosol. Concomitant with this translocation is a change in alternative splice site selection (38) of the interleukin-1β converting enzyme homologue 1 (ICH-1, caspase 2) gene. Its alternative exon 9 is repressed by SC35 and SF2/ASF (70). Since both proteins leave the nucleus after the ischemic event, this translocation could be responsible for the change in splice site selection. The molecular mechanism is still unclear. A negative regulatory element in the intron downstream of exon 9 has been identified and shown to bind to polypyrimidine track-binding protein. It is assumed that SC35 and SF2/ASF act indirectly on this element, for example by sequestration (71). Studies in primary neuronal cultures show that a similar translocation effect can be evoked by increasing the intracellular calcium concentration (38). As some of the regulatory proteins that accumulate in the cytosol are hyperphosphorylated, it is likely that a calcium-dependent kinase is involved in this regulation. More than two pathways seem to be responsible for the stress-mediated translocation of regulatory proteins, since in the ischemia model no activation of the MKK-p38 pathway is observed, which is induced by osmotic shock or UV light that leads to an accumulation of splicing factors in the cytosol of fibroblasts (61).

CONCLUSIONS

The examples compiled here show that alternative splice site selection can be altered by the cell in response to a signal received. Since the formation of protein complexes on the pre-mRNA is responsible for splice site selection, the cell can regulate alternative splicing by altering the composition of these proteins. This can be achieved by altering the interaction of splicing regulatory proteins with RNA or other proteins through phosphorylation or by changing the concentration of these proteins by *de novo* protein synthesis, phosphorylation-dependent release from nuclear storage pools or alteration in the intracellular localization. Several model systems show that phosphorylation of splicing regulatory proteins is at least in part controlled by the established phosphorylation signaling pathways. However, many gaps still remain to be filled.

Given the prevalence of missplicing in human disease, the elucidation of the signaling pathways is an important goal. Because of the combinatorial nature of splice site selection, a combination of bioinformatic (72) and array approaches (73,74) will be needed to work out exactly the signal transduction pathways and their target exons.

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