
Alternative splicing in *C. elegans**

Alan M. Zahler[§], Department of Molecular, Cellular and Developmental Biology, Center for Molecular Biology of RNA, Sinsheimer Laboratories, University of California, Santa Cruz, CA 95064 USA

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Abstract

Alternative splicing is a common mechanism for the generation of multiple isoforms of proteins. It can function to expand the proteome of an organism and can serve as a way to turn off gene expression post-transcriptionally. This review focuses on splicing and its regulation in *C. elegans*. The fully-sequenced *C. elegans* genome combined with its elegant genetics offers unique advantages for exploring alternative splicing regulation in metazoans. The topics covered in this review include constitutive splicing factors, identification of alternatively spliced genes, examples of alternative splicing in *C. elegans*, and alternative splicing regulation. Key genes whose regulated alternative splicing are reviewed include *let-2*, *unc-32*, *unc-52*, *egl-15* and *xol-1*. Factors involved in alternative splicing that are discussed include *mec-8*, *smu-1*, *smu-2*, *fox-1*, *exc-7* and *unc-75*.

1. The Importance of alternative splicing

Advances in animal genome sequencing have led to many questions concerning the very nature and number of genes and how they help to promote the diversity of cellular and organismal functions. One of the interesting disappointments of the analysis of the human genome sequence is that we may possess less than 25,000 genes (International Human Genome Sequencing Consortium, 2004), only one third more than the number of predicted

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[§]To whom correspondence should be addressed. E-mail: zahler@biology.ucsc.edu

genes in *C. elegans*. How can we explain all the complexity and wonder of human biology with such a limited gene set? One way to explain this paradox is to point out that the number of possible proteins from the genome can far exceed the possible number of genes if a large percentage of the genes have the ability to encode multiple proteins. This expansion of the proteome can be accomplished through alternative precursor messenger RNA (pre-mRNA) splicing, which can allow one gene to encode multiple proteins. Comparison of cDNAs with genomic sequence has provided evidence for extensive alternative splicing of human genes (Modrek et al., 2001). Many different studies predict that from 40 to 60% of human genes are alternatively spliced (Croft et al., 2000; Hide et al., 2001; Kan et al., 2001; Modrek et al., 2001). Alternative splicing occurs in *C. elegans* and plays an important role in development. This chapter builds upon a previous review in *C. elegans* II of constitutive splicing signals in *C. elegans* (Blumenthal and Steward, 1997).

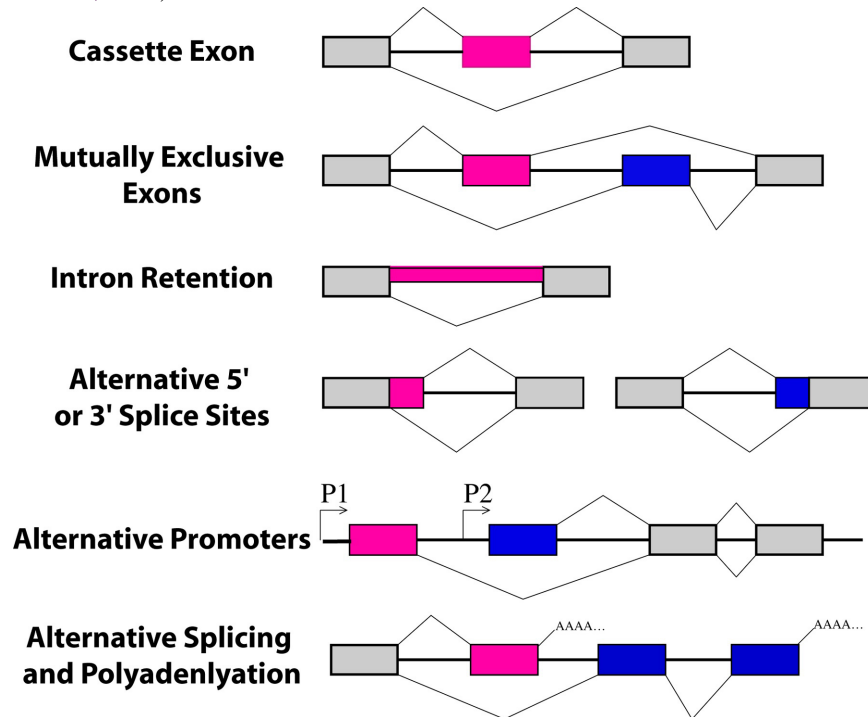


Figure 1. Types of alternative splicing. In these graphics, exons are represented by boxes and introns by lines. Exon regions included in the messages by alternative splicing are colored while constitutive exons are shown in gray. Promoters are indicated with arrows and polyadenylation sites with AAAA.

