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Alternative Pre-mRNA Splicing and Neuronal Function

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ABSTRACT

The protein output of a gene is often regulated by splicing the primary RNA transcript into multiple mRNAs that differ in their coding exon sequences. These alternative splicing patterns are found in all kinds of genes and tissues. However in the nervous system, proteins involved in two processes show particularly high levels of molecular diversity created by alternative splicing. These are proteins that determine the formation of neuronal connections during development and proteins that mediate cell excitation. Although some systems of splicing are highly complex, work on simpler model systems has started to identify the molecular components that determine these splicing switches. This review describes how alternative splicing is central to the control of neuronal function, and what is currently known about its mechanisms of regulation. How errors in splicing might contribute to diseases of the nervous system is also discussed.

The recent genomic description of ~35,000 human genes and the earlier Drosophila genome sequence with ~13,600 genes generated much discussion of how a complex organism can be described by so few genes (Adams et al., 2000; Claverie, 2001; Consortium, 2001; Venter et al., 2001). It is clear that the protein complexity of an organism far outstrips the number of transcription units (Black, 2000; Graveley, 2001). The most common means of producing multiple proteins from one gene is through the alternative splicing of the gene's pre-mRNA. The processing of a primary gene transcript can be altered in the inclusion of exons, or the position of individual splice sites or polyadenylation sites to produce a variety of transcripts that differ in their encoded polypeptides (Figure 1). Such mRNA sequence alterations make crucial changes in protein activity that are precisely regulated by the cellular environment. Alternative splicing is particularly common in the mammalian nervous system. Proteins involved in all aspects of neuronal development and function are diversified in this manner. From hundreds of examples, we focus on a few systems where either the regulatory mechanisms or the effects of altered splicing on protein function are better understood.

Cell-Cell Interactions and Neuronal Differentiation

The most complex patterns of splicing so far described are in molecules important for the differentiation of neurons and the formation of their intricate connections. The DSCAM and Neurexin transcripts show a spectacular variety of splicing patterns numbering in the thousands and have been reviewed elsewhere (Black, 2000; Grabowski and Black, 2001; Graveley, 2001; Missler et al., 1998; Missler and Sudhof, 1998). DSCAM plays a role in axon guidance in the developing Drosophila nervous system (Schmucker et al., 2000). Neurexin may act later during

synaptic differentiation (Scheiffele et al., 2000). N-cadherins are another example of a diverse family of related proteins that are thought to be involved in the formation of neuronal connections (Wu and Maniatis, 1999; Wu and Maniatis, 2000). How the splicing variation changes the activity of these proteins is not well understood, although an Eph Receptor provides an interesting precedent for different spliced forms of a protein mediating cell attraction or repulsion (Holmberg et al., 2000). The role of splicing in altering cell adhesion and synaptic development has been most thoroughly studied in the Agrin and NCAM transcripts.

Agrin is an extracellular matrix protein, identified as an essential mediator of synapse formation at the neuromuscular junction (Bowe and Fallon, 1995; Hoch, 1999). The clustering of muscle acetylcholine receptors at the neuromuscular synapse is induced by agrin secreted by the neuronal growth cone. Although muscle cells also make agrin, only the neuronal protein has clustering activity. Neuronal agrin mRNA contains several exons absent from muscle-expressed agrin (the Y and Z exons). The Y exon confers heparin binding on the protein, whereas the Z exons (exons 32 and 33) increase the affinity of agrin for α dystroglycan and confer clustering activity on the protein (Campanelli et al., 1996; Gesemann et al., 1996; Hopf and Hoch, 1996; O'Toole et al., 1996). Mice with the Z exons selectively knocked out of the Agrin gene are defective in neuromuscular synaptogenesis. In contrast, synaptogenesis is normal when the Y exon is selectively removed (Burgess, 1999). Thus, the agrin splicing phenotype of neurons and muscle cells controls synaptic differentiation at the neuromuscular junction.

Neural Cell Adhesion Molecule (NCAM) is thought to be involved both in maintaining cell-cell interactions in the adult nervous system and in stimulating axonal outgrowth during development (Walsh and Doherty, 1997). There are numerous spliced isoforms of NCAM

(Santoni et al., 1989). Two groups of proteins, NCAM 140 and NCAM 180, differ in the inclusion of a large exon (exon 18) encoding a 266 amino acid intracellular domain. NCAM exon 18 is included in neuronal NCAM but skipped in muscle and this inclusion is controlled by a number of cis-acting RNA regulatory sequences (Cote and Chabot, 1997; Cote et al., 1999; Tacke and Goridis, 1991). NCAM can stimulate neurite outgrowth in neurons plated over cells expressing particular NCAM isoforms (Walsh and Doherty, 1997). This activity is regulated by alternative splicing. NCAM 180, containing the exon 18 domain, is found at sites of cell-cell contact in the adult, and interacts with the cytoskeleton. However, NCAM 180 is poor at stimulating neurite outgrowth compared to isoforms with a small intracellular domain (such as NCAM 140). This outgrowth-promoting activity is also inhibited by another of the many regulated NCAM exons, a short 30 nucleotide exon named VASE. VASE modifies the fourth extracellular immunoglobulin domain of the protein, and may alter a direct contact between the developing axon and the cellular substrate. Thus, NCAM activity is modulated both inside and outside the cell by different alternative exons.

In addition to affecting neuronal interactions during development, the splicing phenotype of a cell also contributes to its identity as a mature neuron. Transcripts from the gene for the transcription factor NSF/REST are spliced to make two different proteins. In non-neuronal cells, the NRSF/REST transcripts encode a transcriptional repressor of neuron-specific genes (Chong, 1995; Schoenherr and Anderson, 1995). In neurons, an alternative splicing event produces a truncated NRSF/REST protein that apparently releases the NRSF/REST repression (Palm et al., 1998) (Shimojo et al., 1999).

The first description of neuronal alternative splicing was in the gene encoding the calcitonin/CGRP neuropeptides, whose transcripts are spliced to produce the calcitonin mRNA in thyroid and the CGRP mRNA in neurons (Rosenfeld et al., 1983). This system has proven to be an exemplar of numerous alternatively spliced neuropeptide transcripts (Angers and DesGroseillers, 1998; Buck et al., 1987; Seong et al., 1999). In Limnaea, Aplysia, C. elegans and other invertebrates, splicing of the FMRFa neuropeptide transcript determines the function of neurons controlling heartbeat, egg laying, copulation and other activity (Benjamin and Burke, 1994; Burke et al., 1992; Rosoff et al., 1992; Santama et al., 1995; van Golen et al., 1995). Alternative splicing of the FMRFa transcript switches expression between families of peptides that have different and sometimes antagonistic effects. The production of these splice variants is precisely controlled such that individual neurons within a particular ganglion can express completely different sets of peptides.

