STAR family RNA-binding protein ASD-2 regulates developmental switching of mutually exclusive alternative splicing in vivo

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Alternative splicing of pre-mRNAs greatly contributes to the spatiotemporal diversity of gene expression in metazoans. However, the molecular basis of developmental regulation and the precise sequence of alternative pre-mRNA processing in vivo are poorly understood. In the present study, we focus on the developmental switching of the mutually exclusive alternative splicing of the *let-2* gene of *Caenorhabditis elegans* from the exon 9 form in embryos to the exon 10 form in adults. By visualizing the usage of the *let-2* mutually exclusive exons through differential expression of green fluorescent protein (GFP) and red fluorescent protein (RFP), we isolated several switching-defective mutants and identified the *alternative splicing defective-2* (*asd-2*) gene, encoding a novel member of the evolutionarily conserved STAR (signal transduction activators of RNA) family of RNA-binding proteins. Comparison of the amounts of partially spliced *let-2* RNAs in synchronized wild-type and *asd-2* mutant worms suggested that either of the introns downstream from the *let-2* mutually exclusive exons is removed prior to the removal of the upstream ones, and that *asd-2* promotes biased excision of intron 10 in the late larval stages. We propose that the developmental switching between alternative sequences of intron removal determines the ratio between the mature *let-2* mRNA isoforms.

Alternative pre-mRNA splicing is a crucial step of gene expression and provides proteome diversity from a limited number of genes in metazoan. Recent global studies have demonstrated that as many as two thirds of human genes have multiple alternative isoforms of mature mRNAs (Modrek and Lee 2002; Johnson et al. 2003; Blencowe 2006), and that deregulation of alternative splicing may cause hereditary diseases and cancer (Faustino and Cooper 2003; Venables 2004; Licatalosi and Darnell 2006). Many alternative splicing events are controlled in tissue- and cell-type-dependent manners (Black 2003; Matlin et al. 2005), and a variety of auxiliary *trans*-acting factors and *cis*-acting elements involved in the regulation of alternative splicing have been identified through biochemical, genetic, and bioinformatic analyses.

Mutually exclusive alternative splicing is a strictly regulated form of alternative splicing; only one out of two or more candidate exons are used in the mature mRNA isoform (Smith 2005). This type of alternative splicing raises two main questions; what ensures selection of only one out of multiple exons at one time, and how the selection of the exons is switched. The most extreme example of the mutually exclusive alternative splicing is in the *Dscam* gene of *Drosophila*. It has multiple clusters of variable exons and potentially produces >38,000 different mRNA and protein isoforms (Schmucker et al. 2000). Graveley raised a model that interaction between the only one docking site and one of multiple selector sequences that reside in the introns upstream of the variable exons allows only one variable exon out of 48 alternative exons in the exon 6 cluster to be selected (Graveley 2005). It has been demonstrated that mutually exclusive selection of two exons of the α-tropomyosin and the α-actinin genes in vertebrates is enforced by the absolute incompatibility of the two adjacent exons due to the close proximity of the branchpoint(s) for the downstream exon to the donor site of the upstream exon (Smith and Nadal-Ginard 1989; Southby

et al. 1999). The mutually exclusive selection of duplicated exons in the human *JNK1* gene is considered to be ensured by the incompatibility between the U2-type major intron and the U12-type minor intron (Letunic et al. 2002). Mutually exclusive exons may not necessarily be strictly regulated, but nonsense-mediated mRNA decay (NMD) may contribute to the apparent fidelity of the mutually exclusive selection when neither of the exons is a multiple of three nucleotides (Jones et al. 2001b).

Mutually exclusive alternative splicing is often regulated in a tissue-specific manner. Several *trans*-acting factors and multiple *cis*-elements have been demonstrated to regulate the mutually exclusive exons of the FGFR2, α-actinin, and α- and β-tropomyosin genes in the cultured cells (Chen et al. 1999; Jones et al. 2001a; Muh et al. 2002; Baraniak et al. 2003, 2006; Gromak et al. 2003a,b; Expert-Bezancon et al. 2004; Lin and Tarn 2005; Wagner et al. 2005; Crawford and Patton 2006). However, the mechanisms of the tissue-specific mutually exclusive alternative splicing are to be evaluated in vivo.

To pave the way to identify regulators of alternative splicing in living organisms, we recently developed a transgenic reporter worm system that visualizes the tissue-specific expression profiles of each of the mutually exclusive exons through differential expression of green fluorescent protein (GFP) and red fluorescent protein (RFP) (Kuroyanagi et al. 2006). With this system, we demonstrated that the evolutionarily conserved Fox-1 subfamily of RNA-binding proteins ASD-1 (for Alterna-

tive Splicing Defective-1) and FOX-1 regulate tissue-specific alternative splicing of the fibroblast growth factor (FGF) receptor gene in *Caenorhabditis elegans* (Kuroyanagi et al. 2006). This work provided an example of tissue-specific regulation of mutually exclusive exons in vivo, and provided evidence that the Fox-1-mediated regulation of alternative splicing (Jin et al. 2003) is conserved between vertebrates and nematodes.

The *let-2* gene, encoding α2 (IV) collagen of *C. elegans*, has a unique property in that selection of its mutually exclusive exon 9 and exon 10 in body wall muscles undergoes dramatic switching along with the larval development (see Fig. 1A); in embryos, an mRNA isoform with exon 9 is exclusively expressed, while in late larval and adult stages, an mRNA isoform with exon 10 predominates (Sibley et al. 1993; Graham et al. 1997). This developmental regulation of *let-2* alternative splicing is evolutionarily conserved in two distantly separated nematodes, *C. elegans* and *Ascaris* (Sibley et al. 1993; Pettitt and Kingston 1994), and it is speculated that switching of exon 9 and exon 10 alters the characteristics of basement membranes during nematode development. In the present study, we applied the transgenic alternative splicing reporter system to analyze the developmentally regulated switching mechanism of the mutually exclusive exons of the *let-2* gene, and identified a novel member of the highly conserved STAR (signal transduction activators of RNA) family RNA-binding proteins, ASD-2 (for Alternative Splicing Defective-2), as a regulator of the *let-2* alternative splicing.
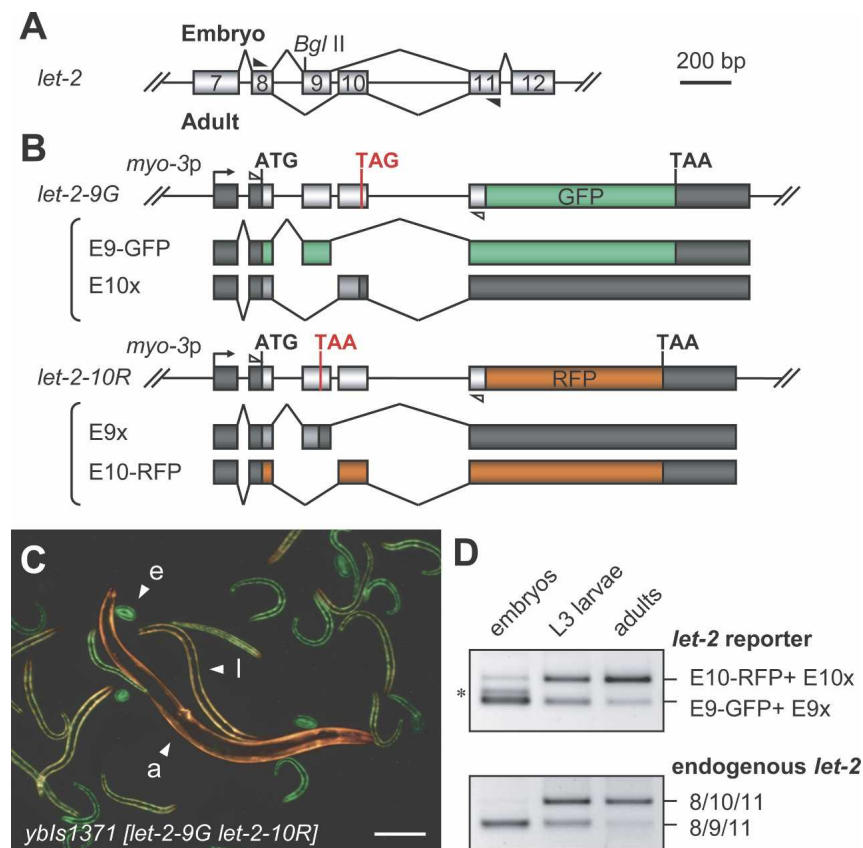

Figure 1. Visualization of the developmentally regulated mutually exclusive alternative splicing patterns of the *let-2* gene. (*A*) Schematic view of the alternatively spliced regions of the endogenous *let-2* gene. Boxes indicate exons. Closed triangles indicate positions and directions of the PCR primers used to amplify the cDNA fragments from the endogenous *let-2* mRNAs in *D* and Figure 2B. (*B*) Structure of a pair of the *let-2* reporter minigenes, *let-2*-9G and *let-2*-10R, and mRNAs derived from the minigenes. Predicted ORFs are colored in green (E9-GFP), red (E10-GFP), or gray (E10x and E9x). Open triangles indicate positions and directions of the PCR primers used to amplify the cDNAs derived from the *let-2* reporter mRNAs in *D* and Figure 2B. (*C*) A microphotograph of worms with a transgenic allele *ybIs1371* [*myo-3::let-2*-9G *myo-3::let-2*-10R], expressing the *let-2* reporters under the *myo-3* promoter. Arrowheads indicate an embryo (e), an L3 larva (l), and a young adult (a). Bar, 100 μm. (*D*) RT–PCR analysis of the *let-2* reporter (*top*) and the endogenous *let-2* (*bottom*). Note that BglII digested the PCR products containing exon 9 into halves. An asterisk denotes a nonspecific band.

