

Splicing in the Inner Ear: a Familiar Tune, but What Are the Instruments?

Minireview

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cSlo Splicing: A Theme with 576 Variations

Alternative pre-mRNA splicing is a central mechanism of gene regulation in eukaryotic cells. Genetic regulation at this level is especially common in the vertebrate nervous system, where many proteins important for neuronal development or function are made in multiple isoforms through alternative patterns of splicing. Proteins that contribute to the excitability of neural tissue often show splicing regulation that is quite complex in both the number of different mRNAs produced and their region-specific expression. These include proteins involved in neurotransmitter storage and release, neurotransmitter receptors, and ion channels responsible for generating and propagating an action potential. Some of the alternate protein products of these genes have been shown to vary in their physiological properties. However, in most cases, a sense of the larger biological role of alternative splicing is lacking. One would like to know: How does the protein diversity created by splicing help an individual type of cell carry out its particular function?

Two papers in a recent issue of *Neuron* describe a system where physiology is again regulated through alternative splicing, but in this case the role of this regulation in cell function is clear: the splicing reaction is tuning the individual hair cells of the avian cochlea to specific sound frequencies (Navaratnam et al., 1997; Rosenblatt et al., 1997).

In the inner ear of the chick, hair cells are distributed along the length of the basilar papilla, a layer of cells within the tubular cochlea that is homologous to the mammalian organ of Corti (Hudspeth, 1989). Individual hair cells respond most strongly to a specific frequency of sound and form a tonotopic gradient along the basilar papilla, with cells at the apical end tuned to low frequency sounds (down to approximately 50 Hz), and cells at the basal end responding to high frequency sounds (up to about 5000 Hz). One component in the tuning of the cells stems from the mechanical properties of the hair cells themselves and of the basilar membrane upon which they rest. In the cochlea of reptiles and birds, there is also an electrical component to the tuning. Here, the oscillation of membrane potential during cell stimulation has a specific resonant frequency that matches the frequency of sound to which the cell most strongly responds (Hudspeth, 1989).

Electrophysiological studies of chick and turtle hair cells show that electrical tuning is due in part to alterations in the kinetic properties of calcium-activated potassium channels. These are membrane proteins containing potassium (K^+) selective pores whose gating is controlled by both voltage and intracellular calcium (Ca^{2+}) concentration. Mechanical stimulation of the hair

cell causes a cation influx that depolarizes the membrane and activates voltage-gated calcium channel proteins. The opening of these channels results in a calcium influx and a further depolarization that opens the calcium activated potassium channel. The opening of this channel allows a K^+ efflux from the cell, repolarizing the membrane. The timing of these Ca^{2+} activated K^+ channels determines the rate at which a stimulated cell can repolarize and hence the frequency at which the membrane potential can oscillate. Electrophysiology indicated that the kinetics of the Ca^{2+} activated K^+ channel varied between individual hair cells, but it was not clear how these channel properties were regulated (Hudspeth, 1989).

Several groups have now cloned cDNAs encoding the calcium activated potassium channel expressed in the chick cochlea (Jiang et al., 1997; Navaratnam et al., 1997; Rosenblatt et al., 1997). This protein is encoded by the chicken homolog of the *Drosophila Slowpoke* gene (*Slo*). Homologs of *Slo* were previously cloned from humans and mice and are expressed widely in the nervous system. These genes were shown to produce multiple splice variants of the *Slo* protein that differed in their kinetic properties. In particular, the sensitivity of the protein to calcium was altered by certain alternatively spliced segments of mRNA sequence (Butler et al., 1993; Tseng et al., 1994). The new studies on the chicken *Slo* gene (*cSlo*) show that the number of splice variants produced from this gene is very large and that the repertoire of variants expressed in hair cells changes with cell position along the basilar papilla (Navaratnam et al., 1997; Rosenblatt et al., 1997).