Many genes are alternatively spliced in tissue-specific, developmentally-regulated, and hormone-responsive manners, providing an additional mechanism for regulation of gene expression (Bandman, 1992; Caceres and Kornblihtt, 2002; Grabowski, 1998; Grabowski and Black, 2001; Singh, 2002; Stamm et al., 1994). Types of alternative splicing include the use of alternative 5' splice sites, alternative 3' splice sites, cassette exons, retained introns and mutually exclusive exons (Figure 1). Alternative splicing often leads to changes in the primary amino acid sequence of the protein, sometimes subtle and sometimes quite dramatic. Alternative splicing can control where the site of 3' end cleavage and polyadenylation occurs, indicating a strong interaction between the splicing and 3' end formation machineries. Alternative splicing can lead to messages with premature termination codons, for example by including a cassette exon with an in-frame stop codon. These premature termination codon-containing messages can be substrates for the nonsense-mediated decay (NMD) pathway in *C. elegans* (Mango, 2001). Evidence for this was first demonstrated in *C. elegans* in a genetic screen for alternative splicing factors. Morrison et al. showed that two of the genes encoding SR protein splicing factors have alternative isoforms with premature termination codons. These alternatively spliced forms were preferentially stabilized in suppressor with morphogenetic effects on genitalia (*smg*) mutants (Morrison et al., 1997). This use of alternative splicing to generate NMD substrates is very common in mammals (Lewis et al., 2003). In humans, polypyrimidine tract binding protein (PTB), which has multiple roles in mRNA processing including alternative splicing regulation, 3' end formation, and stability, regulates its own abundance by influencing the splicing of its own message. In this negative feedback loop, PTB promotes skipping of its own exon 11, leading to an isoform with an in-frame stop codon that is a substrate for NMD (Wollerton et al., 2004). In addition to turning off transcription to downregulate gene expression, alternative splicing is commonly used to downregulate gene expression by producing messages with truncated open reading frames that are rapidly degraded by NMD.

2. Splicing of precursors to messenger RNA

Alternative splicing is regulated at the sites on the pre-mRNA where the splicing machinery assembles. The two transesterification reaction steps of pre-mRNA splicing occur in a large multi-component ribonucleoprotein complex, the spliceosome (Burge et al., 1999; Caceres and Krainer, 1997; Kramer, 1996). The spliceosome assembles onto the pre-mRNA via an ordered binding of its subunits, the uridine-rich small nuclear ribonucleoprotein complexes (U snRNPs). SnRNP assembly is guided by RNA-RNA interactions between the U snRNAs and the pre-mRNA and the snRNAs with each other. First U1 binds to the pre-mRNA guided by base-pairing interactions between its 5' end and the 5' splice site. U2 binding is guided by base pairing interactions with the branchpoint sequence in the intron. Then the U4/U6-U5 tri-snRNP is recruited to the forming spliceosome. Extensive rearrangements of RNA-RNA interactions then occur in the spliceosome (Ares and Weiser, 1994; Madhani and Guthrie, 1994). There is evidence from recent studies that the spliceosomal components can be found together in a pre-assembled penta-snRNP complex (Stevens et al., 2002). The mammalian penta-snRNP has been shown to assemble onto 5' splice sites and undergo rearrangements that replace U1 interactions with U5 interactions (Malca et al., 2003). The functional importance of the pre-assembled penta-snRNP compared to models of step-wise spliceosome assembly on the substrate pre-mRNA is still being studied.