Results

*Transgenic reporter system visualizes
the developmentally regulated mutually exclusive
alternative splicing of the* let-2 *gene in vivo*

In order to monitor the selection of the *let-2* mutually
exclusive exons (Fig. 1A) in vivo, we constructed a pair of
reporter minigenes, *let-2-9G* and *let-2-10R* (Fig. 1B). The
minigenes carry the same *let-2* genomic DNA fragment
spanning from exon 8 to exon 11 connected in-frame to
cDNAs for fluorescent proteins, and termination codons
are artificially introduced into exon 10 of *let-2-9G* and
exon 9 of *let-2-10R* (Fig. 1B). We expected that, from the
let-2-9G minigene, selection of exon 9 would lead to ex-
pression of an mRNA encoding a GFP fusion protein
(E9-GFP), while selection of exon 10 would result in a
nonproductive mRNA (E10x) due to the termination
codon (Fig. 1B). In the same way, selection of exon 10
would lead to expression of an mRNA encoding an RFP
fusion protein (E10-RFP) and selection of exon 9 would
result in a nonproductive mRNA (E9x) from the *let-2-
10R* minigene (Fig. 1B).

The *let-2* reporter successfully visualized the alterna-
tive exon usage. We drove expression of the *let-2* reporter
minigenes under the body wall muscle-specific *myo-3*
promoter, since the endogenous *let-2* is primarily ex-
pressed in the body wall muscles (Graham et al. 1997).
As expected, expression of the *let-2* reporter in the body
wall muscles gradually and almost completely switched
from GFP to RFP along with the development; embryos
exclusively express E9-GFP and elder worms express
E10-RFP (Fig. 1C). RT–PCR analyses of mRNA isoforms
derived from the minigenes confirmed that the alterna-
tive exons are selected mutually exclusively to produce
the E9-GFP and E10x mRNA isoforms from the *let-2-9G*
minigene, and the E9x and E10-RFP isoforms from the
let-2-10R minigene (data not shown). Consistent with
the color switch of the *let-2* reporter worms, the compo-
sition of the reporter mRNAs gradually switched from
the exon 9 forms to the exon 10 forms in parallel with
that of the endogenous *let-2* mRNAs along with the de-
velopmental stage (Fig. 1D). These results indicate that
the *let-2* reporter visualizes the developmental switch-
ing of the *let-2* mutually exclusive exons in vivo.

asd-2 *mutants are defective in the developmental
switching of alternative splicing of both the* let-2
reporter and the endogenous let-2 *gene*

To identify factors regulating the *let-2* alternative splic-
ing in vivo, we screened for mutants defective in the
developmental switching of the *let-2* alternative splicing
reporter expression in the body wall muscles. We muta-
genized the transgenic reporter allele *ybIs1371* (Fig. 1C)
and screened ~5 × 10^4 genomes for F2 progeny that re-
main green until adulthood or that turn red as younger
larvae. We isolated several independent mutant alleles,
all of which remain green during larval development but
eventually turn red in the senile stage (Fig. 2A; data not

shown). RT–PCR analysis of the *let-2* reporter confirmed
that the mutants are defective in the switching to the
exon 10 forms in the late larval stage (Fig. 2B, top panel)
in parallel with the defect in the color switch. Further-
more, the mutants are also defective in the exon switch-
ing of the endogenous *let-2* mRNAs (Fig. 2B, bottom
panel), indicating that the *let-2* reporter reflected the ex-
pression of the endogenous *let-2* gene and allowed the
isolation of alternative splicing defective mutants.

Single nucleotide polymorphism (SNP)-based chromo-
some mapping and genomic DNA sequencing suggested
that the mutants are alleles of a single uncharacterized
gene, T21G5.5 (see below). RNAi of the gene reproduced
the color phenotype (Fig. 2C), confirming that the defect
in the switching of the *let-2* reporter expression resulted
from loss or reduction of function of the gene. We there-
fore named it *alternative splicing defective-2* (*asd-2*).
Figure 2D represents a schematic structure of the *asd-2*
gene and summarizes the mutations identified in the
asd-2 mutant alleles. Expressed sequence tag (EST)
analysis has predicted that the *asd-2* gene produces two
protein isoforms, ASD-2a and ASD-2b, that differ in the
N terminus sequence due to alternative use of the first
exons (Fig. 2D), and we confirmed expression of both of
the isoforms by cloning RT–PCR products (deposited to
GenBank under accession nos. EF630625 and EF630626).

*ASD-2 is a novel member of the evolutionarily
conserved STAR family*

ASD-2 belongs to the evolutionarily conserved STAR
family of RNA-binding proteins (Vernet and Artzt 1997).
The family members share a highly conserved tripartite
GSG (for GRP33, Sam68, GLD-1)/STAR domain (Jones
and Schedl 1995; Vernet and Artzt 1997) composed of a
single KH-type RNA-binding domain and two flanking
domains called QUA1 and QUA2 (Fig. 3A). QUA1 is re-
quired and sufficient for homodimer formation of *C. el-
egans* GLD-1 and mouse QKI (Chen and Richard 1998;
Ryder et al. 2004). QUA2 is involved in the RNA binding
together with the KH domain for GLD-1 and human SF1
(Liu et al. 2001; Ryder et al. 2004; Lehmann-Blount and
Williamson 2005). Figure 3B shows the phylogenetic tree
of the GSG/STAR domains of the STAR family proteins
from *C. elegans* and mammals and *Drosophila* Held Out
Wings (HOW). ASD-2 GSG/STAR domain has 68%,
66%, and 74% identity with those of GLD-1, QKI, and
HOW, respectively, while the ASD-2 KH-QUA2 do-
mains have only 37% identity to those of human SF1.
These sequence analyses indicated that ASD-2 belongs
to the GLD-1 subfamily that includes QKI and HOW.

Mutations identified in the *asd-2* mutants affect con-
served residues in the GSG/STAR domain or the entire
structure of the ASD-2b protein. Five missense muta-
tions were assigned to the conserved residues within the
GSG/STAR domain (Fig. 3A). *yb1422* causes G125E sub-
stitution within the QUA1 domain. *yb1415*, *yb1470*, and
yb1419 cause R154C, H193Y, and L202F substitutions
within the KH RNA-binding domain, respectively.
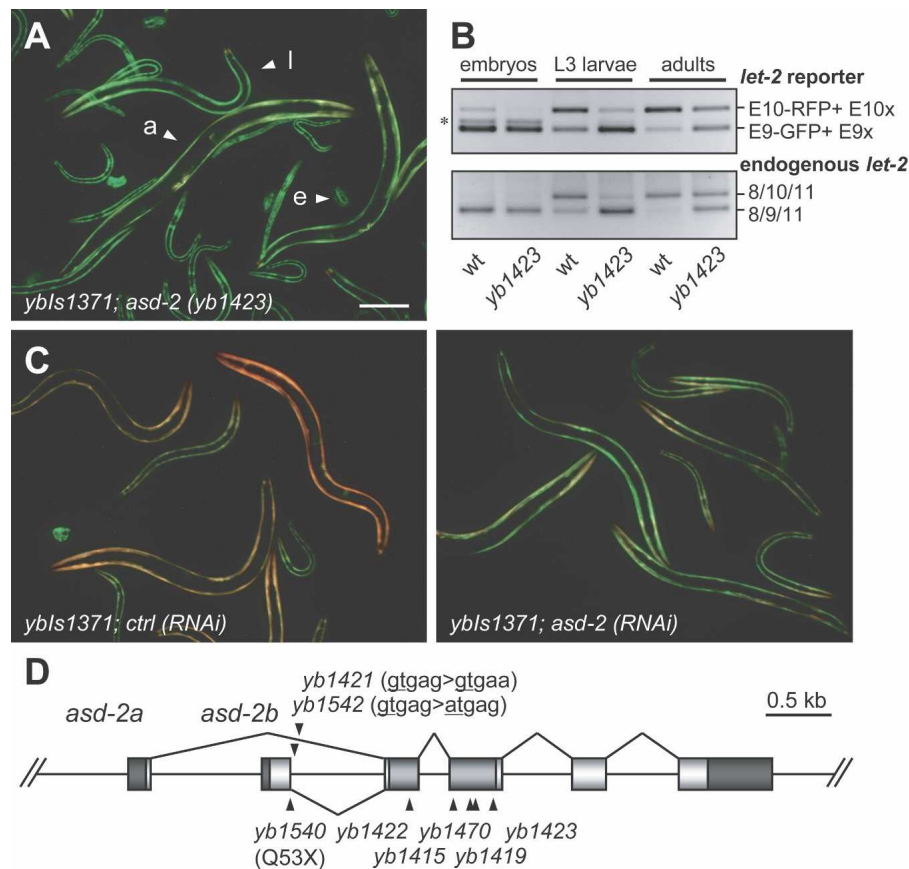*yb1423* causes E244K substitution within the QUA2 do-