The variation in the splicing of *cSlo* transcripts along the basilar papilla parallels changes in the resonant frequencies of the cells. Using RT/PCR assays on single hair cells, the Hudspeth group showed that the level of inclusion of one alternatively spliced segment (SRKR in the protein sequence) was highest at the apical end of the basilar papilla and decreased in a rough gradient as one moved basally, with some cells at the basal end not including the spliced segment at all (Rosenblatt et al., 1997). In situ PCR experiments support the apical localization of variants with this insert. The Oberholtzer group tested individual hair cells for a wide range of alternatively spliced segments of *cSlo*, and found several positions in the mRNA that are highly variable from cell to cell (Navaratnam et al., 1997). Again, the inclusion or exclusion of these alternative segments seems to depend on the relative position of the hair cell. However, there is a notable disagreement between the two papers, as the Oberholtzer group reports the absence of the SRKR insert in a cell isolated from an apical portion of the cochlea and its nearly complete inclusion in a cell located more basally. This discrepancy points to the difficulty in quantifying single cell PCR and highlights the need for careful, more complete studies. Despite these inconsistencies, it is clear that different hair cells within the cochlea differ widely in their expressed variants of *cSlo* transcripts. Coupled with data that the activity of these channels is essential in determining the resonant frequency of the cell, and that splicing alters this

activity, these results indicate that hair cells are electrically tuned in part through variation in the splicing of the *cSlo* transcript (Hudspeth, 1989; Tseng et al., 1994).

Hair cells with different response frequencies express different versions of the cSlo channel depending upon their position along the basilar papilla. How response frequency is determined by the protein sequence is not at all clear. The sequence variation in the protein is very complex: *cSlo* transcripts contain at least eight positions of sequence heterogeneity derived from alternative splicing. These positions are distributed over the length of the protein sequence including both the N and C termini, and in some positions more than two variant sequences have been identified. If all possible combinations of sequence variation are used, then at least 576 proteins are encoded by this gene. The mouse and human genes have additional positions of variation that, if present in the chicken, would drive the diversity still higher. Functional diversity of the channels is further increased by combining different variants: *cSlo* functions as a protein tetramer, and individual hair cells can express multiple splice variants. Through the assembly of multimeric complexes, the cell has a huge array of channel properties available to suit its particular needs.

Extensive further work will be required to elucidate how cSlo channel properties are changed by the inclusion or exclusion of specific sequence segments. This is potentially analyzable through the characterization of recombinant cSlo channels. However, because hair cells express mixtures of different channels, it will be useful to develop methods for testing both the splicing pattern and the channel properties of individual cells. Such a combination of patch-clamping with single cell RT/PCR has been successful in other systems (see Wang and Grabowski, 1996, and references therein).

Regulating Splicing: Who Plays the Melody?

Perhaps more difficult than understanding the relation of cSlo channel structure to hair cell function will be understanding the regulation of channel expression. One group judges from the sequences of the cDNAs that most of the alternative segments in the mRNA are small cassette exons rather than sequences added to the ends of exons through the shifting of individual splice sites (Rosenblatt et al., 1997). Although it requires confirmation by genomic DNA analysis, this is the most common pattern of alternative splicing.

The introns to be excised from a pre-mRNA are defined by the assembly of a large spliceosome complex onto the splice site sequences at the intron termini. This assembly process can be altered by regulatory proteins to define different introns under different cellular conditions. The best understood systems of alternative splicing are in *Drosophila melanogaster* (Wang and Manley, 1997). The splicing of several transcripts in the *Drosophila* sex determination pathway is controlled by sex-specific regulatory proteins. These sex specific proteins combine with generally expressed proteins to alter the assembly of the spliceosome at certain splice sites. For example, the Sex-lethal protein (Sxl) is a negative regulator of splicing that is expressed only in female flies where it binds to sequence elements in certain pre-mRNAs. This binding prevents spliceosome assembly at adjacent splice sites, resulting in alternative sites being chosen by the spliceosome, and producing a female-specific splicing pattern for the regulated mRNA. In contrast,

the female specific Transformer protein (Tra) positively regulates the splicing of several pre-mRNAs, including the *doublesex* transcript (*dsx*). In combination with other proteins, Tra binds to sequence elements in *dsx* exon 4 and stimulates binding of the essential splicing factor U2AF to the upstream 3' splice site. Through this cooperative assembly with non-sex-specific factors, Tra protein activates the splicing of *dsx* exon 4 in female flies.