Several classes of splicing factor proteins have been identified as essential for metazoan spliceosome assembly yet are not integral components of the snRNPs (Kramer, 1996). The SR protein family of splicing factors is required for the earliest interactions of U1 snRNP with the pre-mRNA and for subsequent spliceosome assembly steps (Fu, 1995; Graveley, 2000; Manley and Tacke, 1996). SR proteins have redundant functions in promoting constitutive splicing, but certain family members have distinct roles in promoting specific alternative splice site usage. SR protein family members have distinct binding specificities for sequences within exons that serve as splicing enhancer elements (Cartegni and Krainer, 2002). U2 auxiliary factor (U2AF) is a heterodimeric splicing factor composed of 35 and 65 kD subunits. It binds to the polypyrimidine tract and recognizes the AG dinucleotide at the 3' end of the intron to promote U2 snRNA interactions with the intron branchpoint sequence (Merendino et al., 1999; Wu et al., 1999; Zorio and Blumenthal, 1999b). U2AF, SR proteins and U1 snRNP assemble onto the pre-mRNA in an ATP-independent manner to form the early (E) complex (Michaud and Reed, 1991; Staknis and Reed, 1994). It is important to note that assembly of the E complex essentially identifies both the 5' end 3' splice site for further spliceosome assembly, and thus control of this assembly can be a way of regulating alternative splice site choice. Splicing factors interact with the pre-mRNA, with other splicing factors and with protein components of the snRNPs (Burge et al., 1999; Caceres and Krainer, 1997; Kramer, 1996). This cooperative assembly increases the local concentration of the snRNAs in the vicinity of splice sites and therefore is likely to promote the RNA-RNA interactions required for spliceosome assembly.

3. Models for alternative splicing regulation

From research over the past 20 years, some general themes have emerged for alternative splicing regulation, although the exact mechanisms still need to be determined. Alternatively spliced exons often have weak consensus sequences at the 5' and 3' ends of the introns, suggesting that additional signals are required for recognition of the exon by the splicing machinery (Lopez, 1998). *Cis*-acting pre-mRNA sequences responsible for regulation of splicing have been identified for many genes (Cooper and Mattox, 1997). These regions are found in exons or in introns and can be enhancers or silencers of splice site usage (Figure 2). These sequence motifs serve as binding sites for protein factors that can enhance or inhibit the ability of the spliceosome to recognize the exons. The exonic elements not only encode amino acids but also regulate their own ability to be spliced into the mature message. *Trans*-acting splicing factors that interact with splicing regulatory elements in exons have been identified. Subsets of the SR proteins bind with regulatory sequences important for splicing control (Gontarek and Derse, 1996; Kanopka et al., 1996; Lavigne et al., 1993; Nagel et al., 1998; Ramchatesingh et al., 1995; Staknis and Reed, 1994; Sun et al., 1993). Heterogeneous nuclear ribonucleoprotein (hnRNP) A/B family members can bind to high-affinity sequences in exons and inhibit splicing through blocking SR proteins from binding to the exons (Caputi et al., 1999; Zhu et al., 2001).

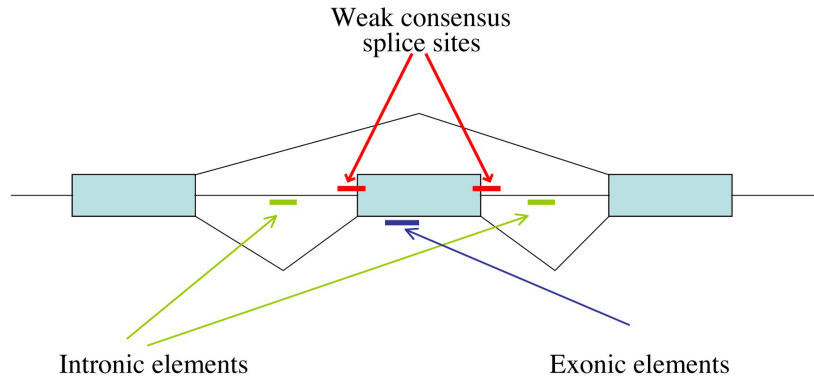


Figure 2. Locations of regions on the pre-mRNA that can affect alternative splicing. Some combination of these regulatory regions can usually be found. Weaker consensus splice sites surrounding the alternative exon, exonic regulatory regions and intronic regulatory regions are indicated.