Neurotransmission: splicing control of neurotransmitter receptors and ion channels

Nearly all the proteins involved in neurotransmission exhibit subtle but important functional variation brought about by alternative splicing. The electrical properties of a neuron or other excitable cell are carefully tuned to coordinate many different ion channels and receptors, and to match the properties of cells within a circuit. To meet these needs, the proteins involved in these processes exhibit great diversity. Functional diversity is generated by a variety of mechanisms including extensive alternative splicing. Changes in the splicing of an ion channel can regulate its voltage and ligand dependence, its activation and inactivation kinetics, and its coupling to intracellular signaling pathways. Some of these properties are set during the

differentiation of a neuron, while other properties can be altered in a mature neuron in response to particular stimulus.

AMPA Receptors

AMPA type glutamate receptors are the major excitatory neurotransmitter receptor in the central nervous system. They are encoded by a multigene family GluR1, GluR2, GluR3, GluR4, GluRA and GluRD. Each of these subtypes is generated in two spliced isoforms containing either the flip or the flop exon. In electrophysiological measurements of recombinant channels, Flop isoforms of GluR2 exhibited faster desensitization kinetics compared to the Flip isoforms (Koike et al., 2000). Single cell studies have correlated AMPA receptor function to splicing pattern in a variety of neuronal cell types. In nonpyramidal neurons of the cortex where AMPA receptors exhibit faster desensitization kinetics, flop isoforms predominate (Lambolez et al., 1996). Conversely, slower GluR desensitization is seen in pyramidal cells, where the flip isoforms are more abundant. The flip and flop variants of AMPA receptors also vary in their sensitivity to the allosteric regulator cyclothiazide (Partin et al., 1995). AMPA receptors were one of the first examples of electrophysiology being regulated by alternative splicing. This has become a very common theme in many different ion channels.

NMDA R1 receptor

NMDA receptors are another major class of glutamate-gated ion channels important in synaptic plasticity and neuronal development. There are multiple spliced forms of the NMDA R1 receptor (Zukin, 1995). The best understood splicing derived changes in NMDA receptor activity result from the regulated inclusion of exons 5 and 21. Exon 5 encodes what is called the N1 peptide cassette within the extracellular domain of the protein. Exon 5 inclusion has complex effects on the physiology of the receptor. The N1 cassette increases the current amplitude and alters receptor potentiation by polyamines and zinc (Rumbaugh et al., 2000; Traynelis et al., 1998; Zukin, 1995). Exon 21 encodes what is called the C1 cassette within the intracellular domain of the protein. Exon 22 contains two different 3' splice sites whose use determines the inclusion of the C2 cassette. C1 and C2 mediate targeting of the receptor to the plasma membrane, and allow the association of the receptor with Neurofilament L. C1 also contains a phosphorylation site and a binding site for calcium/calmodulin (Ehlers et al., 1998; Hisatsune et al., 1997; Zukin, 1995). There have been several interesting studies of the role of the C1 and C2 cassettes in targeting the receptor to the plasma membrane. C1 contains an ER retention signal that blocks export of the R1 receptor alone to the plasma. If the R1 Receptor is assembled with the R2 subunit in the ER, this masks the retention signal allowing expression on the cell surface. Either the exclusion of the C1 cassette by splicing or the inclusion of C2 will allow R1 receptor transport to the synapse without R2 expression. There are also a number of interesting reports of the splicing of these NMDA exons being altered by cell excitation and other stimuli (Musshoff et al., 2000; Rafiki et al., 1998; Vallano et al., 1999; Vallano et al., 1996; Vezzani et al., 1995; Xie and Black, 2001). Exons 5 and 21 show divergent patterns of inclusion within the brain. Exon 5 is more strongly included in hind brain structures such as the cerebellum where Exon 21 is low.

Conversely, Exon 21 is high in the cortex and Exon 5 is low. The splicing regulator NAPOR1 is implicated in maintaining this pattern by repressing Exon 5 and activating Exon 21(Zhang et al., 2002).

Dopamine Receptors

Dopamine receptors are G protein-coupled receptors of diverse sub-types that mediate particular physiological responses to the neurotransmitter dopamine. The D2 and D3 dopamine receptors have multiple spliced isoforms that vary according to cell type (Dal Toso, 1989; Giros B, 1989; Grandy DK and JB, 1989). Two isoforms of the D2 receptor, D2L and D2S, differentially affect adenylyl cyclase activity and hence downstream signaling (Guiramand et al., 1995). The D2L cassette exon is within the third cytoplasmic loop of the receptor and is thought to mediate the interaction of the receptor with the G protein alpha subunit. Other G protein coupled receptors show splicing variation that modulates downstream signaling. Notably the pituitary adenylyl cyclase-activating polypeptide (PACAP) receptor is produced in several forms that vary in their ability to stimulate adenylyl cyclase and phospholipase C (Journot et al., 1994).

GABA receptors

Synaptic inhibition is often mediated by the neurotransmitter GABA that binds the $GABA_A$ receptor. The $GABA_A$ receptor is a ligand-gated chloride channel assembled from different combinations of subunits. Alternative splicing of the γ2 subunit controls the binding of benzodiazepine agonists to the receptor (Wafford et al., 1993). The γ2L and γ2S forms differ in

the inclusion of a short eight amino acid exon within an intracellular loop domain of the protein. γ2L knock out mice, which express only the γ2S form, have increased affinity for benzodiazepines, and show increased sensitivity in behavioral responses to these agonists compared to parent strains (Quinlan et al., 2000). The γ2L exon is thus thought to make the receptor less sensitive to benzodiazepine. This eight amino acid segment also contains a protein kinase C phosphorylation site that is believed to modulate the channel response to ethanol (Krishek, 1994; Whiting et al., 1990). The γ2 exon has been extensively studied biochemically and is the best understood ion channel exon from the standpoint of its mechanisms of regulation(Ashiya and Grabowski, 1997; Zhang et al., 1996; Zhang et al., 1999).

Voltage-dependent Potassium and Sodium Channels

Voltage-dependent potassium and sodium channels mediate action potential propagation and determine many aspects of the electrical activity in neurons (Coetzee et al., 1999; Hille, 1992). These proteins are encoded by large families of related genes. Alternative splicing within these transcripts generates still more functional diversity. The best understood of these proteins is the Shaker Potassium Channel. Drosophila Shaker is differentially processed at both the N and C termini through the use of alternative promoters, splice sites and poly-A sites (Schwarz et al., 1988; Sutcliffe and Milner, 1988). Shaker channels in other organisms show additional sites of alternative splicing (Kim et al., 1997). Since Shaker protein exists as a tetramer, the combination of variants produces a wide variety of functionally distinct channels. In Drosophila Shaker, the variable processing leads to distinct inactivation kinetics for the channel. Fast inactivation kinetics are altered by the choice of amino terminal sequences. The different carboxyl termini

create differences in the rate of recovery from inactivation (Hoshi et al., 1990; Hoshi et al., 1991; Iverson and Rudy, 1990; Iverson et al., 1988; Timpe et al., 1988).