Among the vertebrates, alternative splicing has been mainly studied in mammalian cell lines (Wang and Manley, 1997). These systems of tissue-specific splicing appear different in some ways from the sex-specific splicing of *Drosophila*. Splicing factors highly specific for cell-type, that could serve roles similar to the sex specific Sxl and Tra proteins, have not yet been identified. However, the more generally expressed proteins, with which Tra and Sxl cooperate, do have homologs in mammalian cells and in the chicken. These include an important class of regulators called SR proteins that have been implicated in many aspects of constitutive and regulated splicing. In vertebrates, as in the *Drosophila* examples, it is thought that combinations of regulatory proteins bind to pre-mRNAs to affect the assembly of the spliceosomal components. However, in vertebrates these combinations of factors appear highly diverse.

Combinatorial control of splicing is particularly evident in the examples of neural specific splicing that have been analysed. These include transcripts that show altered splicing patterns between neurons and non-neuronal cells such as the c-src and Calcitonin/CGRP transcripts, or between different types of neurons such as the $\gamma 2$ subunit of the GABA_A receptor (Wang and Manley, 1997). The mechanistic details of these regulatory systems are almost completely unknown, but all are affected by a variety of intronic and exonic sequence elements that are presumed bound by a large variety of protein factors. Proteins identified as potential regulators of these transcripts are widely expressed rather than specific to a cell type (Ashiya and Grabowski, 1997; Min et al., 1997). The tissue specificity of the splicing appears to be generated through the complex combination of sequence elements and proteins that individually have small effects. How these regulatory factors ultimately interact with the spliceosomal components is also not understood.

The cSlo channel has potential for generating hundreds or even thousands of different splice variants. Other systems of alternative splicing in the nervous system are equally if not more complicated. The examples of neural specific splicing whose regulation is starting to be analysed are relatively simple, usually with only two possible splicing patterns that are observed in easily accessible cells. Those who study these systems are apt to find systems such as the cSlo channel a daunting prospect. An accordionist who has never seen another instrument may appreciate the structural complexity and precision in the sound of an orchestra; but can he understand how the sounds were generated? Similarly, with our rudimentary understanding of the regulation of spliceosome assembly, we can admire the complexity of the *cSlo* system, but it is difficult to even speculate on the mechanisms that precisely define the splicing of *cSlo* to a few patterns out of hundreds of possible choices. A much clearer view is needed of how simple

exons are regulated before we can understand these more baroque systems.

**Signaling for a Splicing Pattern:
Who Is the Conductor?**

To understand *cSlo* regulation, we first need a map that correlates the inclusion of each alternative exon with hair cell position along the apical to basal axis of the cochlea. Because of the frequency map, it seems likely that individual exons with a defined effect on the channel will show a gradient of inclusion, exhibiting high levels of insertion at one end of the axis and low levels at the other. It is also possible that different exons will show inverse patterns of inclusion. With a better understanding of the locations and physiological effects of specific splice variants, it will be very interesting to do developmental studies of when and where certain splice variants begin to be expressed in the hair cells. Initial experiments in this regard show an interesting change in the expression pattern of one splice variant during development (Rosenblatt et al., 1997). The developmental timing of exon inclusion will be important in identifying the intercellular signals that determine the *cSlo* splicing pattern and in understanding how those signals are transmitted via intracellular pathways to the splicing apparatus.