Splicing factors important for tissue-specific regulation of vertebrate splicing often assemble into multicomponent complexes on intronic splicing regulatory elements. The downstream control sequence (DCS), found in the intron downstream of the human neural-specific c-src N1 exon, is one such example. Factors that bind to the DCS regulate N1 splicing; these include hnRNP H, hnRNP F, KH-type splicing regulatory protein (KSRP) and a neural-specific homolog of PTB (nPTB; Chou et al., 1999; Markovtsov et al., 2000; Min et al., 1995; Min et al., 1997). Comparison of the factors that assemble onto this element from neuronal cell nuclear extract vs. epithelial cell nuclear extract indicate that a subset of these proteins bind from both extracts. Factors in the neuronal extract promote assembly of a different complex that is required to activate splicing of the neural-specific exon. PTB was identified by its ability to bind to polypyrimidine tracts. It has been shown to regulate the alternative splicing of its own message, as well as others including cardiac troponin T, alpha-actinin, fibroblast growth factor receptor R2, calcitonin/CGRP, and alpha tropomyosin pre-mRNAs (Wagner and Garcia-Blanco, 2001; Wollerton et al., 2001). In these systems, PTB binding to both introns flanking an exon promotes exon skipping (Wagner and Garcia-Blanco, 2001). CUG binding protein and related family members known as CELF proteins interact with CUG repeats in introns to regulate splicing (Ladd et al., 2001; Philips et al., 1998). An antagonistic interaction between one of the CELF proteins, ETR-3, and PTB regulates troponin t alternative splicing (Charlet-B et al., 2002). The nova-1 protein is important for regulation of alternative splicing in the nervous system. This neuronal protein binds the sequence UCAY (Jensen, Dredge, et al., 2000; Jensen, Musunuru, et al., 2000). A balance between nova activity and PTB is important in regulation of alternative splicing in neurons (Polydorides et al., 2000). The mechanism by which binding of these factors to intronic elements regulates exon inclusion is not clear. Genomic analysis indicates that homologs of all of the major vertebrate splicing factors discussed above can also be found in *C. elegans* (AMZ, unpublished observations).

4. Constitutive splicing factors in *C. elegans*

The spliceosomal snRNAs of *C. elegans* are very similar to their yeast and mammalian counterparts (Thomas et al., 1990). The importance of U1 snRNA base-pairing with the 5' splice site is demonstrated by two extragenic allele-specific suppressors of mutations of the canonical G that begins *C. elegans* introns. *sup-6* and *sup-39* encode two of the 12 U1 snRNA genes in *C. elegans* and were identified in screens as suppressors that can allow mutated splice donors to be recognized by the splicing machinery (Roller et al., 2000; Run et al., 1996). The suppressor mutations are compensatory mutations in the 5' end of U1 snRNA genes (Zahler et al., 2004). These U1 snRNA suppressors are informational suppressors, allele-specific gene-nonspecific suppressors, and join the amber suppressors and the *smg* genes as members of this group (Mount and Anderson, 2000).

C. elegans contains seven different members of the SR protein family called the *rsp* genes (Kawano et al., 2000; Longman et al., 2000; Morrison et al., 1997). Family members are expressed in all cells at all stages of development (Kawano et al., 2000). There is functional overlap among the family members as studies suggest that, with the exception of *rsp-3*, multiple SR proteins need to be targeted by RNA interference in order for a phenotype to become evident (Kawano et al., 2000; Longman et al., 2000; Longman et al., 2001). The phenomenon of trans splicing is common in nematode species (see Trans-splicing and operons). Using splicing competent embryonic extracts from parasitic nematode *Ascaris lumbricoides*, Sanford and Bruzik have shown that SR proteins are essential for the trans splicing reaction in nematodes and that the phosphorylation state and activity level of these proteins change during early development (Sanford and Bruzik, 1999a; Sanford and Bruzik, 1999b).