Figure 2. The *asd-2* mutants defective in the developmental switching of the *let-2* alternative splicing. (*A*) A microphotograph of a mutant allele, *ybIs1371; asd-2 (yb1423)*. Arrowheads indicate an embryo (e), an L3 larva (l), and a young adult (a). (*B*) RT–PCR analysis of the *let-2* reporter (*top*) and the endogenous *let-2* (*bottom*). The genotypic backgrounds and the stages of the worms are indicated. An asterisk denotes a nonspecific band. (wt) Wild type. (*C*) RNAi of the *asd-2* gene. Microphotographs of the F1 progeny of the *ybIs1371* reporter worms treated with buffer alone (*left* panel) or with *asd-2* dsRNA (*right* panel). Bar in *A*, 100 μm. (*D*) Schematic representation of the *asd-2* gene. Boxes indicate exons. The ORFs are in white. The GSG/STAR domain is in light gray. UTRs are in dark gray. Mutations identified in the mutant alleles are indicated.

main. Putative *asd-2b*-specific null mutations were identified in the other three alleles. *yb1540* causes nonsense mutation in the first exon of the *asd-2b* isoform (Fig. 2D). *yb1421* and *yb1542* have base substitutions at the splice donor site in the first intron of the *asd-2b* isoform (Fig. 2D). These results suggested that the RNA-binding property of the ASD-2b isoform is responsible for the developmental switching of the *let-2* mutually exclusive exons in the body wall muscles.

ASD-2b regulates the switching of the let-2 reporter in the body wall muscles

We then analyzed the expression profiles of the *asd-2a* and *asd-2b* isoforms. We constructed transcriptional fusion minigenes, *asd-2a::GFP* and *asd-2b::RFP* (Fig. 4A). These reporters showed differential tissue-specific expression profiles throughout development (Fig. 4B). Expression of *asd-2a::GFP* was detected in hypodermis and pharyngeal muscles. On the other hand, expression of *asd-2b::RFP* was observed in the body wall muscles and

the pharyngeal muscles, consistent with the idea that *asd-2b* is responsible for the regulation of the *let-2* alternative splicing in the body wall muscles.

Next, we asked whether expression of ASD-2b in the body wall muscle rescues the color phenotype of the *asd-2* mutant. Overexpression of ASD-2b under the body wall muscle-specific *myo-3* promoter overrescued the *asd-2 (yb1423)* mutant, and enforced E10-RFP expression even in early developmental stages (Fig. 4C). Figure 4D summarizes the effects of the *asd-2* expression on the *let-2* reporter. We concluded that the ASD-2b isoform regulates the *let-2* alternative splicing reporter in the body wall muscles.

ASD-2b regulates the switching of the let-2 alternative exons via a short element in intron 10

Then, we searched for a *cis*-acting element for the ASD-2b function in the *let-2* pre-mRNA processing. Kabat et al. (2006) have recently reported that evolutionarily conserved intronic stretches are likely to be involved in the
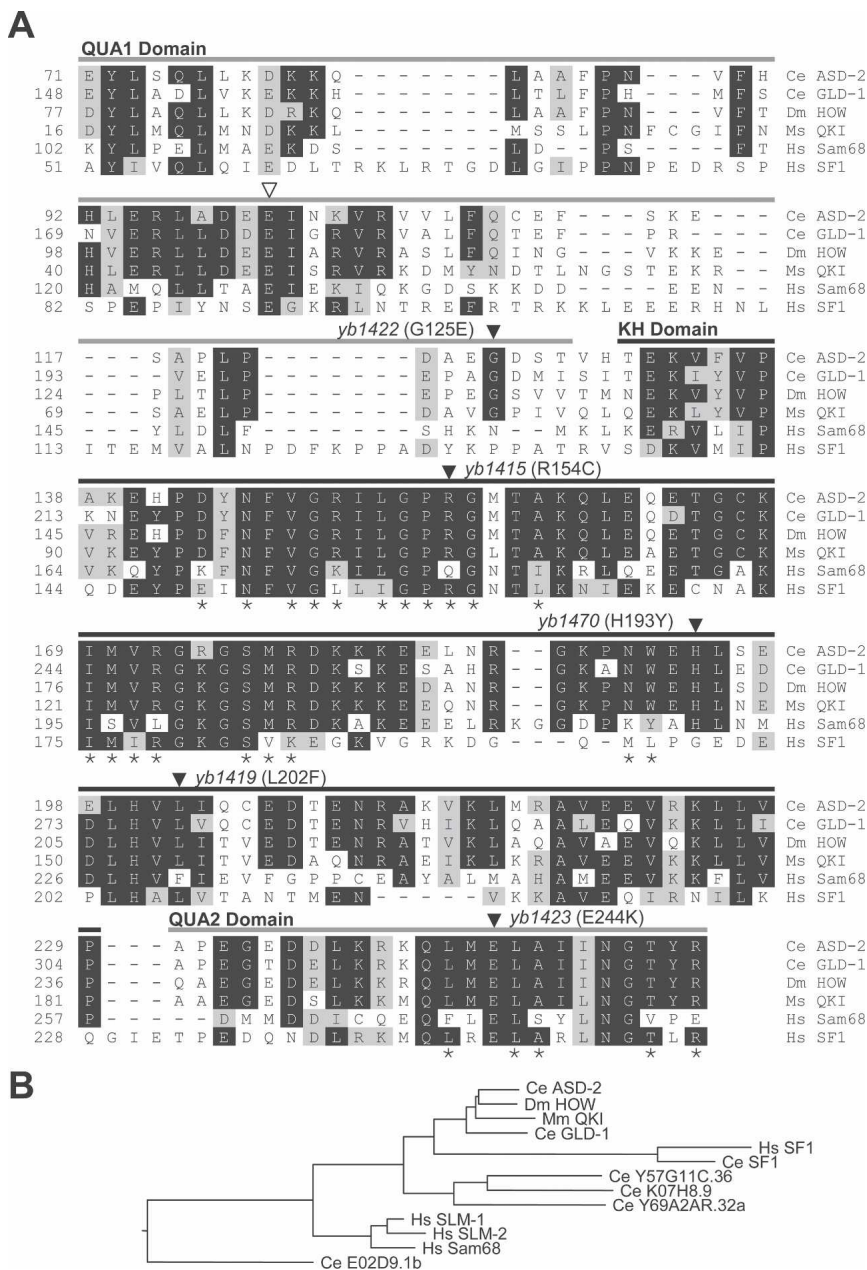
Figure 3. GSG/STAR domain of ASD-2 and other STAR family proteins. (*A*) Amino acid sequence alignment of the GSG/STAR domains. Identical amino acid residues are shaded in black. Amino acid residues with similar properties are shaded in gray. The GSG/STAR domain and the QUA1 and QUA2 domains are indicated with black and gray lines, respectively, *above* the sequence alignment. Closed triangles indicate the positions of the missense mutations identified in *asd-2* mutant alleles. An open triangle indicates the position of the E48 residue of mouse QKI essential for dimerization (Chen and Richard 1998). Asterisks under the sequences indicate the human SF1 amino acid residues contacting the branchpoint RNA 5′-AUACUAACAA-3′ (Liu et al. 2001). (Ce GLD-1) *C. elegans* GLD-1 (Francis et al. 1995); (Dm HOW) *Drosophila* HOW (Zaffran et al. 1997); (Ms QKI) mouse QKI (Ebersole et al. 1996); (Hs Sam68) human Sam68 (Matter et al. 2002); (Hs SF1) human SF1 (Kramer and Utans 1991). (*B*) Phylogenetic tree of the GSG/STAR domains from the STAR family proteins. (Ce) *C. elegans*; (Dm) *Drosophila*; (Ms) mouse; (Hs) human.

regulation of alternative pre-mRNA splicing in nematodes. Furthermore, they demonstrated that deletion of short stretches within *let-2* intron 10 affected the alternative splicing regulation (Kabat et al. 2006). We therefore constructed a pair of modified *let-2* reporter minigenes (M1) with a deletion of a short sequence in intron 10 (Fig. 5A), and observed that the M1 mutant reporter continued expressing E9-GFP in the late larval stages (Fig. 5B). Since the sequence deleted in the M1 minigenes contained a half-site of the QKI response element (QRE, NACUAAY-N$_{1-20}$-UAAY), a consensus sequence of mouse QKI (Galarneau and Richard 2005), we modified two residues in the short stretch, CUAACUCUAAC, in another pair of modified *let-2* reporter minigenes (M2) (Fig. 5A), and found that disruption of the stretch also phenocopied the *asd-2* mutants (Fig. 5C). Furthermore, the M2 mutant reporter no longer responded to the overexpression of the ASD-2b cDNA (Fig. 5D). We also constructed a pair of minigenes (M3) in which a conserved UGCAUG element is disrupted (Fig. 5A), and found that the M3 mutant reporter responded to ASD-2b overexpression (Fig. 5E) as did the wild-type (WT) reporter (Fig. 4C). These results indicated that the QRE-like stretch is essential for ASD-2b to enforce expression of E10-RFP.

