Switch-like regulation of tissue-specific alternative pre-mRNA processing patterns revealed by customized fluorescence reporters

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Alternative processing of precursor mRNAs (pre-mRNAs), including alternative transcription start sites, alternative splicing and alternative polyadenylation, is the major source of protein diversity and plays crucial roles in development, differentiation and diseases in higher eukaryotes. It is estimated from microarray analyses and deep sequencing of mRNAs from synchronized worms that up to 25% of protein-coding genes in *Caenorhabditis elegans* undergo alternative pre-mRNA processing and that many of them are subject to developmental regulation. Recent progress in visualizing the alternative pre-mRNA processing patterns in living worms with custom-designed fluorescence reporters has enabled genetic analyses of the regulatory mechanisms for alternative processing events of interest in vivo. Expression of the tissue-specific isoforms of actin depolymerising factor (ADF)/cofilin, UNC-60A and UNC-60B, is regulated by a combination of alternative splicing and alternative polyadenylation of pre-mRNAs from a single gene *unc-60*. We recently found that muscle-specific splicing regulators ASD-2 and SUP-12 cooperatively switch the pre-mRNA processing patterns of the *unc-60* gene in body wall muscles. Here I summarize the bichromatic fluorescence reporter system utilized for visualizing the tissue-specific alternative processing patterns of the *unc-60* pre-mRNA. I also discuss the model for the coordinated regulation of the UNC-60B-type pre-mRNA processing in body wall muscles by ASD-2 and SUP-12.

Introduction

Newly synthesized pre-mRNAs are considered to be processed cotranscriptionally in eukaryotes.[1] The sites of transcription initiation, splicing and polyadenylation should be tightly regulated for proper gene expression. Yet, a single gene can produce multiple mRNA and protein isoforms by the combination of alternative promoters, alternative splicing and alternative polyadenylation.[2-4] A variety of *cis*-regulatory elements and tissue-specific *trans*-acting factors involved in the regulation of alternative splicing have been identified in higher eukaryotes by biochemical and bioinformatic approaches.[5-7]

In *C. elegans*, it is now estimated by a recent genome-wide analysis that up to 25% of the protein-coding genes undergo alternative splicing.[8] Mutations mapped to isoform-specific exons or isoform-specific rescue experiments suggested isoform-specific functions for some genes.[9-12] Some splicing regulators have been identified by their genetic interactions with mutations in other genes.[13-22] But the tissue-specific splicing regulation was not subjected to genetic screening probably because specific morphological or behavioral phenotypes were not expected.[23-25] The fluorescence alternative splicing reporter system, which is based on transgenic expression of multiple fluorescent proteins according to alternative pre-mRNA splicing patterns of reporter minigenes carrying genomic fragments of interest, has successfully visualized a variety of tissue-specific and developmentally regulated alternative splicing patterns in vivo and enabled forward and reverse genetic screenings of

the regulators required for the splicing regulation.[26-32] Here I discuss the findings regarding the muscle-specific regulation of the *unc-60* pre-mRNA processing by ASD-2 and SUP-12.[30]

Structure of the *unc-60* Gene

The *unc-60* gene, generating two mRNA isoforms UNC-60A and UNC-60B, has a common first exon followed by two separate series of downstream exons, 2A through 5A for UNC-60A and 2B through 5B for UNC-60B (**Fig. 1A**).[33] The unique structure of the *unc-60* gene suggested that the choice between exons 2A-5A and exons 2B-5B in combination with alternative polyadenylation at the 3' end of exon 5A or exon 5B determine the fate of the *unc-60* pre-mRNA. The structure of the gene also suggested that the splice donor site for exon 1 should be preserved during the course of transcription until the splice acceptor site for exon 2B is available for splicing in situations where the UNC-60B mRNA is generated.

The UNC-60A and UNC-60B mRNAs share only the initiation codon harbored in the common first exon. Yet the UNC-60A and UNC-60B proteins share 38% amino acid sequence identity.[33] The UNC-60 isoforms have distinct biochemical properties in the regulation of actin dynamics[34,35] and different in vivo functions during development and in muscle organization.[36,37] Immunohistochemical staining demonstrated that UNC-60A is predominantly expressed in non-muscle tissues, while UNC-60B is mainly detected in body wall muscles,[36-38] suggesting that alternative processing of the *unc-60* pre-mRNA is regulated in a tissue-specific manner.