C. elegans has a homolog of each U2AF subunit, *uaf-1* and *uaf-2* (Zorio and Blumenthal, 1999a; Zorio et al., 1997). Using the short defined polypyrimidine tract at the 3' end of *C. elegans* introns (Blumenthal and Steward, 1997), studies in this organism were able to demonstrate the specificity with which the U2AF heterodimer subunits recognize the polypyrimidine tract and 3' splice site at the end of the intron. The U2AF65 homolog, *uaf-1*, interacts with the polypyrimidine tract while the U2AF35 homolog *uaf-2* recognizes the AG dinucleotide at the 3' end of the intron (Zorio and Blumenthal, 1999b). This showed that even though the AG at the 3' end of the intron is involved in the second transesterification reaction of splicing, it also has an important role in early spliceosome assembly. *UAF-1* regulates its own expression. The *uaf-1* message is alternatively spliced to contain an unusual exon with an in-frame stop codon that should be a subject for nonsense mediated decay. This exon is unusual because it contains 10 matches to the *C. elegans* 3' splice acceptor sequence UUUUCAG/R, the sequence recognized directly by *C. elegans* U2AF (Zorio and Blumenthal, 1999b; Zorio et al., 1997). MacMorris et al. showed that these splice acceptor repeats cause the pre-mRNA to be retained in the nucleus and thus not become a substrate for translation or nonsense-mediated decay. Experiments in which the UUUUCAG/R repeats were placed in a GFP transgene indicate that *UAF-1* is responsible for the nuclear retention of its message (MacMorris et al., 1999).

5. Identification of alternatively spliced genes in *C. elegans*

Intron and exon structure in *C. elegans* is similar to that found in other higher eukaryotes (Blumenthal and Steward, 1997). The completed genome sequence and >200,000 expressed sequence tag (EST) complementary DNA (cDNA) sequences are available in public databases such as Wormbase at <http://www.wormbase.org/> (*C. elegans* Sequencing Consortium, 1998; Stein et al., 2001). Computational approaches have been developed to identify alternatively spliced *C. elegans* genes by comparing cDNA and genomic sequences (Kent and Zahler, 2000). This led to the initial identification of 844 alternatively spliced genes. Regularly updated lists of alternatively spliced genes can be found at the Intronerator web site, <http://genome-test.cse.ucsc.edu/Intronerator/>. Wormbase annotations of genes indicate alternatively spliced isoforms. These are generated by hand annotation based on cDNA and EST data as well as experimental data reported by researchers. As of release WS132, there are 2,562 alternatively spliced genes annotated in Wormbase, accounting for 13% of annotated *C. elegans* genes. This is likely an underestimate of the total percentage of *C. elegans* genes that are alternatively spliced as these values are dependent on EST and cDNA coverage of the genome and at the current time 25% of the 19,726 annotated *C. elegans* coding sequences have no cDNA transcriptional evidence (Wormbase WS132 release notes). Genes with alternative isoforms are denoted by a lower case letter after the assigned gene name, for example T01D7.1a. Many of the genes with alternative isoforms are generated by using alternative promoters with unique first exons that splice into a common second exon. Even though these alternative promoters will lead to alternative 5' exons spliced to common downstream exons (Figure 1), the generation of the unique first exon is not regulated at the level of alternative splicing but instead by transcription.

6. Some examples of alternatively spliced *C. elegans* genes

There are four genes in *C. elegans* that encode the α subunit of the vacuolar ATPase, suggesting a mechanism for regulating the activity of this pump (Oka et al., 2001). One of these genes, encoded by *unc-32*, can be alternatively spliced to yield six different isoforms, adding even more complexity (Figure 3A). There are three different mutually exclusive exons for exon 4, and two for exon 7 leading to six possible transcripts (Pujol et al., 2001). In the study by Pujol et al., GFP was fused in frame to exon 4B of an *unc-32* genomic clone and transfected into animals. The expression of GFP was limited to neurons suggesting that splicing of exon 4B is neural-specific (Pujol et al., 2001).