To examine whether ASD-2 directly binds to the QRE-like stretch in vitro, we performed an electrophoretic mobility shift assay (EMSA) with RNA probes harboring the intact stretch or the mutation as in the M2 mutant reporter (Fig. 5F). The recombinant protein of the ASD-2 GSG/STAR domain (ASD-2 STAR) shifted the mobility
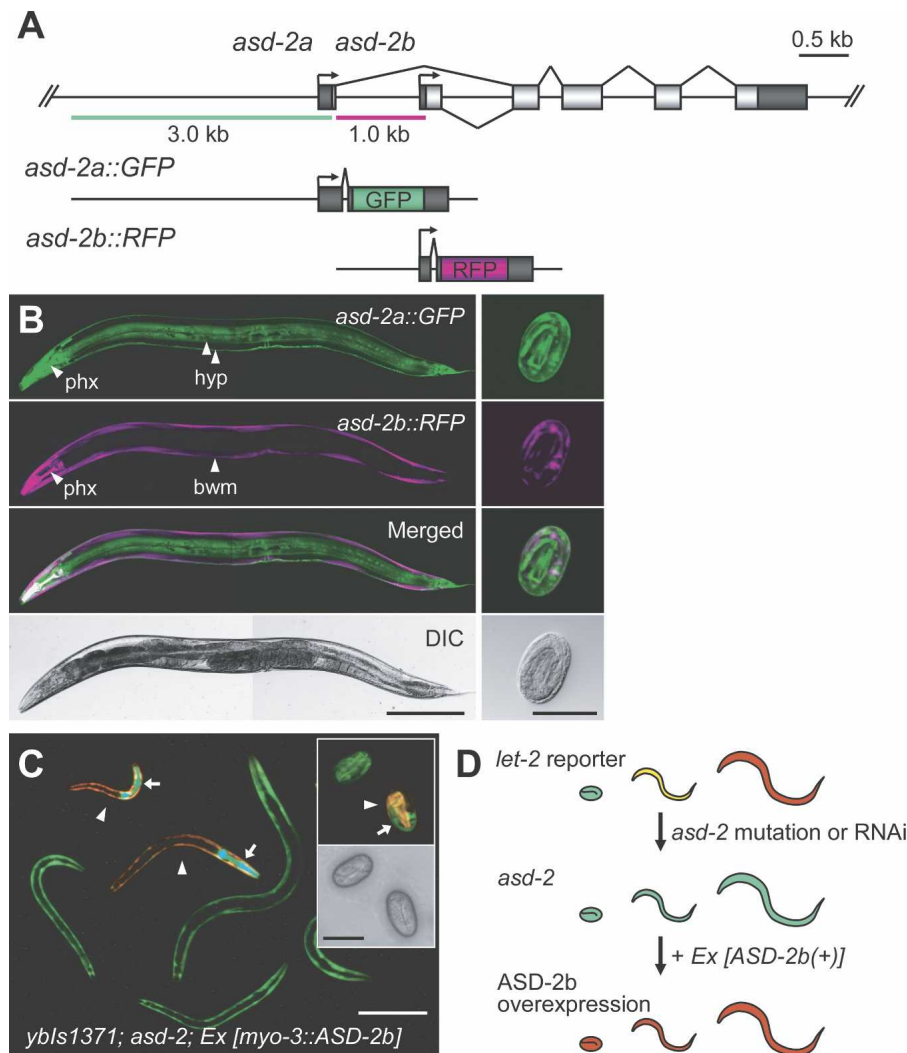
Figure 4. Expression and function of *asd-2b* in the body wall muscles. (*A*) Schematic representation of the *asd-2* transcriptional fusion minigenes *asd-2a*::*GFP* and *asd-2b*::*RFP*. Green and magenta lines indicate the *asd-2a* promoter and the *asd-2b* promoter regions, respectively, used in the constructs. (*B*) Confocal images of the expression patterns of the *asd-2* transcriptional fusions in an adult (*left* column) and a late embryo (*right* column). Expression of *asd-2a*::*GFP* (green) is detected in hypodermis (hyp) and pharyngeal muscles (phx). Expression of *asd-2b*::*RFP* (magenta) is detected in the body wall muscles (bwm) and the pharyngeal muscles (phx). (*Bottom*) A DIC image of the same field. Bars: *left* column, 100 μm; *right* column, 50 μm. (*C*) Microphotograph of the *let-2* reporter allele *ybIs1371* in the *asd-2 (yb1423)* mutant background carrying an extrachromosomal array for ASD-2b expression under the *myo-3* promoter. Arrowheads indicate young larvae and an embryo (shown in *inset*) carrying the array. Arrows indicate the pharynges of transgenic worms expressing GFP from *myo-2*::*GFP*, a coinjected marker of transformation. Bars: 100 μm; *inset*, 50 μm. (*D*) Summary of the effects of *asd-2* expression on the *let-2* reporter.

of the wild-type RNA probe (WT) in a dose-dependent manner (Fig. 5F, lanes 2–6), but not of the M2 mutant probe (Fig. 5F, lanes 8–11), demonstrating the direct and specific binding of ASD-2 to the QRE-like stretch in vitro.