Visualization of the Tissue-Specific Alternative Pre-mRNA Processing Patterns of the *unc-60* Gene

In order to visualize the binary processing patterns of the *unc-60* transcript in vivo, a pair of reporter minigenes was constructed (see **Fig. 1B** for details). The unique property of the *unc-60* reporter is that the minigenes are asymmetric, i.e.,

the two minigenes carry distinct portions from the *unc-60* gene. The bichromatic and trichromatic reporter minigenes utilized in other studies for visualizing in vivo selection patterns of mutually exclusive exons or cassette exons were symmetric pairs,[28,31,32] a symmetric trio[31] or a single construct.[26] Because the UNC-60A- and UNC-60B-type mRNAs end at the distinct exons, the symmetric-pair type was not applicable and the asymmetric pair was designed. The *unc-60E1-E2A-RFP* cassette (**Fig. 1B**, top panel), carrying the short genomic fragment spanning from exon 1 through exon 2A, was designed to focus on the excision of the only intron between exon 1 and exon 2A (hereafter referred to as intron 1A) via expression of RFP-fusion protein (UNC-60A-RFP) based on the assumption that intron 1A should be retained in situations where UNC-60B is expressed. On the other hand, the *unc-60E1-E3B-GFP* cassette (**Fig. 1B**, bottom panel) was designed to monitor the UNC-60B-type processing via expression of GFP-fusion protein (UNC-60B-GFP).

When the *unc-60* reporter minigene pair was expressed under the control of the *unc-51* promoter, UNC-60A-RFP and UNC-60B-GFP were expressed in a mutually exclusive manner; UNC-60A-RFP was expressed in non-muscle tissues such as the nervous system, intestine and hypodermis, while UNC-60B-GFP was expressed in body wall muscles and pharyngeal muscles (**Fig. 1C and D**). The expression pattern indicated that the *unc-60* reporter pre-mRNAs were processed in a tissue-specific manner that is consistent with the previous results of immunohistochemical staining of the endogenous UNC-60 proteins.[36-38]

The *unc-60* reporter driven under the control of the *myo-3* promoter (**Fig. 1E**) was utilized for further genetic analyses of the muscle-specific processing regulation. Reverse genetic screening of candidate RNA-binding proteins revealed that muscle-specific RNA-binding proteins ASD-2 and SUP-12 are required for the expression of UNC-60B-GFP in body wall muscles.[30] Directed mutagenesis of short stretches in the pairs of the reporter minigenes revealed CUAAC repeats and a UGUGUG stretch residing near the

putative branch site for intron 1A as the crucial *cis*-elements for the UNC-60B-type processing in body wall muscles (**Fig. 1E–F**). Electrophoretic mobility shift assays (EMSAs) revealed that ASD-2 and SUP-12 specifically recognize the CUAAC repeats and the UGUGUG stretch, respectively, to cooperatively bind to intron 1A.[30] Thus, once the alternative processing patterns of the gene of interest is successfully visualized in vivo with the fluorescence reporter system, the standard genetic tools can be applied to elucidate the regulatory mechanisms in *C. elegans*.[29]

Models of the Muscle-Specific Switching of the *unc-60* Pre-mRNA Processing Patterns by ASD-2 and SUP-12

Figure 2 illustrates the models of the tissue-specific pre-mRNA processing of the *unc-60* gene. In non-muscle tissues (**Fig. 2**, top), the introns are excised during or after transcription and the UNC-60A mRNA is produced. The order of removal of the four introns does not appear to be strictly regulated.[30] In muscles (**Fig. 2**, bottom), ASD-2 and SUP-12 cooperatively bind to the CUAAC repeats and the UGUGUG stretch, respectively, in intron 1A to repress its excision during the transcription and processing of the UNC-60A region. Intron 3A and intron 4A appear to be immediately removed upon transcription.[30] When exon 2B is transcribed and become available, the preserved donor site for exon 1 is readily spliced to exon 2B and the pre-mRNA is committed to the UNC-60B isoform.

The orders of the intron excision in the models presented here and described in more detail in ref. 30 are suggested by comparison of the partially-spliced RNA species from the endogenous *unc-60* gene in N2 and the *sup-12* mutant. The detected partially-spliced RNAs in the steady-state may not necessarily be the processing intermediates for the mature mRNAs but instead be dead-end products of aberrant processing. Yet, the differences in the repertoires of the partially-spliced *unc-60* RNAs were consistent with the differences in the amounts of the mature UNC-60 mRNAs between the wild type and the *sup-12* mutant,[30] suggesting that

the RNA species are the actual processing intermediates.