Alternative splicing can be used to transcribe two distinct genes from a single promoter, in a way that accomplishes the same end but is distinct from trans-splicing of operons. This occurs for the *cha-1* and *unc-17* genes. *cha-1* encodes choline acetyl-transferase while *unc-17* encodes a synaptic vesicle-associated acetylcholine transporter. Analysis of transcripts of these two genes involved in acetylcholine metabolism indicate that they share a common promoter and 5' untranslated exon (Figure 3B). The *unc-17* transcript is nested within the long first intron of *cha-1* (Alfonso et al., 1994). In this case, two proteins with related functions but with no peptide sequences in common are produced as a result of alternative splicing of a common mRNA precursor.

The fibroblast growth factor receptor homolog *egl-15* is alternatively spliced to yield five different C-termini. In addition, the gene has two distinct mutually exclusive central exons, 5A and 5B (Figure 3C). These two exons encode a domain in the extracellular portion of the protein that gives ligand specificity to the receptor. Exon 5A is required for the response to the FGF chemoattractant EGL-17, which guides migrating sex myoblasts to their final

positions. Exon 5B is required for the essential functions mediated by this receptor through interaction with the ligand LET-756 (Goodman et al., 2003). In this case of alternative splicing, two mutually exclusive exons allow one gene to code for a receptor tyrosine kinase with two distinct functional specificities.

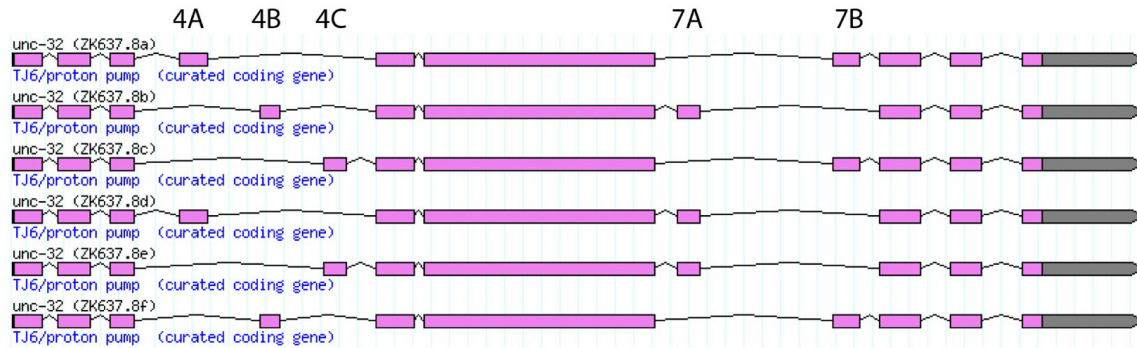
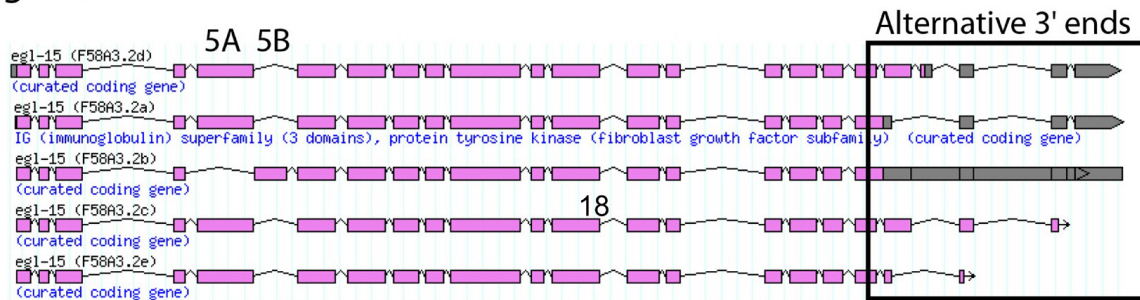
A.*unc-32***B.***cha-1/unc-17***C.***egl-15*

Figure 3. Examples of *C. elegans* alternatively spliced genes. Images are screen shots taken from the Wormbase genome browser. (A) *unc-32*. The three alternative exon 4s and two alternative exon 7s are indicated. (B) *cha-1* and *unc-17* share a common promoter and 5' untranslated exon. (C) *egl-15*. The two mutually exclusive exons, 5A and 5B, which provide functional specificity, are indicated, as are the five different 3' ends generated by alternative splicing. Both exon 5 isoforms have been detected with each of the 3' end isoforms, so there are 10 alternatively spliced isoforms of this gene (Goodman et al., 2003). Only five of the isoforms are indicated in the graphic.