asd-2 promotes removal of let-2 intron 10 in the late larval stages

Next, we examined the processing steps of the endogenous *let-2* pre-mRNA. When we look at exon 8 to exon 11 of the *let-2* pre-mRNA, mutually exclusive inclusion of exon 9 or exon 10 requires sequential removal of two flanking introns. As intron 9 (30 nucleotides) is too short to be excised due to steric interference between the donor and the possible branch site (Smith et al. 1993; Southby et al. 1999), we can expect four introns to be removed in the first splicing, and the subsequent second splicing to produce either of the two mature *let-2* mRNAs (Fig. 6A). To gain insight into the processing course of the endogenous *let-2* pre-mRNA, we tried to detect and compare the amounts of the four possible processing intermediates or partially spliced RNAs (Fig. 6A) in the synchronized wild-type and *asd-2* mutant worms.
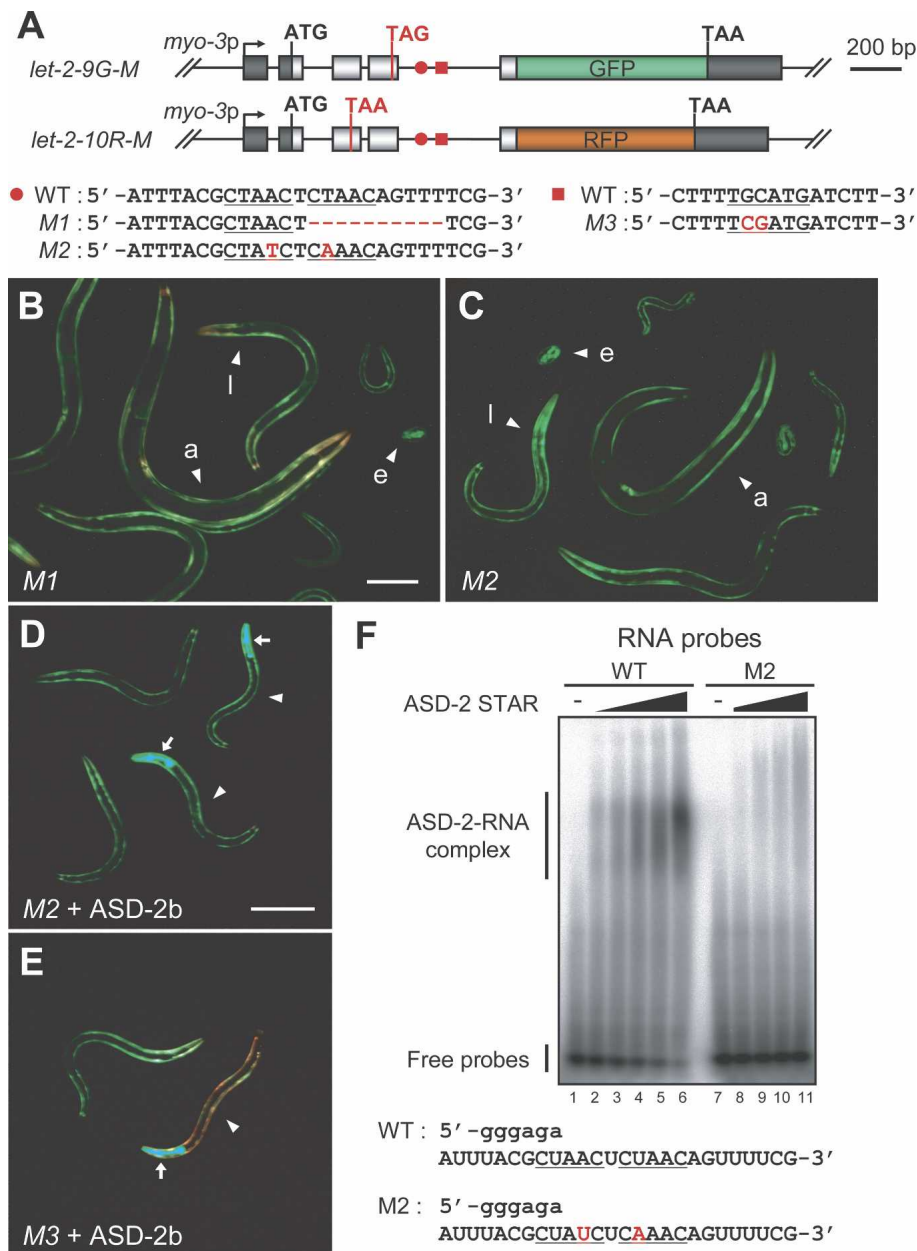
Figure 5. A regulatory element essential for the developmental switching of the *let-2* alternative splicing by ASD-2b. (*A*) Schematic representation (*top*) and sequences (*bottom*) of the modified *let-2* reporter minigenes *M1*, *M2*, and *M3*. A red circle and a red square indicate the positions of the mutation sites for *M1/M2* and *M3*, respectively. Sequences fitting the consensus of QRE and a UGCAUG element are underlined. Mutated sequences are indicated in red. Dashed line indicates deletion. (*B,C*) Transgenic worms expressing a pair of the *let-2-M1* minigenes (*B*) and a pair of the *let-2-M2* minigenes (*C*). Arrowheads indicate an embryo (e), an L3 larva (l), and a young adult (a). Bar in *B*, 100 μm. (*D,E*) Transgenic worms overexpressing ASD-2b in body wall muscles, and carrying a pair of either the *let-2-M2* minigenes (*D*) or the *let-2-M3* minigenes (*E*). Arrowheads indicate young larvae carrying the extrachromosomal array for ASD-2b expression with a pharyngeal GFP marker (arrows). Bar in *D*, 100 μm. (*F*) EMSA. Twofold dilution series of the recombinant ASD-2 STAR protein is used. Sequences of the wild-type and M2 probes are indicated at the *bottom*. The QRE-like sequences are underlined. Mutation sites are indicated in red.

To quantify the amounts of the *let-2* processing intermediates, we prepared two pairs of PCR primers to amplify the complementary DNA fragments for the *let-2* RNAs retaining intron(s). One primer set was designed on exon 8 and intron 10 to amplify partially spliced RNAs in which either of the upstream flanking introns was removed (Fig. 6B, left panel). The primer set amplified the unspliced pre-mRNA (8–9–10–11) and partially spliced RNAs in which intron 8 was excised (8/9–10–11) or the intron between exon 8 and exon 10 was excised (8/10–11). The other primer set was designed on intron 8 and exon 11 to amplify partially spliced RNAs in which
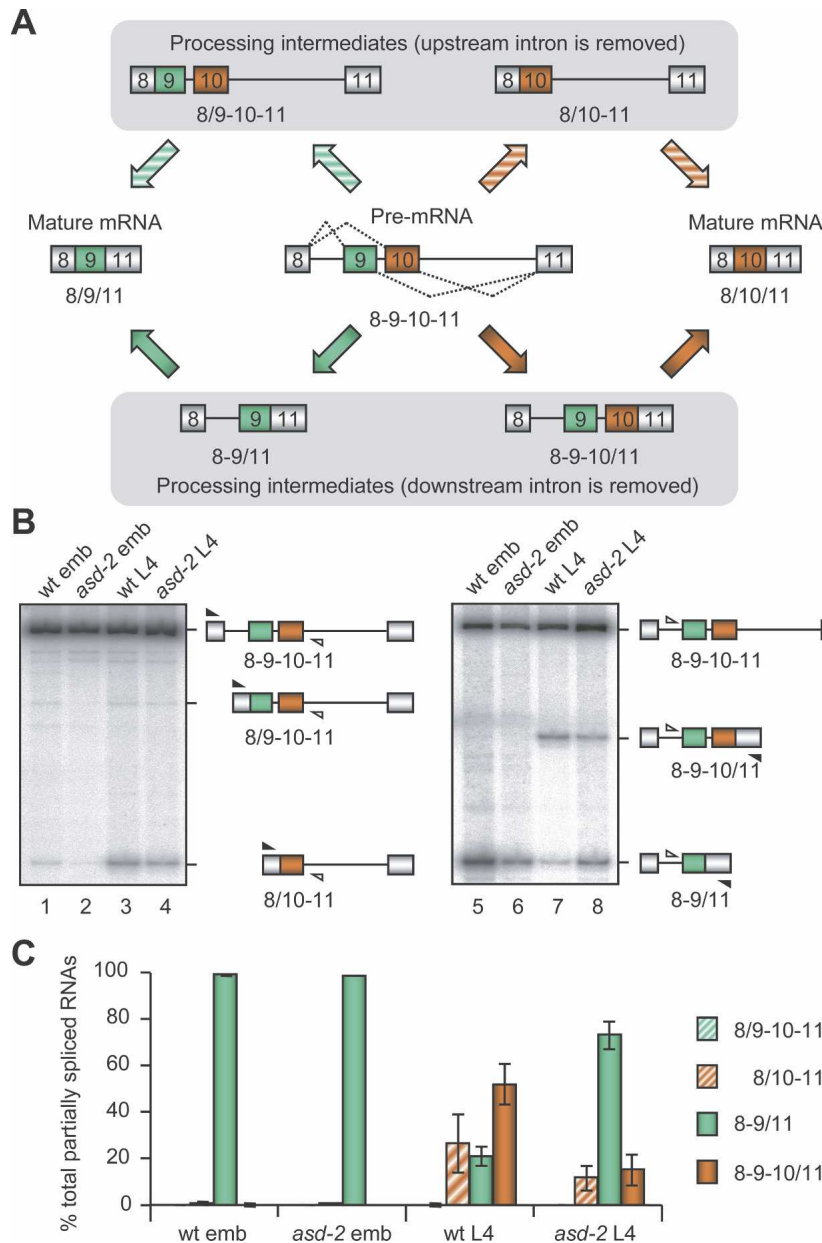
Figure 6. Analysis of partially spliced *let-2* RNAs as the intermediates of the alternative pre-mRNA processing. (*A*) A model of two-step pre-mRNA processing ensuring the *let-2* mutually exclusive alternative splicing. Four possible courses leading to the production of the two mature mRNAs are indicated. One of four introns (dotted lines) flanking the mutually exclusive exons is removed in the first splicing to generate one of the four possible processing intermediates (shaded in gray), and the remaining intron is removed in the second splicing. We assumed that intron 9 is too short to be excised. (*B*) Radioactive RT–PCR detection of the partially spliced *let-2* RNAs in the synchronized embryos (emb) and L4 larvae (L4) of the wild-type (wt) and the *asd-2 (yb1423)* mutant worms. Two pairs of the PCR primers were used to detect the *let-2* RNAs in which either of the upstream introns was removed (*left* panel), and either of the downstream introns was removed (*right* panel). The structure of each PCR product was analyzed by cloning and sequencing, and is schematically indicated. Closed and open triangles indicate the positions of the exonic and the intronic primers, respectively. (*C*) The relative amount of each of the partially spliced RNAs. The amount of each of the RNA species was calculated by normalization to the amount of the unspliced pre-mRNA. $n = 1$ for *asd-2* embryos; $n = 3$ for others. Error bars indicate standard error of mean (SEM).

either of the downstream flanking introns was removed (Fig. 6B, right panel). The primer set amplified the pre-mRNA (8–9–10–11) as well as partially spliced RNAs in which intron 10 was removed (8–9–10/11), or the intron between exon 9 and exon 11 was removed (8–9/11).