That differential splicing is responsible for the different currents observed by electrophysiology was shown for the C-terminal variants using an elegant reporter system (Iverson et al., 1997; Mottes and Iverson, 1995). The alternatively spliced segments of Shaker were fused to LacZ, such that β- Galactosidase would be expressed only if certain splicing patterns were used. These reporter genes were expressed in transgenic flies and the flies were stained for β-Gal. It was found that the choice of C-terminal splicing pattern, as reported by tissue staining, correlated with the electrophysiology for each tissue. Similar splicing reporters have been used to good effect in other systems (Adams et al., 1997). Mammalian potassium channels also show functional variation derived from alternative splicing (Liu and Kaczmarek, 1998).

Similar to potassium channels, sodium channels show extensive alternative splicing in both vertebrates and Drosophila. An extreme example is the channel encoded by the Drosophila Paralytic (Para) gene whose transcripts encode over a thousand different polypeptides through alternative splicing and many more through RNA editing events (Graveley, 2001; Thackeray and Ganetzky, 1994; Thackeray and Ganetzky, 1995). Genetic studies have uncovered modifiers of Para splicing including one fascinating gene, mle^{napte}, encoding an RNA Helicase, whose mutation has drastic effects on several Para exons(Reenan et al., 2000). For Shaker, Para and other potassium and sodium channels, electrophysiology is intricately adjusted through the differential use of numerous spliced isoforms. These genetic studies are promising approaches

both for uncovering the regulators of this splicing and to understand the roles of individual isoforms.

Calcium Channels

Calcium channels are multi-subunit voltage-gated channels that play diverse cellular roles regulating calcium-dependent genes, controlling firing patterns, and affecting neurotransmitter release at the synapse. The N and P/Q type channels function in part to couple cellular excitation to neurotransmitter release. The α_1 subunit of the channel forms the ion pore and is encoded in multiple genes. Two extracellular loops of the α_{1B} subunit, found in N-type channels, show variation due to alternative splicing. In one loop, insertion of a small cassette exon encoding just two amino acids, ET (glutamic acid, threonine), confers slower activation kinetics on the channel (Lin et al., 1999). The inclusion of the ET exon is tissue specific. In the central nervous system, most of the α_{1B} subunits lack the ET exon, whereas the predominant form in sympathetic and sensory ganglia contains the ET segment (Lin et al., 1997). P and Q type channels apparently both contain the α_{1A} subunit but differ in their physiology due to cell type specific changes in the splicing of the α_{1A} transcript (Beam, 1999) (Bourinet et al., 1999). The inclusion of a single valine in the domain I-II linker of the Q channel gives it slower inactivation kinetics than the P channel. The inclusion of the dipeptide NP in domain 4 of α_{1A} makes the Q channel less sensitive to ω-agatoxin IVA than the P channel. Changes in splicing also alter the regulation of the channel by G proteins. The calcium channel illustrates how very small peptide cassettes, sometimes encoding a single amino acid, can determine important changes in protein activity.

Calcium and voltage activated potassium channels

The Ca^{++} and voltage activated K^{+} Channel (also called BK or *slo* channel) provides an example of how the controlled production of subtly different spliced forms of a protein is important for modulating the function of a particular cell type (Vergara et al., 1998) (Black, 1998; Jones et al., 1999a). Potassium gating by BK channels requires both a voltage change across the membrane and the presence of intracellular calcium. These channels contribute to shaping the repolarization phase of action potentials in many cell types (Coetzee et al., 1999; Lingle et al., 1996). More than 500 different BK transcripts are potentially generated by the combinatorial inclusion of numerous cassette exons. These different exon cassettes specify changes in the channel physiology by altering its calcium or voltage dependence, its conductance, or other properties. Changes in BK channel kinetics control, in part, the tuning of hair cells in the inner ear to transduce particular sound frequencies (Black, 1998; Fettiplace R, 1999). Several groups have shown that individual hair cells along the tonotopic gradient of the Cochlea express different repertoires of BK channel splice variants (Jones et al., 1999b; Navaratnam et al., 1997; Rosenblatt et al., 1997). This splicing allows hair cells tuned for low or high frequency responses to have the channels appropriate for these frequencies. BK channels have also been extensively studied in adrenal chromaffin cells. In these cells, specific changes in excitation induced by stress hormones were shown to derive from BK channel alternative splicing (Xie and McCobb, 1998). The hormonally activated inclusion of the STREX exon increases the sensitivity of the channel to calcium and leads to a higher rate of action potential spiking in the chromaffin cells (Solaro et al., 1995). It seems likely that, similar to hair cells, the

tuning of action potential kinetics in neurons will also involve the controlled expression of particular splice variants for multiple ion channel proteins.

Mechanisms of alternative splicing control

The molecular mechanisms that control changes in splice site choice are only beginning to emerge (Lopez, 1998; Smith and Valcarcel, 2000). We will discuss a few model systems directly related to the nervous system. Additional model systems are discussed in other chapters of this volume. Many transcripts undergo changes in splicing during neuronal development or are spliced differently in different types of neurons. In addition to these changes with development or cell type, some splicing patterns are regulated in mature cells in response to particular stimuli, and may vary from cell to cell even within a defined population of neurons (Thomas et al., 1993; Wang and Grabowski, 1996). Thus, the splicing apparatus must be able to respond to myriad signals in controlling production of the thousands of alternatively spliced transcripts.

Each intron in a pre-mRNA is excised by a particle called the spliceosome that must assemble onto the splice sites, pair them across the intron, and finally catalyze the intron excision and exon ligation. Splice sites adhere to defined consensus sequences: the 5' splice site at the 5' end of the intron and the 3' splice site with its associated branch point and polypyrimidine tract at the 3' end of the intron (Burge, 1999). The spliceosome is made up of 5 small nuclear ribonucleoproteins (snRNPs), U1, U2, U4, U5, and U6 and a number of accessory proteins.

Spliceosome assembly is a stepwise process requiring the sequential binding and disruption of multiple RNA and protein contacts (Staley and Guthrie, 1998). Alternative splicing patterns result from changes in the assembly of spliceosomes. These changes are thought to be primarily regulated during the early steps of spliceosome assembly, such as the recognition of the 5' splice site by the U1 snRNP and the 3' splice site by the U2 auxiliary factor U2AF. Splicing patterns can be altered in a variety of ways (Lopez, 1998). It is clear that the transcription reaction itself as well as the secondary structure of the pre-mRNA can both affect splice site choice. However, here we will discuss the best understood systems of neuronal splicing regulation where the hnRNP structure of the mRNA precursor seems to be the primary determinant of the change in splicing.