Repression of the Acceptor Site for Exon 2A is the Key Event to Switch the Processing Patterns of the Entire Pre-mRNA

Expression of the UNC-60A protein instead of UNC-60B in body wall muscles in the *asd-2* and *sup-12* mutants has been demonstrated by immunohistochemical staining and suppression of the uncoordinated (Unc) phenotype of the *unc-60B*-specific mutant.[19,30] The findings indicated that ASD-2 and SUP-12 switch the processing patterns of the entire *unc-60* pre-mRNA from UNC-60A-type to UNC-60B-type in body wall muscles. This raises a question about whether ASD-2 and SUP-12 repress excision of only intron 1A as demonstrated by the *unc-60E1-E2A-RFP* reporter shown in **Figure 1B** (top) or they also bind to other unspecified site(s) and repress splicing of exons 3A, 4A and/or 5A to inhibit aberrant splicing between exon 1 and these exons. Disruption of the CUAAC repeats (*M1*) or the UGUGUG stretch (*M2*) in intron 1A of the *unc-60E1-E3B-GFP* reporter led to proper expression of the mature full-length UNC-60A mRNA (**Fig. 1B**, bottom, **Fig. 1E**), indicating that splicing of exons 3A, 4A and 5A is unaffected in body wall muscles in the wild-type background. Therefore, the repression of the acceptor site for exon 2A via the CUAAC repeats and the UGUGUG stretch in intron 1A is the crucial event to switch the processing patterns of the entire *unc-60E1-E3B-GFP* cassette from UNC-60A-type to UNC-60B-type as shown in **Figure 2**.

Does Polyadenylation at Exon 5A Need to be Repressed in Muscles?

The remaining question is whether proper regulation of the alternative polyadenylation at exon 5A is crucial for the UNC-60B-type pre-mRNA processing; we do not have a conclusive answer.

If the *unc-60* pre-mRNA is cleaved at the 3' end of exon 5A before the acceptor site for exon 2B is committed to splicing, exon 1 would not be spliced to exon 2B. Although it is likely that ASD-2, SUP-12 or other muscle-specific factors may play roles in repressing the polyadenylation, the muscle-specific repression of the cleavage at exon 5A might be unnecessary considering the following situations. The polyadenylation signal (PAS) for exon 5A is not the canonical motif AAUAAA but appears to be a variant PAS; the cleavage and polyadenylation site is just about 90-nucleotide upstream of the acceptor site for exon 2B;[39] exon 2B appears to be readily spliced to exon 1 when available.[30]

If the cleavage and polyadenylation at exon 5A is tightly repressed specifically in muscles, the mature mRNAs produced in muscles of the *asd-2* and *sup-12* mutants would include all the exons, i.e., exons 1 through 5A and 2B through 5B, where the region from exon 5A through exon 2B behaves as one exon because there left no available donor site in this region. This type of mRNAs may be retained in the nucleus due to the potential acceptor site for exon 2B or may be rapidly degraded by nonsense-mediated mRNA decay (NMD) due to its long 3' untranslated region (UTR). However, such an mRNA isoform was not detected even in the NMD-deficient *smg-2; sup-12* double mutant (unpublished observation), suggesting that exon 5A is consequently cleaved and polyadenylated even in muscles of the *sup-12* mutant.

Cooperative Regulation of the *unc-60* Pre-mRNA Processing by ASD-2 and SUP-12

The *asd-2* gene has two tissue-specific promoters and therefore has two distinct first exons. Transcriptional fusion reporters revealed that transcription from exon 1a and exon 1b is driven in hypodermis and pharynx and in body wall muscles and pharynx, respectively.[28] The ASD-2a and ASD-2b protein isoforms share most of the amino acid sequences including the evolutionarily conserved signal transduction and activation of RNA (STAR) domain.[28] Ectopic expression of either ASD-2a or ASD-2b turned the *let-2* alternative splicing reporter expression,[28] indicating that both ASD-2a and ASD-2b are capable of regulating alternative splicing. Western

blotting and immunohistochemical staining of the wild-type and *asd-2b*-specific allele of the *asd-2* mutants revealed that ASD-2b is the major isoform in *C. elegans* and is specifically localized to the nuclei of body wall muscles.[30]

The mammalian ortholog of ASD-2, QKI, has been shown to form a homodimer[40] and recognize a bipartite consensus sequence NACUAAY-N$_{1-20}$-UAAY.[41] The *Drosophila* ortholog HOW also forms a homodimer, which is enhanced upon phosphorylation.[42] Considering the evolutionarily conserved amino acid sequences of the STAR domain that mediates the dimerization and RNA-binding[28,42] as well as the conservation of the CUAAC repeats in intron 1A of the *unc-60* gene in the genus *Caenorhabditis*,[30] it is reasonable to suggest that ASD-2b also forms a homodimer when binding to the CUAAC repeats (**Fig. 1F and 2**, bottom).