The normalized amounts of the four partially spliced RNAs are shown in Figure 6C. In both the wild-type and the *asd-2* mutant embryos, the 8–9/11 form, which is committed to the exon 9 mRNA isoform, was almost exclusively detected (Fig. 6B [lanes 1,2,5,6], C), consistent with the exclusive expression of the exon 9 mRNA in the embryos. In parallel with the developmental switching to the exon 10 mRNA isoform in the wild-type L4 larvae, we observed switching in the major form of the partially spliced RNAs from the 8–9/11 form to

the 8–9–10/11 form, one of two possible intermediates leading to the exon 10 mRNA (Fig. 6B [lane 7], C). On the other hand, the amount of the 8–9/11 form still predominated over that of the 8–9–10/11 form in the L4 larvae of the *asd-2* mutant (Fig. 6B [lane 8], C), consistent with its switching-defective phenotype. These results indicated that *asd-2* is required for selective removal of intron 10 in this stage. We also observed substantial increase in the amount of the 8/10–11 form in the L4 larvae independent of the *asd-2* functions (Fig. 6B [lanes 3,4], C), which may contribute to the fraction of the exon 10 mRNA in the adult stage (Fig. 2B) and the color change of the *let-2* reporter in the *asd-2* senile adults.

The 8–9/11 form, the only intermediate detected in the embryos, will further be processed exclusively into

the exon 9 mRNA (Fig. 6A). However, the 8–9–10/11 form, the major intermediate in the wild-type L4 larvae, has two remaining splice acceptors, for exon 9 and exon 10, and may not necessarily be processed into the exon 10 mRNA. We therefore analyzed the fate of a pre-mRNA from a modified *let-2* reporter minigene that carried only exon 8 to exon 10, and observed a selective use of the exon 10 splice acceptor (Fig. 7A [top panel], B [lane 1]). We reasoned that the selective use of the exon 10 acceptor (TTTTT<u>AG</u>) was due to the relative weakness of the exon 9 splice acceptor (ATTCT<u>AG</u>), since the exon 9 acceptor became predominantly selected (Fig. 7A [bottom panel], B [lane 2]) when the reporter was further modified, so that the exon 9 acceptor matched the consensus of U2AF65 binding for *C. elegans* (TTTTC<u>AG</u>) (Hollins et al. 2005). These results are schematically summarized in Figure 7C.

Discussion

In the present study, we isolated *asd-2* mutants defective in the switching of the developmentally regulated mutually exclusive alternative exons in the *let-2* mRNAs, and compared the amounts of the intermediates of the post-transcriptional *let-2* pre-mRNA processing between the wild-type and the *asd-2* mutant.

Course of mutually exclusive alternative splicing of let-2 pre-mRNA and the switching mechanism by ASD-2

The unique property of the developmental switching and the isolation of the mutants of the *let-2* alternative splicing allowed us to analyze the relative amounts of the partially spliced RNAs (Fig. 6). In the case of Nova-2-regulated cassette exons in mice, Ule et al. (2006) ob-
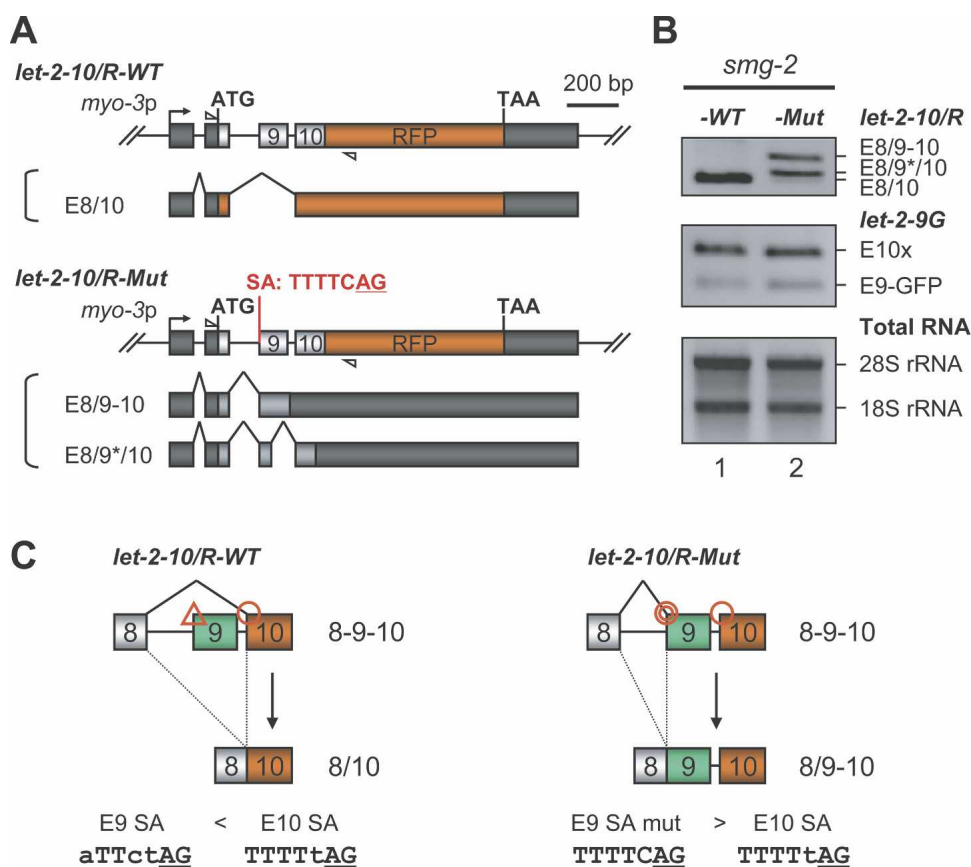

Figure 7. Biased selection between the splice acceptors for exon 9 and exon 10. (*A*) Structure of the *let-2* reporter minigenes *let-2-10/R-WT* (*top*) and *let-2-10/R-Mut* (*bottom*), and mRNAs derived from the minigenes. The *let-2* genomic DNA fragment spanning from exon 8 to exon 10 is connected in-frame to RFP cDNA. The splice acceptor (SA) for exon 9 (aTTct<u>AG</u>) is mutagenized to TTTTC<u>AG</u> in *let-2-10/R-Mut*. Structure of the mRNAs was confirmed by cloning and sequencing the RT–PCR products. Predicted ORFs are colored in red (E8/10-RFP) or gray (E8/9–10 and E8/9*/10). A cryptic donor site within exon 9 is used in the E8/9*/10 form. Open triangles indicate positions and directions of the PCR primers used to amplify the cDNAs in *B*. (*B*) RT–PCR analysis of the mRNAs derived from the *let-2-10/R* minigenes (*top*) and coinjected *let-2-9G* (*middle*). (*Bottom*) Total RNAs prepared from mixed-stage population. (Lane *1*) *let-2-10/R-WT*. (Lane *2*) *let-2-10/R-Mut.* Either of the *let-2-10/R* minigenes was coinjected with *let-2-9G* (Fig. 1B) into the *smg-2 (yb979)* mutant (Kuroyanagi et al. 2007), defective in NMD, to avoid degradation of the nonproductive mRNAs. Note that the E8/10-RFP form was exclusively produced from *let-2-10/R-WT*, while the E8/9–10 and E8/9*/10 forms were preferentially produced from *let-2-10/R-Mut*. (*C*) Schematic models of the biased selection of the exon 10 acceptor in *let-2-10/R-WT* pre-mRNA (*left* panel) and the exon 9 acceptor in *let-2-10/R-Mut* pre-mRNA (*right* panel). Note that the relatively stronger acceptor is selected in each case.

served biased accumulation of a partially spliced RNA in which either of the upstream or the downstream flanking introns was removed in the exon-including condition, either in the wild-type or the Nova-2 knock-out brain. In general, the steady-state amount of the processing intermediate is determined by the rate of production (removal of an intron in the first splicing) and the rate of degradation (mainly the subsequent splicing), and therefore it does not necessarily reflect the rate of production of the mature mRNA isoform produced from the intermediate. Nevertheless, we found a dramatic switching in the major species of the partially spliced *let-2* RNAs during larval development in parallel with the switching from the exon 9 to the exon 10 isoforms of the mature *let-2* mRNA (Fig. 6C), suggesting that the major species of the partially spliced RNAs is the processing intermediate of the major course of the pre-mRNA processing. Furthermore, we observed a defect in the switching in the major species of the partially spliced *let-2* RNAs in L4 larvae of the *asd-2* mutant (Fig. 6C), consistent with the defect of the *asd-2* mutant to produce the exon 10 mRNA. Assuming that *asd-2* mutation does not affect the rate of the second splicing, the result indicates that *asd-2* is required for the production of the major 8–9–10/11 form in the wild-type L4 larvae, and strongly suggests that the 8–9–10/11 form is the intermediate in the course of the *let-2* pre-mRNA processing by which most of the exon 10 mRNA is produced in the later stages.