RNA sequence elements in the pre-mRNA can direct changes in splicing pattern by activating or repressing nearby spliceosome assembly (Figure 2). Regulatory sequences that activate splicing at particular sites are called exonic or intronic splicing enhancers, depending on where they are located. There are also splicing repressor elements, or silencers, that inhibit splicing. The tissue-specific splicing of a transcript is often under a combination of positive and negative control (Modafferi and Black, 1999; Smith and Valcarcel, 2000; Zhang et al., 1996). These regulatory elements are bound by sequence specific RNA binding proteins that induce changes in spliceosome assembly. Regulatory elements and proteins have been identified in a number of systems. However, there is limited understanding of how they actually work.

Splicing repression

We will focus on two mRNAs exhibiting neural specific splicing patterns that have been analyzed biochemically. In most neurons, transcripts for the tyrosine kinase c-*src* include an exon (N1) that is skipped in non-neuronal tissue. In contrast, a regulated exon in the $GABA_A$ receptor γ2, is spliced differentially between different neuronal subsets.

Regulated exons often have features, such as short exon length and weak splice sites, that prevent their efficient recognition by general splicing factors and thus contribute to the general repression of these exons. Reducing the length of a normally constitutive exon below ~50 nucleotides can cause exon skipping (Dominski and Kole, 1991). This is believed to result from the loss of exon bridging interactions that promote splice site recognition in a process called exon definition (Berget, 1995). Many regulated exons including the *c-src* N1 exon (18 nucleotides) and the $GABA_A$ receptor γ2 exon (24 nucleotides) are well below this length. When the N1 exon is lengthened to ~100 nucleotides it becomes constitutively spliced (Black, 1991). The splice sites in the $GABA_A$ receptor γ2 exon are also suboptimal, and mutations that strengthen the 5' splice site result in efficient splicing in non-neural cells (Zhang et al., 1996). Similar effects of exon length and splice site strength have been seen in many other regulated exons (Smith and Valcarcel, 2000).

Another common feature of c-src and $GABA_A$ splicing, as well as most other characterized systems of tissue specific splicing in mammals, is the involvement of the polypyrimidine tract binding protein (PTB; also called hnRNP I) (Valcarcel and Gebauer, 1997; Wagner and Garcia-Blanco, 2001). PTB contains four RRM domains (Conte et al., 2000; Varani and Nagai, 1998), and binds to pyrimidine rich RNA elements that act as splicing repressor or silencer elements (Valcarcel and Gebauer, 1997; Wagner and Garcia-Blanco, 2001).

Neuronally regulated exons of *c-src*, $GABA_A$ receptor γ2, clathrin light chain B (CLCB) and NMDA R1 exon 5 all contain PTB binding repressor elements within the polypyrimidine tract of their 3' splice sites (Ashiya and Grabowski, 1997; Chan and Black, 1995; Zhang et al., 1999). Regulated exons in nonneural systems also can have PTB binding sites in their polypyrimidine tracts (Gooding et al., 1998; Mulligan et al., 1992; Valcarcel and Gebauer, 1997; Wagner and Garcia-Blanco, 2001). The region surrounding a regulated exon often contains additional PTB binding sites. Mutating these binding sites or depleting PTB from an in vitro splicing system generally activates splicing of a repressed exon. PTB thus seems to be required for splicing repression.

Different arrangements of PTB binding sites has led to two models for PTB mediated splicing repression (Figure 3). For the $GABA_A$ receptor γ2 exon, PTB binds to an extended polypyrimidine tract starting about 100 nucleotides upstream of the 3' splice site and extending through the exon. Since the branch site is embedded in this polypyrimidine tract, it is thought to be the target of negative regulation by PTB.

Repression of the c-src N1 exon is dependent upon distributed PTB binding sites on both sides of the N1 exon (Chan and Black, 1995; Chan and Black, 1997; Chou et al., 2000). Mutating the binding sites downstream of the exon reduces PTB binding to the upstream 3' splice site and activates splicing in vitro. The PTB molecules binding to these separated sites are thought to interact and sequester the N1 exon in a loop. However, the contacts that bridge this loop are not clear. Interestingly, the downstream binding sites for PTB are located within an intronic splicing enhancer (see below). One possibility is that the arrangement of PTB sites is needed to prevent splicing activation by this enhancer. Although they have not yet been shown to bind PTB,

similar pyrimidine rich repressor elements control the splicing of neuronally regulated exons in β Amyloid pre-mRNA (Bergsdorf et al., 2000; Shibata et al., 1996).

Several muscle specific exons are also repressed by PTB in a similar manner to src N1 and $GABA_A$ γ2 (Smith and Valcarcel, 2000; Valcarcel and Gebauer, 1997). These two patterns of repression by PTB are also reminiscent of models for splicing repression by the Drosophila sxl protein (Lopez, 1998). In repressing splicing of the tra transcript, sxl appears primarily to block a 3' splice site, whereas for an exon in the *sxl* transcript itself, sxl protein binds on both sides of the exon to repress its splicing.

An important question for exons repressed by PTB is how the tissue specificity of splicing is generated. The exons in the $GABA_A$ γ2 receptor and *c-src* are repressed by PTB in non-neural cells. There are several skeletal muscle-specific exons that are repressed by PTB in non-muscle tissues. One common feature in these systems is that PTB is thought to maintain repression of a highly tissue-specific exon outside of one particular tissue. Thus, the tissue specificity of splicing apparently derives from the controlled loss of PTB repression.

PTB is widely expressed, although there are tissue specific differences in its expression. In most cell lines (HeLa, for example), PTB is expressed as several spliced isoforms. These isoforms have different activity in some assays and their ratio changes in different cell lines (Christopher Smith personal communication and (Wagner et al., 1999)). In nuclear extracts from rat brain (Ashiya and Grabowski, 1997) and a neural cell line (Chan and Black, 1997), these isoforms are reduced, and a PTB-like protein is present, termed neural or brain enriched PTB (nPTB or brPTB) (Ashiya and Grabowski, 1997; Chan and Black, 1997; Markovtsov et al., 2000; Polydorides et al., 2000). nPTB shows reduced splicing repression activity on the N1 exon

(Markovtsov et al., 2000). Interestingly, a switch in PTB and nPTB expression occurs during rat cerebellar development that correlates with the loss of splicing repression in several transcripts (Zhang et al., 1999).

Although changes in PTB expression likely contribute to the release of splicing repression, they are not the only mechanism. Some cell lines with abundant PTB, and little nPTB, can splice the *c-src* N1 exon (Markovtsov et al., 2000). There must be additional factors that affect splicing repression by PTB. In a neural extract where PTB does not repress c-src N1 splicing, PTB is present and binds to the N1 3' splice site in the absence of ATP (Chou et al., 2000). However, this PTB binding is lost when ATP is added to initiate the splicing reaction. In HeLa extracts, where N1 is repressed, PTB remains bound even in the presence of ATP. This ATP dependence of PTB dissociation may imply an active removal process where the protein is specifically detached from particular sites to allow splicing to proceed (Chou et al., 2000).