SUP-12 represses the acceptor sites for *unc-60* exon 2A[30] and *egl-15* exon 5B[27] in a muscle-specific manner. In these cases, the presence of SUP-12 alone is not sufficient for repressing the splice sites but the partner regulators ASD-2b and the RBFOX family, respectively, are required for the proper regulation.[27,30] Although SUP-12 has been shown to preferentially recognize the (U)GUGUG stretch in these cases,[27,30] EMSAs revealed that SUP-12 can also bind to other unspecified site(s) in the *unc-60* intron 1A probe.[30] Therefore, the cooperation with the partner proteins is crucial for SUP-12 to specifically and efficiently regulate its target pre-mRNAs.

The full-length ASD-2b protein and the full-length SUP-12 protein cooperatively bind to *unc-60* intron 1A.[30] However, the STAR domain of ASD-2 and the RNA recognition motif (RRM) domain of SUP-12 did not exhibit cooperativity although they can specifically recognize the CUAAC repeats and the UGUGUG stretch, respectively, like the full-length proteins (unpublished observation). Considering that full-length ASD-2b and full-length SUP-12 can interact even in the absence of the target RNA,[30] the portions other than the RNA-binding domains may contribute to the protein-protein interaction required for the cooperativity. Similarly, full-length SUP-12 can interact
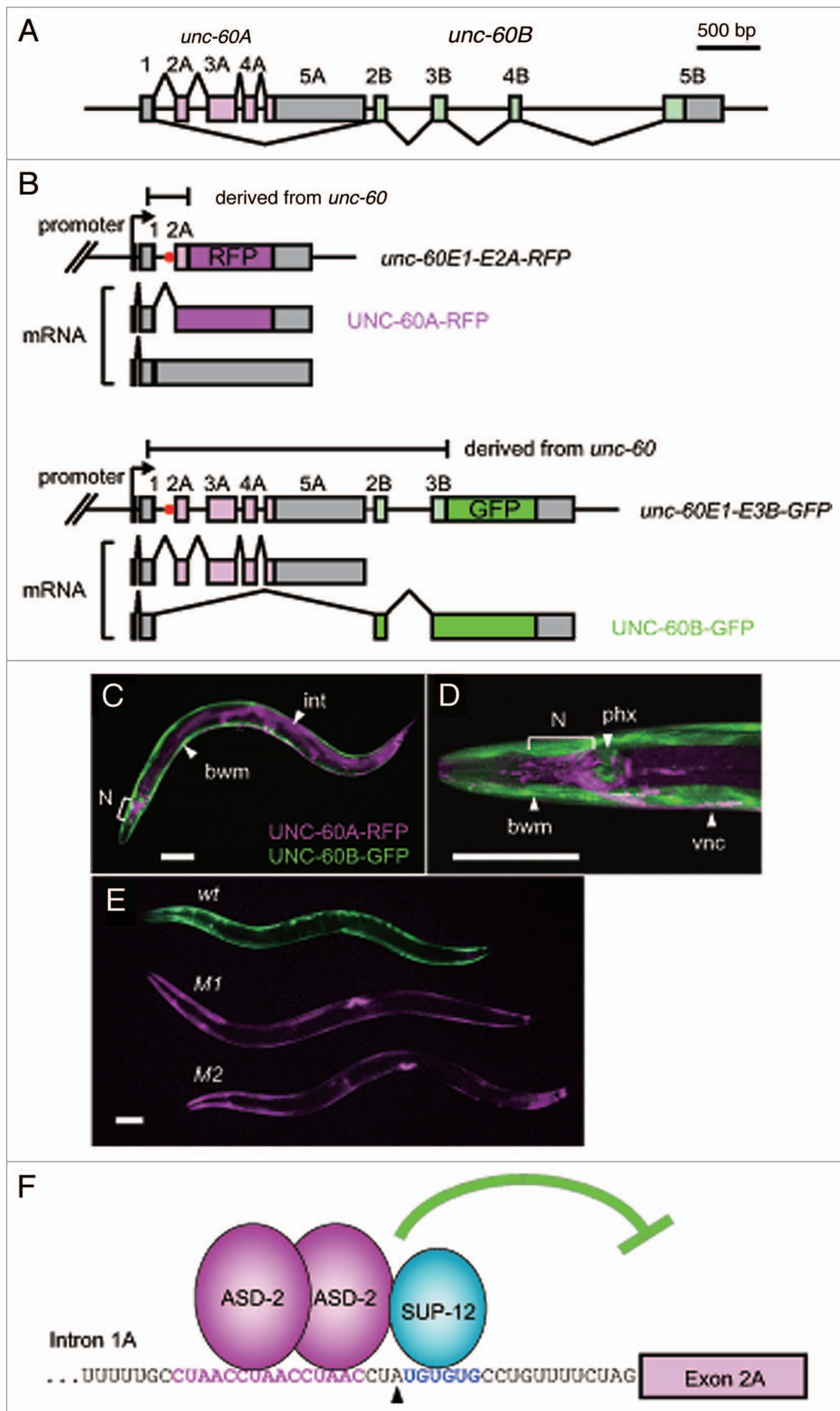
Figure 1. For figure legend, see page 5.