Figure 8 illustrates a model of the processing courses of the *let-2* pre-mRNA and the mode of switching regulation by ASD-2 raised by the present study. The unique feature of the *let-2* alternative splicing is that either of the two introns downstream from the mutually exclusive exons is removed in the first splicing out of four possible flanking introns (Fig. 6A). In the embryonic stage, or when *asd-2* is inactive (Fig. 8, left panel), the splice donor of exon 9 (Fig. 8, green circle, <u>GT</u>ACTG) is preferentially selected to produce the 8–9/11 intermediate, since the splice donor of exon 10 (Fig. 8, green triangle, <u>GC</u>AAGA) is noncanonical and weaker (Farrer et al. 2002; Kabat et al. 2006), leading to the production of

the exon 9 mRNA. On the other hand, when *asd-2* is active in the late larval stages (Fig. 8, right panel), ASD-2 binds to the QRE-like *cis*-acting element in intron 10 to promote the selection of the weak exon 10 splice donor, leading to the production of the 8–9–10/11 intermediate. Thereafter, the splice acceptor of exon 10 (Fig. 8, red circle, TTTTT<u>AG</u>) is preferentially selected, as that of exon 9 (Fig. 8, red triangle, ATTCT<u>AG</u>) is weaker.

The model described above indicates that the fate of the pre-mRNA, whether to be the exon 9 or exon 10 mRNA, is primarily determined by the removal of a flanking intron in the first splicing. The model also indicates that the mutually exclusive nature of the *let-2* alternative exons is ensured by the ordered removal of the flanking introns and the asymmetric strength of the splice donors and the acceptors of the two alternative exons, as well as by the steric interference of the two exons. In the present study, we observed that endogenous *asd-2b* was expressed in embryos (Fig. 4B) and that the *let-2* exon 10 mRNA isoform was eventually produced in the adult stage, even in the *asd-2* mutant background. These observations suggest a possibility that besides ASD-2, other regulators, such as modulators of the ASD-2 function and/or other *trans*-factors, are involved in the *let-2* alternative splicing. Regulation of the order of the intron removal may also be involved in the developmental switching as implicated by the substantial increase in the 8/10–11 intermediate in the L4 stage (Fig. 6C). It would be an interesting challenge to elucidate all of the factors involved in the precise control of developmental switching of the *let-2* mutually exclusive alternative splicing in vivo.

STAR family RNA-binding protein ASD-2b regulates alternative splicing in *C. elegans*

STAR family proteins are known as key regulators of the various aspects of biological processes through multiple functions in the RNA metabolism. In the STAR family, the GLD-1 subfamily has been shown to play multiple roles through direct binding to the target RNAs. The *Quaking* gene generates three isoforms of proteins by alternative splicing. The nuclear isoform QKI-5 regulates alternative splicing of the myelin-associated glycoprotein (MAG) gene (Wu et al. 2002), while the balance of the nuclear and cytoplasmic isoforms QKI-6 and QKI-7 regulates the nuclear export of the myelin basic protein (MBP) mRNA (Larocque et al. 2002). *Drosophila how* encodes two alternative isoforms and the balance between the opposing activities of the two isoforms regulates tendon cell differentiation through the level of the transcription factor Stripe (Nabel-Rosen et al. 1999, 2002). Unlike vertebrates and insects, *C. elegans* has two members of the GLD-1 subfamily of genes. The cytoplasmic protein GLD-1 (Jones et al. 1996) regulates the germ cell development and sexual identity by regulating mRNA translation and stability (Jan et al. 1999; Lee and Schedl 2001, 2004; Schumacher et al. 2005). Our genetic studies demonstrate that ASD-2b regulates alternative splicing in the body wall muscles. Although the molecu-
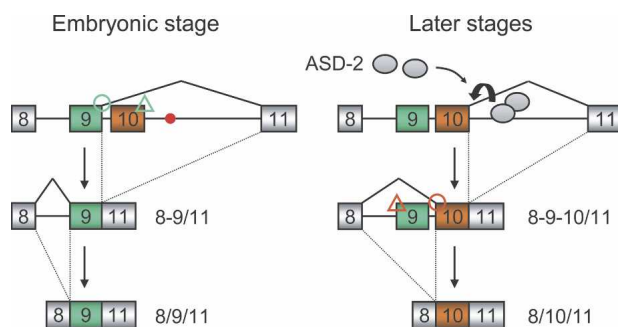

Figure 8. Models of the sequences of the *let-2* alternative splicing and the mechanism of exon switching by ASD-2. (*Left* panel) A model of exon 9 selection in the embryonic stage or when *asd-2* is inactive. (*Right* panel) A model of exon 10 selection when *asd-2* is active in the late larval stages. Red dot indicates the ASD-2-binding site. See the text for detail.

lar function of the ASD-2a isoform should be characterized further, ASD-2a may play essential roles in the nematode development, since the *asd-2* deletion allele *ok483*, in which common exon 3 and exon 4 are deleted, is lethal (WormBase, http://www.wormbase.org), while the *asd-2b*-specific mutants look otherwise normal and only show the color phenotype. The multiple nuclear and cytoplasmic isoforms produced by alternative splicing and/or gene duplication may have conferred the multiple functions in mRNA metabolisms to the subfamily.

Sam68 and its paralogs, SLM1 and SLM2 of mammals constitute of the Sam68/SLM subfamily in the STAR family (Fig. 3B; Di Fruscio et al. 1999). The subfamily has been shown to mediate the signal transduction to RNA metabolism by modulating the RNA-binding properties upon phosphorylation (Stoss et al. 2001; Matter et al. 2002; Haegebarth et al. 2004; Paronetto et al. 2007). Although signals regulating the GLD-1 subfamily are to be elucidated, regulation of the RNA-binding property may be involved in the regulation of the *let-2* alternative splicing by ASD-2b in vivo.

RNA-binding specificity of ASD-2

We identified the essential QRE-like *cis*-element for ASD-2b to regulate *let-2* alternative splicing. Several studies have identified the direct binding sites of the GLD-1 subfamily and addressed the consensus of the binding sequences. Ryder et al. (2004) demonstrated that GLD-1 preforms a dimer, and specifically binds to the UACU(C/A)A stretch(es) in the target mRNAs. They also showed that QKI recognizes the consensus NA(A>C)UAA by extensive mutagenesis of the GLD-1 consensus sequence (Ryder and Williamson 2004). Enrichment of the high-affinity RNA ligands using systematic evolution of ligands by exponential enrichment (SELEX) defined the QRE as a bipartite consensus sequence NACUAAY-$N_{1–20}$-UAAY, consisting of a core site (NACUAAY) and a tetra-nucleotide half site (UAAY) (Galarneau and Richard 2005). Recently, the primary sequence and the secondary structure requirement for *Drosophila* HOW response element (HRE) has been determined as the ACUAA stretch in the loop (Israeli et al. 2007). The results from these studies indicate that the GSG/STAR domains of the GLD-1 subfamily share not only the high amino acid sequence identity, but also the consensus binding sequence determined as QRE, although the sequence that binds with the highest affinity may differ among the GLD-1 subfamily members (Saccomanno et al. 1999; Ryder and Williamson 2004).

The *cis*-element we identified in this study consists of direct repeats of the penta-nucleotide stretch, CUAAC, which matches five out of six nucleotides of the QRE core site. The tetra-nucleotide QRE half site, which can be either upstream of or downstream from the core site, has been shown to be required for the high-affinity binding (Galarneau and Richard 2005) and the requirement of the half site in the proximity has also been suggested for GLD-1 (Ryder et al. 2004). It is likely that the direct repeats of the penta-nucleotide compensated the require-

ment of the complete core site for *let-2* regulation by ASD-2 as in the case of QRE-1 in the 3′-untranslated region (UTR) of MBP mRNA, where multiple clusters of the half site constitute QRE (Galarneau and Richard 2005). QKI and GLD-1 form homodimers via the QUA1 domain (Chen and Richard 1998; Ryder et al. 2004) and the E48 residue of QKI, conserved among the GLD-1 subfamily proteins (Fig. 3A), is essential for the dimer formation and the function of the *Quaking* gene (Chen and Richard 1998). We identified a substitution of a conserved residue in the QUA1 domain in the *asd-2 (yb1422)* mutant (Fig. 3A), suggesting the functional importance of the QUA1 domain for the ASD-2 function. ASD-2 may also form a homodimer via the conserved QUA1 domain, and each subunit may contact one CUAAC stretch in binding to the *let-2* pre-mRNA. Comparison of the dimer formation activities and the RNA-binding properties of the GSG/STAR domains derived from the various *asd-2* mutants isolated in this study would lead to further understanding of the RNA-recognition by the STAR family proteins.

Recent global and comparative analyses of the alternatively spliced exons have demonstrated that the (A)CTAAC stretch, which resembles the QRE, is enriched in the introns flanking the alternative exons in both mammals and nematodes, suggesting that the stretch is an intronic alternative splicing regulator (Kabat et al. 2006; Sugnet et al. 2006; Das et al. 2007; Yeo et al. 2007). The GLD-1 subfamily may be involved in the regulation of these genes, although the requirement of the (A)CTAAC stretch and the function of the GLD-1 subfamily are to be experimentally addressed.