7. Regulation of alternative splicing in *C. elegans*

Splicing studies in *C. elegans* have demonstrated the importance of varying strengths among splice site consensus sequences to allow for splicing regulation. 5' splice site strength is determined by the extent of complementarity of the splice donor region to the 5' end of U1 snRNA. The alpha 2(IV) collagen gene *let-2* is alternatively spliced to yield two forms of the protein containing one of two mutually exclusive exons, exon 9 or exon 10 (Figure 4A). There is developmental regulation of this alternative splicing; 95% of embryonic LET-2

messages contain exon 9 while in adults 90% contain exon 10. The larval stages show a gradual shift in usage between these isoforms, indicating developmental control of alternative splicing (Sibley et al., 1993). The intron downstream of *let-2* exon 10 begins with a rare GC dinucleotide instead of the canonical GU. Only 0.5% of introns in *C. elegans* begin with a GC dinucleotide (Farrer et al., 2002). In *Ascaris*, which maintains the same developmentally regulated alternative splicing of this alpha 2 (IV) collagen gene, the same intron begins with GU (Pettitt and Kingston, 1994). The GC splice donor serves as a weak consensus sequence that allows for the proper regulation of alternative splicing. Replacement of this sequence with a GU donor (such as the one from *Ascaris*) that has weak overall base-pairing with the 5' end of U1 snRNA, allows for maintenance of splicing regulation. Replacing the GC donor with a moderate or strong consensus GU donor abolishes the splicing regulation and leads to high usage of exon 10 in embryos (Farrer et al., 2002). The presence of a weak splice donor for exon 10 allows developmentally regulated factors to control the recognition of this exon by the splicing machinery. The identity of these factors is not yet known, but our group has identified an 11 base sequence element in the intron between exons 10 and 11 that is required for efficient inclusion of exon 10 in late larval stages. This element may serve as an interacting site for developmentally regulated splicing factors (Tracy Farrer and AMZ, unpublished observation).

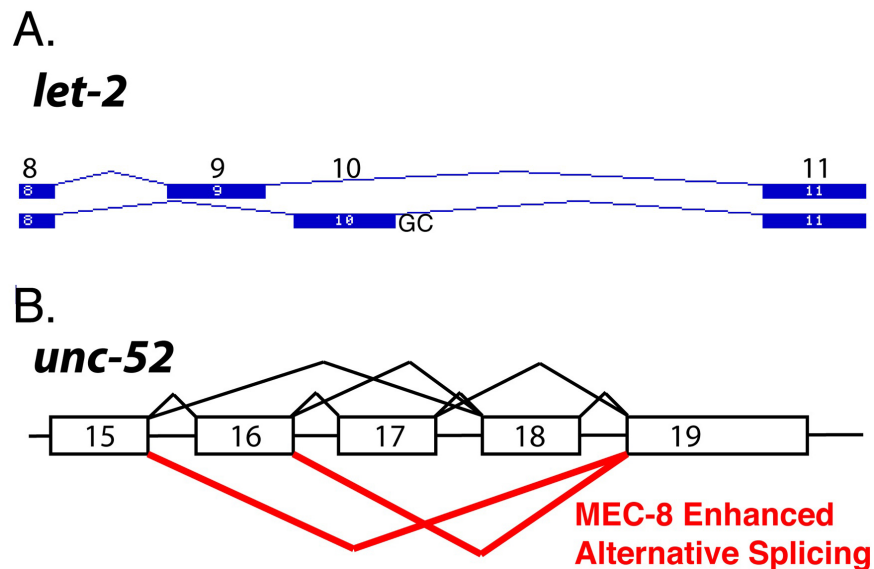


Figure 4. Alternative splicing regulation in *C. elegans* genes. (A) Alternative splicing of the *let-2* gene containing mutually exclusive exons 9 and 10. The intron downstream of exon 10 begins with a GC dinucleotide important for splicing regulation. (B) Diagram of alternative splicing of the *unc-52* gene. Every combination of exon inclusion and skipping of exons 16, 17 and 18 has been detected. MEC-8 enhances the splicing of two of the isoforms, 15-19 and 15-16-19, indicated in red (Lundquist et al., 1996).