with full-length ASD-1 and FOX-1 in the absence of the target RNA[27] and full-length SUP-12 and full-length ASD-1 or FOX-1 cooperatively bind to *egl-15* intron 4.[27] In contrast to the highly conserved RNA-binding domains, the N- and C-terminal portions of ASD-2, SUP-12, ASD-1 or FOX-1 are not conserved in the amino acid sequences among the orthologs from vertebrates, insects and nematodes[19,28,43] but share similarities in the amino acid composition; they are rich in alanine (A) and glutamine (Q) residues (**Fig. 3**). As these A/Q-rich regions are hydrophobic, they may contribute to the specific protein-protein interaction in a certain or any combination in the cooperative recognition of the target RNAs.

Pre-mRNA Processing of the Cholinergic Gene Locus

Another example of a locus with a structure similar to the *unc-60* gene is the cholinergic gene locus consisting of *unc-17*, encoding vesicular acetylcholine transporter (VAChT), and *cha-1*, encoding choline acetyltransferase (ChAT).[44] The two genes share a common 5' untranslated exon, and the other three exons of the *unc-17* gene reside in the first intron of the *cha-1* gene.[44] The structure of the locus suggests the switch-like regulation of the pre-mRNA processing like the *unc-60* gene. Interestingly, the products of the two genes function in sequential steps in the metabolism of the neurotransmitter acetylcholine. In contrast to the *unc-60* gene that produce the UNC-60A and UNC-60B isoforms in distinct tissues in a mutually exclusive manner, both the UNC-17 and CHA-1 mRNAs and
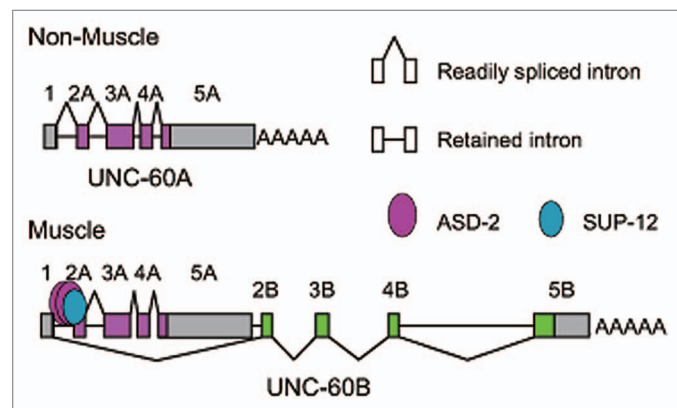

Figure 2. Models of the alternative processing of the *unc-60* pre-mRNAs in non-muscle tissues (top) and muscles (bottom). See the main text and ref. 30 for details.
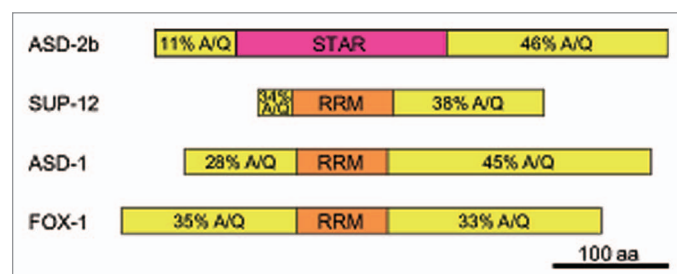
Figure 3. The N-terminal and C-terminal portions of ASD-2b, SUP-12, ASD-1 and FOX-1 are rich in the alanine and glutamine residues. The protein structures are schematically shown with boxes. The STAR domain and the RRM domains are colored in magenta and orange, respectively. The total contents of the alanine (A) and glutamine (Q) residues in the N-terminal and C-terminal portions of each protein are indicated.

proteins should be produced in the same cholinergic neurons. Therefore, not only the binary pre-mRNA processing patterns but the ratio of the amounts of the mature mRNAs should be somehow properly regulated for the cholinergic gene locus.

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