Visualization of the alternative splicing patterns in vivo

In the present study, we utilized a pair of symmetric minigenes, both of which contain four exons and three intervening introns for visualizing the expression profiles of the *let-2* mutually exclusive alternative exons (Fig. 1B). In contrast, for visualizing the tissue-specific expression profiles of the *egl-15* mutually exclusive exons, we utilized a single unified minigene, in which GFP and RFP cDNAs were introduced into the alternative exons (Kuroyanagi et al. 2006). With the unified reporter minigene, we demonstrated that mutually exclusive selection of the *egl-15* alternative exons is regulated by the upstream-most intron, and that the downstream-most intron is dispensable for the regulation (Kuroyanagi et al. 2006). In general, the downstream flanking intron and the downstream constitutive exon should be included in the alternative splicing reporter minigenes, since the *cis*-element(s) in the intron downstream from the alternative exon(s) could be essential for the regulation, as demonstrated in this study. As the *let-2* reporter minigenes are symmetric and contain the intact genomic sequence, except for a modification in one exon in each of the reporter minigenes, we can assume that expression of the reporter reflects the regulation of the endogenous gene, and that the simultaneous modification of the *cis*-ele-

ment(s) in both of the minigene pairs should cause the same effect on the pre-mRNAs derived from these minigenes, as demonstrated in this study. We therefore conclude that a symmetric pair of minigenes is feasible for the visualization of the alternative splicing patterns and for the elucidation of the regulation mechanisms in *C. elegans*.

The utilization of dichromatic alternative splicing reporters has recently been described in other organisms. Carstens and colleagues (Newman et al. 2006) utilized a pair of reporter minigenes for the *FGFR2* mutually exclusive exons IIIb and IIIc with a similar structure to the *let-2* reporter. They demonstrated that the reporter and a fluorescence-activated cell sorter (FACS) facilitated the analysis of the function of various *trans*-factors in the selection of the mutually exclusive exons. Cooper and colleagues (Orengo et al. 2006) utilized another type of a single dichromatic reporter minigene, in which GFP and RFP cDNAs were fused in the different frame so that mutually exclusive expression of GFP and RFP from the single minigene reflected the expression of the alternative isoforms. Garcia-Blanco and colleagues (Bonano et al. 2006) generated knock-in mice that visualized the repression of the *FGFR2* exon IIIb in vivo. The mice carry an RFP-minigene to monitor efficiency of constitutive splicing and a GFP-minigene to monitor repression of *FGFR2* exon IIIb at a single-cell resolution in vivo. The advantage of these dichromatic fluorescent alternative splicing reporters, including those in our study, is that relative abundance of the two alternative isoforms in each cell can be reliably analyzed with a minimal effect of other aspects of gene expression such as a transcription level. Application of these alternative splicing reporters will facilitate expression profiling and experimental identification of *trans*-factors and *cis*-elements of various alternative splicing events.

In conclusion, visualization of the expression profiles of the alternative exons enabled us to isolate the splicing mutants and identify *trans*-acting factors and *cis*-acting elements. Isolation of the splicing mutants enabled us to detect and analyze the partially spliced pre-mRNAs or processing intermediates. These experimental data led to a model of how only one out of two exons is exclusively included in each mRNA and how the factor and elements regulate the fate of the exons. Experimental approaches of visualization of the splicing patterns, genetic analyses of splicing mutants, and detection of the processing intermediates in vivo, together with the comprehensive bioinformatic approaches, would open the way to further elucidate the spatiotemporally regulated fate of pre-mRNAs, or deciphering the splicing codes in metazoans.

Materials and methods

Plasmid construction

We constructed the reporter minigenes essentially as described in the text. To construct the reporter cassettes for *let-2-9G*, *let-2-10R*, and *let-2-10/R-WT*, we amplified the *let-2* genomic DNA fragments spanning from exon 8 to either exon 11 or exon 10 and cloned them into Gateway Entry vectors (Invitrogen) carrying either EGFP (Clontech) or mRFP1 (Campbell et al. 2002) cDNAs using the In-Fusion system (BD Biosciences). We introduced termination codons by using QuickChange (Stratagene). To construct the ASD-2b Entry vector, we amplified the ASD-2b cDNA fragment and cloned it in pENTR-D/TOPO (Invitrogen). We constructed the Expression vectors by homologous recombination between the reporter cassettes and the *myo-3* promoter vector pDEST-myo-3p (Kuroyanagi et al. 2006) by utilizing the Gateway system (Invitrogen). We generated *M1*, *M2*, and *M3* mutant minigenes from *myo-3::let-2-9G* and *myo-3::let-2-10R*, and *myo-3::let-2-10/R-Mut* from *myo-3::let-2-10/R-WT* by mutagenesis utilizing QuickChange (Stratagene). We amplified the genomic fragments of the *asd-2a* and *asd-2b* promoter regions and cloned them into promoterless Expression vectors to construct *asd-2a::GFP* and *asd-2b::RFP*. We confirmed all of the constructs by sequence analysis. Sequences of the primers used in the construction are available in the Supplemental Material.

Worm culture and microscopy

We cultured worms following standard methods (Brenner 1974). We prepared the transgenic lines essentially as described (Mello et al. 1991) using *lin-15 (n765)* as a host. We generated integrant lines by ultraviolet light irradiation (Mitani 1995). We treated *ybIs1371 [myo-3::let-2-9G myo-3::let-2-10R lin-15(+)]* animals with 47 mM ethyl methanesulfonate (EMS) for 4 h at room temperature, and we screened the F2 progeny for mutants with altered expression profiles of the GFP and RFP under a fluorescence stereoscope (MZ16FA; Leica) equipped with a dual-band-pass filter set. We captured the images of the *let-2* reporter worms with CCD cameras (E300 and DP71; Olympus). We used a confocal microscope (Fluoview FV500; Olympus) for image scanning and processed the acquired images with Metamorph (Molecular Devices). We identified the *asd-2* gene by SNP-based mapping essentially as described (Wicks et al. 2001). Briefly, we crossed the mutants with the Hawaiian wild-type strain, CB4856, which has many SNPs compared with the standard wild-type strain N2, and the SNPs of each F2 progeny with the green phenotype were analyzed by either restriction fragment length polymorphisms or direct sequencing of the PCR-amplified genomic fragments. Of ~75 predicted genes within the defined 391-kb region, we amplified and sequenced 13 RNA-related genes. We performed RNAi by soaking L4 hermaphrodites as described previously (Kuroyanagi et al. 2000; Maeda et al. 2001) by using dsRNAs corresponding to nucleotides 164–841 of the ASD-2b ORF.

RT–PCR

We prepared total RNAs and performed RT–PCR analysis essentially as described previously (Kuroyanagi et al. 2006). We digested the RT–PCR products for the *let-2* reporter and the endogenous *let-2* mRNAs with BglII before electrophoresis separation. Sequences of the primers used in the RT–PCR assays are available in the Supplemental Material.

EMSA

We prepared the recombinant protein for the GSG/STAR domain of ASD-2 (ASD-2 STAR), corresponding to amino acids 55–280 of ASD-2b, as a His-tagged protein. We introduced the ASD-2 STAR cDNA fragment into the modified bacterial cold-induction vector (Kuroyanagi et al. 2007) and induced the pro-

tein production following the standard cold-induction procedure (Takara). We purified the His-ASD-2 STAR protein with Ni-NTA agarose (Qiagen). We prepared the ^{32}P-labeled wild-type and M2 RNA probes by in vitro transcription with [α^{32}P]UTP and T7 RNA polymerase (Takara). We incubated the gel-purified RNA probes alone or with increasing concentrations of His-ASD-2 STAR in 25 µL of RNA-binding buffer (20 mM HEPES-KOH at pH 7.9, 320 mM KCl, 5% glycerol, 1% Triton X-100, 1 mM DTT, 0.1 mM PMSF) supplemented with 130 ng/µL *Escherichia coli* tRNA and 50 ng/µL BSA for 30 min at 20°C. Each sample was separated on a nondenaturing polyacrylamide gel and visualized with a phosphorimage analyzer (FLA-3000G; Fuji Film).

Analysis of partially spliced let-2 RNAs

We performed RT with random hexamers. We performed PCR following standard protocols supplemented with [α^{32}P]dCTP, and separated the PCR products on 5% nondenaturing polyacrylamide gels and quantified the signal intensity of each band with a phosphorimage analyzer (FLA-3000G; Fuji Film). We calculated the amount of each processing intermediate by normalization to that of the pre-mRNA in the same reaction, taking their G + C contents into account. We confirmed the identity of each band by cloning and sequence analysis. We performed control PCRs using RT products without reverse transcriptase (shown in Supplemental Fig. 1) to calculate the contamination of genomic DNAs in the total RNA fraction. Sequences of the primers used in the PCRs are available in the Supplemental Material.

Database information

The gene assignment *asd-2* has been approved by the CGC Genetic Map and Nomenclature Curator. The nucleotide sequences of the *asd-2* cDNAs have been deposited in the GenBank/EMBL/DDBJ databases with the accession numbers EF630625 and EF630626.

Acknowledgments

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