Different splicing silencer elements have been found in other systems. The hnRNP A1 protein was identified as a splicing repressor that binds to elements within the repressed exon of several transcripts (Caputi et al., 1999; Chabot et al., 1997; Tange et al., 2001). Similar results are seen with the hnRNP H protein repressing an exon in β-Tropomyosin (Chen et al., 1999; Jacquenet et al., 2001). SnRNPs are also implicated in the negative control of splicing through exonic repressor elements (Kan and Green, 1999; Siebel et al., 1994). Although these systems do not show neuronal regulation, these factors are also likely to affect exons in neuronal transcripts.

Splicing enhancement

Splicing patterns are also positively regulated through splicing enhancers. Exonic enhancers are diverse in sequence but the best characterized are purine rich elements with GAR (R=purine) triplet repeats. GAR elements and other exonic enhancers are bound by proteins of the SR family (Lopez, 1998). These proteins contain one or more RRM type RNA binding domains joined to a carboxy terminal SR domain containing repeated serine-arginine dipeptides. SR proteins are needed for constitutive splicing, and also play important roles in alternative splicing (for reviews, see (Manley and Tacke, 1996) (Graveley, 2000)). The best studied enhancer is in the Drosophila doublesex transcript, where the specific regulatory proteins tra and tra-2 cooperate with SR proteins to assemble an enhancer complex on the regulated exon. The tethering of the SR domain to the exon stimulates spliceosome assembly on the upstream 3' splice site (Graveley and Maniatis, 1998). This induced assembly is thought to be due to interactions between the SR domain of the SR protein and similar domains on the U2AF heterodimer. Phosphorylation of the SR domain also regulates its biochemical properties (Colwill et al., 1996; Du et al., 1998; Prasad et al., 1999; Xiao and Manley, 1998; Yeakley et al., 1999; Yun and Fu, 2000). Through the interactions of the effector (SR) domain with spliceosomal components, SR proteins are thought to be mediators of a range of splicing choices (Graveley, 2000).

Individual enhancers require specific members of the SR family and thus tissue-specific variation in SR protein expression can affect enhancer activity (Hanamura et al., 1998). For example, levels of ASF/SF2, an SR protein that promotes activation of proximal 5' splice sites, vary considerably relative to the protein hnRNP A1, which promotes activation of distal 5' splice sites (Caceres et al., 1994; Mayeda et al., 1993). These proteins have mostly been studied in

non-neuronal transcripts. However, SR proteins as well as the mammalian homolog of Tra-2 are present in neurons, and many neuronal splicing events will involve exonic enhancers controlled by these proteins (Beil et al., 1997).

In addition to exonic enhancers, intronic splicing enhancers have been identified downstream of numerous regulated exons (Black, 1992; Carlo et al., 1996; Del Gatto-Konczak et al., 2000; Huh and Hynes, 1994; Modafferi and Black, 1997; Ryan and Cooper, 1996; Sirand-Pugnet et al., 1995) (Wei et al., 1997). Intronic enhancers are not known to bind SR proteins, and may function differently from exonic enhancers. Several small sequence elements have been shown to have intronic enhancer activity, including a G-rich element found in the c-src, cardiac troponin T, α–Globin, and β–Tropomyosin transcripts (Carlo et al., 1996; McCullough and Berget, 2000; Modafferi and Black, 1997; Sirand-Pugnet et al., 1995), and a UGCAUG element in the c-src, Fibronectin, non-muscle Myosin II, and calcitonin/CGRP transcripts (Black, 1992; Hedjran et al., 1997; Huh and Hynes, 1994; Kawamoto, 1996; Modafferi and Black, 1997). A bioinformatic analysis of the intron sequences downstream from a group of neuronally regulated exons found that UGCAUG was the most common hexamer in these intron regions indicating its likely involvement in further splicing events (Brudno et al., 2001).

The complex intronic enhancer downstream of the *c-src* N1 exon is required for N1 splicing in vivo, and is relatively non-tissue specific, activating splicing in many cell types (Modafferi and Black, 1997; Modafferi and Black, 1999). The tissue specificity of N1 splicing stems from the interaction of the enhancer with the system of PTB mediated repression (see above). The most conserved core of the src N1 enhancer (called the DCS) contains a GGGGG element (G5) and a UGCAUG element flanking a PTB/nPTB binding site. In splicing active

neural extracts, the DCS assembles a complex of proteins including hnRNPs F and H bound to G5, nPTB bound to CUCUCU, and the KH type splicing regulatory protein (KSRP) bound to the UGCAUG (Chou et al., 1999; Markovtsov et al., 2000; Min et al., 1995; Min et al., 1997). The roles for these proteins in splicing enhancement are not yet clear. Antibody inhibition experiments imply a positive role for hnRNPs F and H and KSRP in N1 splicing. However, their interaction with PTB may indicate a function in repression or derepression rather than true splicing enhancement. Reports of G rich and UGCAUG elements activating splicing in vitro is encouraging for identifying the proteins that mediate their enhancement activity (Carlo et al., 2000; Carlo et al., 1996; Guo and Kawamoto, 2000).

Other intronic enhancer proteins were also recently identified. A study of a regulated intron in the *Drosophila* msl2 transcript identified the protein TIA-1 as binding to an intronic enhancer in HeLa extract and promoting U1 snRNP binding to the upstream 5' splice site (Forch et al., 2000). This work is important since this is the only intronic enhancer binding protein shown to affect spliceosome assembly. TIA-1 is also involved in FGF Receptor 2 alternative splicing (Del Gatto-Konczak et al., 2000). Interestingly, TIA-1 is a mammalian protein involved in apoptosis and has homologs in *Drosophila* (Rox-1) and Yeast (NAM8).

Another significant group of intronic enhancer binding factors are the CELF proteins (CUG binding proteins and ETR3 like factors) (Ladd et al., 2001). Several members of this family, including CUG binding protein (CUG-BP), enhance cardiac troponin T exon 5 inclusion when overexpressed in vivo (Philips et al., 1998). The developmental regulation of these proteins is proposed to mediate control of muscle specific splicing events. CUG binding protein also binds to the intronic repressor region of the neural pre-mRNAs CLCB and NMDA R1,

although its functional effect is not known (Zhang et al., 1999). A neuronal member of this family, NAPOR1, was recently shown to have strong effects on the splicing of NMDAR1 exons 5 and 21. Interestingly, NAPOR1 represses Exon 5 but enhances Exon 21 splicing. Thus, these proteins are likely to play important roles in diverse neuronal splicing events(Zhang et al., 2002).