Do changes in splicing coincide with the morphological differentiation of the hair cell? Although *cSlo* type currents become detectable only after hair cell differentiation, it is possible that the splicing of *cSlo* is set when other differentiated properties of the hair cell are determined during development of the inner ear (see Corwin, 1997 and references therein). At the time hair cells differentiate from the supporting cells of the papilla they already exhibit structural differences that determine the mechanical tuning of the cells: cells at the basal end of the papilla possess more and shorter stereocilia than cells at the apical end. How this morphological gradient in the hair cells is established is not known, but it is proposed to result from a concentration gradient of a signaling molecule along the length of the developing cochlea. Morphogen gradients that produce gradients of transcriptional response are well characterized in the *Drosophila* embryo (Rivera and Jackle, 1996). Other types of signal gradients apparently direct aspects of tissue development (Dale, 1997; Pituelo, 1997). The cellular response to this kind of signaling has always been characterized as transcriptional, but gradations in the level of a signaling molecule could also determine levels of alternative exon inclusion. A signaling molecule's effect on splicing could result from a direct alteration of the splicing apparatus or from different levels of induced transcription differentially saturating a limiting splicing factor.

Another interesting possibility is that the variation in splicing is a consequence of the mechanical tuning of the cells. Perhaps the splicing machinery can sense the frequency that gives the strongest mechanical response from the cell and adjust the splicing of the *cSlo* channel appropriately. Is the splicing regulation one voice in a fugue of changes taking place during hair cell differentiation or is the splicing a secondary theme that is developed after the major program of differentiation has occurred? Either possibility requires an understanding of how splicing is able to respond to cellular stimuli.

The transmission of extracellular signals to the splicing apparatus is an area where almost nothing is known. A great deal of work has documented the impact of various second messenger systems on transcription, but the effects of these signaling pathways on splicing are just beginning to be analyzed. Splicing factors of the SR family are dependent on phosphorylation for their activity and protein kinases have been identified that specifically phosphorylate these proteins (Wang and Manley, 1997). However, some of these phosphorylation events are needed for the constitutive splicing of all transcripts. It is not clear whether they can also affect specific splicing patterns in certain pre-mRNAs. Several transcripts have splicing patterns that are altered by growth factor stimulation, but again the mechanisms that transmit these extracellular signals to the splicing machinery are unknown. Interestingly, transient increases in intracellular calcium can induce long lasting changes in the splicing pattern of Ca-ATPase transcripts in neuroblastoma cells. Changes in intracellular calcium concentration that occur during hair cell depolarization may similarly affect the splicing of *cSlo* transcripts; data regarding the timing of changes in *cSlo* splicing relative to the functional development of the hair cells and their innervation may illuminate this issue.

Finding the actual regulatory molecules that determine the inclusion or exclusion of the *cSlo* exons is likely to be difficult. Biochemical techniques for identifying splicing regulatory proteins will depend on the development of cellular extracts that show reliable differences in their splicing of the *cSlo* exons. Due to their limited number, hair cells are not a likely source of these extracts but perhaps other cells can be used. *Drosophila* genetics offers another approach. The kinetics of the *Drosophila* Shaker potassium channel is similarly regulated by alternative splicing. *Shaker/LacZ* fusion genes have been developed that generate LacZ expression dependent upon specific *Shaker* splicing patterns (Mottes and Iverson, 1995). *Drosophila* *Slowpoke* transcripts also show extensive alternative splicing, and similar reporter genes could be constructed to analyze the regulation of the *Slo* alternative exons.

The regulation of *cSlo* channels provides a fascinating example of the use of alternative splicing to create differentiated properties within a single cell type. Given the small number of hair cells and the large number of channel variants, it is possible that no two hair cells are identical in their expressed repertoire of *cSlo* proteins. Such careful tuning of membrane properties via alternative splicing may occur in other cells. All excitable cells require precise coordination in the timing of their membrane proteins and nearly all of these molecules are created in diverse forms through alternative splicing. It may be that the tuning of these proteins to suit specific cellular needs is often orchestrated by this versatile form of genetic regulation.

Selected Reading

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