Genetic analysis in *C. elegans* has led to the identification of splicing regulatory pathways. MEC-8 is a two RNA recognition motif (RRM)-containing, nuclear protein. Mutations in *mec-8* lead to mechanosensory and other defects. Mutations in *mec-8* are synthetically lethal with viable mutations in the *unc-52* gene (Lundquist et al., 1996). UNC-52 is a homolog of the mammalian perlecan protein, an extracellular matrix molecule important in muscle anchorage (Rogalski et al., 1993). These viable *unc-52* mutations occur in the alternatively spliced exons 17 and 18 (Figure 4b). Every possible combination of inclusion or skipping of exons 16, 17 and 18 has been detected (Rogalski et al., 1995). Skipping of exons 17 and 18 is dependent on the function of *mec-8* (Lundquist et al., 1996). MEC-8 protein is found in all nuclei in embryos, but only in a subset of mechanosensory neurons and hypodermal cells in adults (Spike et al., 2002). *unc-52* mutant alleles with stop codons in exons 17 and 18 develop a progressive paralysis at later developmental stages. Null mutations in the *unc-52* gene lead to embryonic lethality (Rogalski et al., 1995). Animals containing both viable mutations in *unc-52* and viable mutations in *mec-8* have a synthetic lethal phenotype that mimics the embryonic lethality of the lethal *unc-52* alleles (Lundquist and Herman, 1994). In *mec-8(+)* animals with viable *unc-52* mutations, there is a loss of full-length UNC-52 production in the later developmental stages, leading to the adult paralysis phenotype. In *mec-8; unc-52* double mutant animals, loss of *mec-8*-enhanced skipping of exons 17 and 18 (containing stop codons) leads to insufficient production of full-length UNC-52 in embryos and thus to the more severe embryonic lethality. Two suppressors of the *mec-8; unc-52* synthetic lethality were identified (Lundquist and Herman, 1994). One of these, *smu-1*, which encodes a nuclear protein that contains five WD motifs, is ubiquitously expressed and is 62% identical to a human spliceosome associated protein fSAP57 (Spike et al., 2001). *smu-2* encodes a *C. elegans* homolog of the RED protein, which has also been shown to be associated with the human spliceosome. SMU-1 and SMU-2 bind to each other; SMU-2

stabilizes SMU-1 but SMU-1 does not stabilize SMU-2. Based on their homology with components of the mammalian spliceosome, it is hypothesized that SMU-1 and SMU-2 might interact with the spliceosome and modulate splice site choice (Spartz et al., 2004).

Alternative splicing factors play a role in *C. elegans* sex determination. In hermaphrodites, the feminizing locus on X (*fox-1*) gene is a regulator of sex determination that acts as a numerator for counting X chromosomes (Hodgkin et al., 1994). *fox-1* encodes an RRM protein that acts post-transcriptionally to inhibit the expression of the *xol-1* gene, the major specifier of male fate. FOX-1 inhibits splicing of the terminal intron of *xol-1* pre-mRNA, preventing production of active XOL-1 (Nicoll et al., 1997).

Tissue specific expression of alternative splicing factors has been demonstrated in *C. elegans*. Two genetically identified neuronal, nuclear RNA binding proteins involved in synaptic transmission have been cloned in *C. elegans*. These are *unc-75*, which is an ortholog of the human CELF family of alternative splicing factors, and *exc-7*, which is a homolog of the *Drosophila* neuronal splicing factor ELAV (Loria et al., 2003). Another ELAV homolog in *C. elegans*, *etr-1*, is expressed only in muscle cells and is essential for development (Milne and Hodgkin, 1999).

8. Conclusions

With its many alternatively spliced genes and evolutionarily conserved alternative splicing factors, *C. elegans* offers a tractable model system for the study of pre-mRNA splicing regulation. With much smaller average intron size compared to vertebrates, a fully sequenced genome, and a highly developed and simple genetics system, research in *C. elegans* has the potential to provide important contributions to our understanding of alternative splicing regulation.

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