A puzzling question for all the analyzed mammalian systems of splicing is how the tissue specificity is generated (Smith and Valcarcel, 2000). The characterized muscle and neuron specific exons all show multiple features of positive and negative control. For example, the src N1 exon is generally repressed by its short length, generally activated by multiple factors affecting both the intronic enhancer and an exonic enhancer, and finally specifically repressed in nonneural cells by PTB. Thus, the control of N1 is the product of a highly combinatorial system of inputs, but the key to its tissue specificity seems to be the loss of PTB repression in neural cells (Modafferi and Black, 1999). Most other systems of splicing regulation show similar combinations of relatively non-tissue-specific factors affecting the level of each splicing pattern (Smith and Valcarcel, 2000).

Neuronal RNA binding proteins

Although splicing patterns are at least partly controlled by combinations of relatively unspecific factors, there are some RNA binding proteins that show highly neural specific expression. The *Drosophila* embryonic lethal abnormal visual system (*elav*) gene encodes a neuron specific RNA binding protein implicated in the regulation of alternative splicing. When *elav* is genetically depleted from *Drosophila* photoreceptor neurons, a decrease is observed in a spliced variant of neuroglian (Koushika et al., 1996). Conversely, this neural form of

neuroglian is generated ectopically when *elav* is expressed in non-neural cells. Similar effects on the splicing of *Drosophila erect wing* transcripts are observed later in development (Koushika et al., 1999). ELAV was recently shown to directly bind the Neurologin transcript (Lisbin et al., 2001). It will be very interesting to determine how it interacts with other regulators and the spliceosome itself.

In mammals there are numerous *elav* family members, some of which are specifically expressed in neurons (Chung, 1996; Gao, 1996). These proteins are most strongly implicated in the control of mRNA stability through their recognition of 3' untranslated regions (Antic and Keene, 1997; Antic et al., 1999; Chung, 1997; Fan, 1998; Ford et al., 1999; Peng et al., 1998). However, given that some *elav* proteins are cytoplasmic while others predominantly nuclear, it seems possible that they will serve a variety of roles in mRNA metabolism.

Two other highly neuron-specific RNA binding proteins are Nova-1 and Nova-2. These are closely related proteins of the hnRNP K homology type (KH type), first identified as autoantigens in patients with paraneoplastic neurologic disease (Darnell, 1996). The Nova proteins are enriched in the cell nucleus of neurons, and like other hnRNP proteins may be involved in multiple processes. However, Nova-1 clearly effects alternative splicing. Nova-1 interacts with nPTB (brPTB) in the yeast two hybrid assay, and recognition elements for both nPTB and Nova-1 are located adjacent to a regulated exon in the glycine receptor pre-mRNA (GlyRα2) (Buchanovich, 1997; Jensen et al., 2000b; Polydorides et al., 2000). Nova-1 knockout mice exhibit postnatal lethality due to the loss of spinal and brainstem neurons. This is accompanied by changes in the splicing of both the GlyRα2 exon and the regulated $GABA_A$ receptor γ2 exon (Jensen et al., 2000a). The partial effect of the knockout suggests that Nova-1

is one of several factors that affect these exons, as might be predicted from the multiple factors implicated in other systems. This genetic knockout approach offers an important route for dissecting these highly combinatorial systems of splicing control (Wang et al., 2001; Wang et al., 1996).

In a more refined genetic system, the mec-8 gene product of C. elegans was defined as a splicing regulator (Lundquist and Herman, 1994; Lundquist et al., 1996). Mec-8 encodes an RNA binding protein with two RRM domains. Mutations in Mec-8 reduce the abundance of several spliced isoforms of UNC52, a proteoglycan important for body wall muscle function. Mec-8 mutations are pleiotropic and also affect the function of mechansensory and chemosensory neurons. It will be very interesting to determine the role of Mec-8 in regulating splicing in these cells.

Splicing and cell excitation.

How signal transduction pathways affect splicing patterns is an area of great interest but little data. The level of many spliced transcripts is altered by particular extracellular stimuli (Berke et al., 2001; Chalfant et al., 1995; Collett and Steele, 1993; Rodger et al., 1998; Shifrin and Neel, 1993; Smith et al., 1997; Wang et al., 1991; Zacharias and Strehler, 1996). In cases where the change in transcript occurs rapidly, a change in splicing must be distinguished from a change in the decay rates of the different spliced forms. In two systems (CD44 and CD45), specific RNA elements within the regulated exon are needed for the splicing to respond to protein kinase C and Ras signaling. (Konig et al., 1998; Lynch and Weiss, 2000). The activation of CD44 exon V5 upon T cell stimulation requires the MEK and ERK kinases (Weg-Remers

et al., 2001). However, the complete path from an initial stimulus to the splicing apparatus is not clear. Significantly, it was recently shown that the protein hnRNP A1 is controlled in its nucleo/cytoplasmic localization by the MKK-p38 signaling pathway (van der Houven van Oordt et al., 2000). Since hnRNP A1 affects the splicing of multiple transcripts, this control of splicing factor localization should prove to be important in determining splicing patterns. Although the direct modification of splicing factors or the induction of splicing regulator genes are also likely mechanisms.

The splicing of NMDA receptors and of BK potassium channels is known to be altered by cell activity (Daoud et al., 1999; Vallano et al., 1999). It was recently shown that depolarization of GH3 pituitary cells and signaling by CaM Kinase IV represses splicing of both the BK channel STREX exon and NMDA exons 5 and 21 (Xie and Black, 2001). Particular sequence elements surrounding the STREX exon respond to the CaMK IV signal. These depolarization-induced changes in the BK channel and NMDA R1 proteins will alter the subsequent excitatory properties of the stimulated cells. Thus, it will be interesting to look for effects of CaMK IV signaling on many other ion channel and neurotransmitter receptor transcripts.

The role of signal transduction pathways in controlling splicing is particularly interesting in neuronal systems, where learning and memory, as well as cellular homeostasis, depend on activity-dependent changes in cell excitation. The mechanisms that control these changes are known to include alterations in transcription and translation (Finkbeiner and Greenberg, 1998; Lisman and Fallon, 1999). However, nearly all the receptor and channel molecules that determine cell electrical excitation are also regulated by alternative splicing. Thus, altering the

splicing of these transcripts in response to electrical activity provides an additional mechanism for inducing long term changes in excitability.

Alternative splicing and neurologic disease

The importance of alternative splicing to gene expression in the nervous system is highlighted by several forms of neurologic disease caused by splicing misregulation or other errors of RNA metabolism. There are many examples of mutations in splice sites or splicing enhancer or silencer elements that disrupt constitutive splicing patterns (Krawczak et al., 1992; Liu et al., 2001; Nakai and Sakamoto, 1994; O'Neill et al., 1998). There are also disease pathologies where the regulation of splicing is known or suspected to be in error (Cooper, 1997; Philips and Cooper, 2000). Several systems provide examples of how seemingly subtle changes in a ratio of spliced isoforms can have disastrous consequences. Mutations in the microtubule associated protein Tau cause the disorder Frontotemporal Dementia with Parkinsonism linked to chromosome 17 (Wilhelmsen, 1999). Spinal Muscular Atrophy (SMA) is a common congenital disorder resulting from mutations in the survival of motor neurons (SMN) gene (Lefebvre et al., 1998; Matera, 1999; Mattaj, 1998; Sendtner, 2001). Both of these systems involve changes in exon inclusion and have been discussed extensively elsewhere (Grabowski and Black, 2001; Lefebvre et al., 1998; Sendtner, 2001; Wilhelmsen, 1999).

Myotonic Dystrophy (MD) provides an interesting picture of how a mutation in a gene unrelated to splicing might lead to a dominant and generalized effect on splicing regulation. MD is an inherited neuromuscular disease exhibiting a complex pathology in both skeletal and

smooth muscle and in other systems (Timchenko, 1999). MD is caused by a CTG triplet expansion mutation in the 3' UTR of the DMPK protein kinase gene. The CTG expansion reduces the expression of DMPK and other genes in adjacent chromatin, and this may lead to portions of the complex disease phenotype (Alwazzan et al., 1999). However, the disease shows autosomal dominant inheritance and this is thought to result from the presence of the expanded CUG repeat in the expressed DMPK RNA. In MD cells, the mutant DMPK RNA becomes aggregated in cell nuclei (Taneja et al., 1995). DMPK is alternatively spliced and one set of spliced mRNAs lacking the CUG repeats is transported to the cytoplasm normally (Groenen et al., 2000; Tiscornia and Mahadevan, 2000). Thus, the CUG expansion will change the ratio of expressed DMPK isoforms and this could, in part, explain the dominant phenotype. However, transgenic mouse experiments argue for a dominant effect from the CUG repeat RNA alone (Mankodi et al., 2000). When the CUG repeat is expressed in the muscle of transgenic mice from a transcript unrelated to DMPK, the mice develop a disease very similar to MD. This is strong evidence for an "RNA gain of function" basis for the disease. The nuclear RNA accumulation is thought to sequester RNA binding proteins, including CUG-BP, hnRNP C, PTB, U2AF, PSF, and Muscleblind, thus changing their effective concentration in the cell (Miller et al., 2000; Timchenko et al., 1996; Tiscornia and Mahadevan, 2000). CUG-BP is a ubiquitous hnRNP protein identified by its binding to CUG repeat RNA, although its preferred RNA binding site may be a somewhat different sequence. CUG-BP is normally distributed between nucleus and cytoplasm, but in DM cells a hypophosphorylated form of the protein accumulates in the nucleus (Roberts et al., 1997). CUG-BP is known to affect splicing of exon 5 in cardiac Troponin T (cTNT), and in DM muscle cells, the inclusion of cTNT exon 5 is increased (Philips et al.,

1998). CUG-BP also affects the translation initiation of C/EBPB mRNA and this process is also altered in DM cells (Timchenko et al., 1999). However, it is not clear how the activity of CUG-BP might be altered by the DM mutation. Troponin exon 5 is thought to be positively regulated by CUG-BP and so the increase in exon 5 splicing is not easily explained by the sequestration of the protein by the CUG repeats. Instead, the protein may be altered in its modification or localization. Moreover, other proteins binding to CUG repeats such as Muscleblind could be altered in activity (Miller et al., 2000). Nevertheless, the genetic dominant nature of the disease is nicely explained by an RNA gain of function, something that is likely to be seen again in other triplet expansion diseases, many of which show neurologic manifestation (Imbert et al., 1996; Pulst et al., 1996; Sanpei et al., 1996).

The currently known diseases that are caused by errors in splicing regulation are likely to be the first of many. Tau and SMN provide demonstrations of how changes in spliced isoform ratios can have highly deleterious consequences in specific cell types. MD points to how triplet expansion mutations can have indirect effects on splicing. There are a number of studies associating changes in alternative splicing with various psychiatric disorders (Huntsman NM, 1998; Le Corre et al., 2000; Vawter et al., 2000; Weiland et al., 2000), although these changes may indirectly result from some other dysfunction. We clearly need to know more about how the vast network of splicing regulatory machinery contributes to function both in individual cells and at the level of integrating larger systems.

Conclusions

We discuss here only a tiny portion of the huge number of important neuronal and glial cell proteins that exhibit multiple splice variants. It is clear that alternative splicing plays a central role in controlling neuronal development and function. However, the most interesting questions remain unanswered. How does protein diversity from alternative splicing contribute to the diversity neuronal function? For BK channels in the cochlea, it seems clear that the multiple splice variants are needed to tune the frequency response of the hair cell. But for most proteins, it is not known why it is important to have a particular ratio of protein isoforms in a particular cell type. More genetic data, such as seen with the agrin knockouts is needed to answer these questions.

Our understanding of the mechanisms of splice site choice is also in its infancy. Very little is known about how regulatory factors actually interact with a spliceosome. How is specificity determined by combinations of common factors and what is the contribution of the tissue specific proteins? Progress on these fronts will require new biochemical assays that allow examination of single or small groups of proteins for their effects on spliceosomal assembly.

How do signaling pathways affect the splicing apparatus and how are inducible changes in splicing mediated? Are splicing regulatory protein genes induced during the primary response to a stimulus? How does the phosphorylation or methylation of splicing factors alter their localization, RNA binding or interactions with other factors? What is the ensemble of splicing changes occurring after a stimulus and how long do they last? Answers to these questions will be important to understanding the mechanisms controlling a neuron's ability to adapt to excitation and its remarkable plastic properties.

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Figure Legends

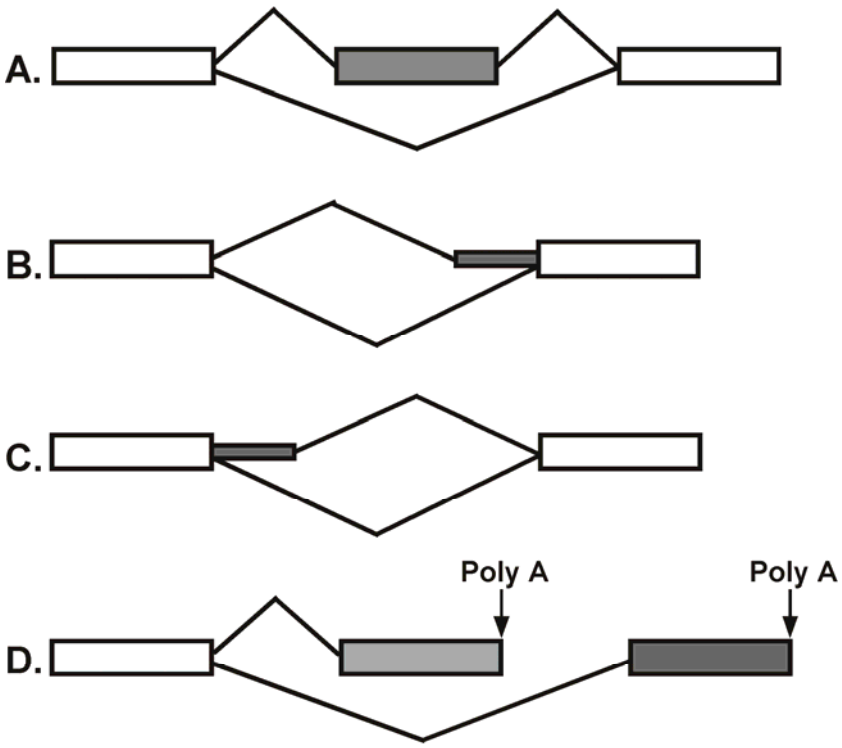

Figure 1.

Figure 1. Four Common Patterns of Alternative Splicing

White boxes represent constitutive exons present in every mRNA product. Gray boxes represent optional sequences that are included or excluded from the mRNA depending on the conditions. Patterns of alternative splicing include: (A) cassette exon; (B) alternative 3’ splice sites; (C) alternative 5’ splice sites; (D) Alternative 3' splice sites combined with alternative polyadenylation sites. These patterns can occur singly or in combination. Other patterns of alternative splicing are not shown.

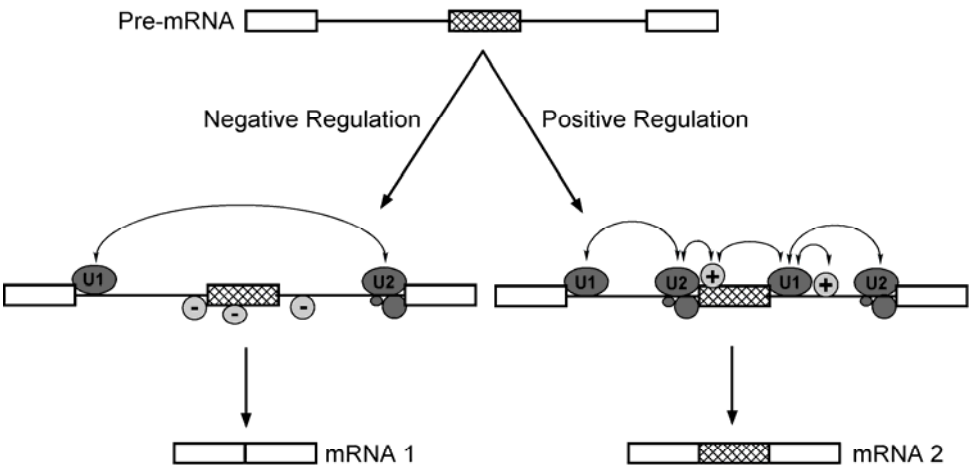

Figure 2.

Figure 2. Positive and Negative Regulation of Splicing

The introns and constitutive (white) or regulated (hatched) exons of a pre-mRNA (top) are defined by where the spliceosome assembles. During early spliceosome assembly the U1 snRNP binds the 5' splice site of an intron and the U2 snRNP, with the auxiliary proteins U2AF and SF1, binds to the branch point/3' splice site region. The binding of these initial components and the assembly of the early spliceosome complexes is thought to define the intron to be excised. The spliceosome can assemble on a single large intron to form a two exon mRNA (exon skipping or exclusion, bottom left). Alternatively, spliceosomes can assemble on each of two smaller introns, to include a new exon in the mRNA (exon inclusion, bottom right). Negative regulatory proteins, such as sxl, PTB or hnRNP A1 (light gray, left pathway), can block spliceosome assembly at particular sites by preventing U1 or U2 binding or by preventing later snRNP assembly. Positive regulatory proteins, such as SR proteins or TIA-1 (light gray, right pathway), enhance spliceosome assembly at sites that are otherwise recognized poorly by the general splicing factors. Systems of alternative splicing are usually controlled by a combination of positive and negative regulatory proteins. Although not shown here, splicing patterns can also be affected by factors such as RNA secondary structure and transcription rate. This figure is adapted from Black (Cell Vol 103 p367 2000).

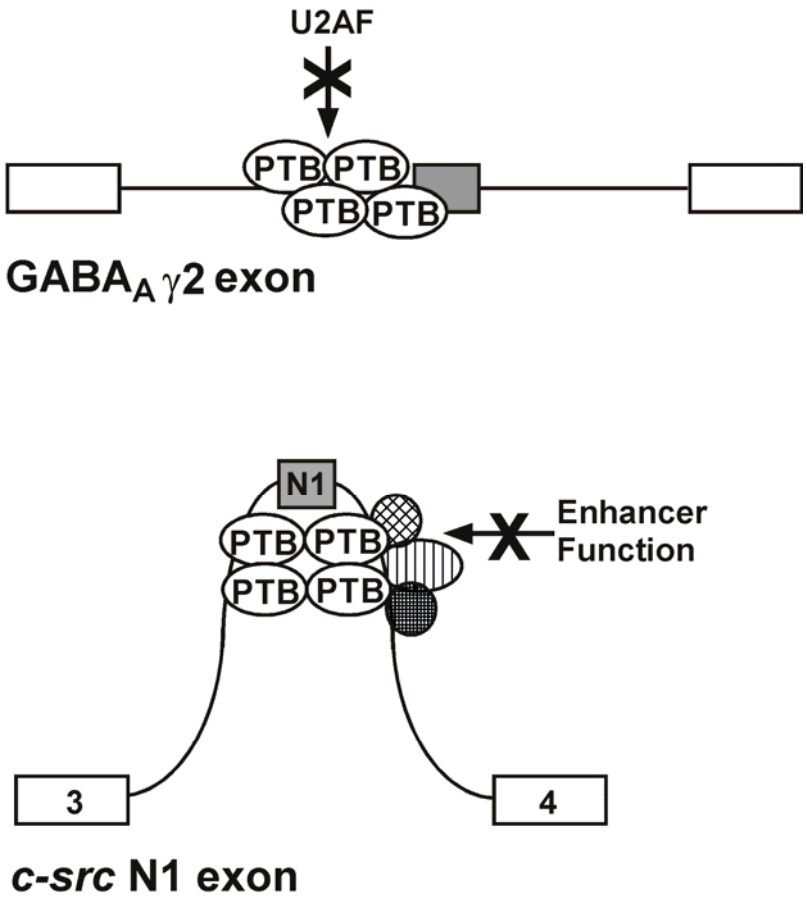

Figure 3.

Figure 3. Models for PTB mediated splicing repression.

In the $GABA_A$ γ2 transcript, multiple PTB proteins are thought to bind tightly to the 3' splice site region and block spliceosome assembly on the alternative exon. For the c-src N1 exon, PTB binding sites on both sides of the exon are required for splicing repression. These are thought to block the function of an intronic splicing enhancer that surrounds the downstream PTB sites, as well as block the upstream 3' splice site. In both cases, PTB binding must be released in neuronal cells to allow splicing to proceed.