The RNA-binding protein SUP-12 controls muscle-specific splicing of the ADF/cofilin pre-mRNA in C. elegans

Akwasi Anyanful, Kanako Ono, Robert C. Johnsen, Hinh Ly, Victor Jensen, David L. Baillie, and Shoichiro Ono

Department of Pathology, Emory University, Atlanta, GA 30322
Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

The online version of this article contains supplemental material.

Acceptor splicing is an essential mechanism for increasing diversity of functionally different gene products. In Caenorhabditis elegans, UNC-60A and UNC-60B, nonmuscle and muscle isoforms of actin depolymerizing factor (ADF)/cofilin, are expressed by alternative splicing of unc-60 and regulate distinct actin-dependent developmental processes. We report that SUP-12, a member of a new family of RNA recognition motif (RRM) proteins, including SEB-4, regulates muscle-specific splicing of unc-60. In unc-60 mutants, expression of UNC-60B is decreased, whereas UNC-60A is up-regulated in muscle. sup-12 mutations strongly suppress muscle defects in unc-60B mutants by allowing expression of UNC-60A in muscle that can substitute for UNC-60B, thus unmasking their functional redundancy. SUP-12 is expressed in muscle and localized to the nuclei in a speckled pattern. The RRM domain of SUP-12 binds to several sites of the unc-60 pre-mRNA including the UG repeats near the 3′-splice site in the first intron. Our results suggest that SUP-12 is a novel tissue-specific splicing factor and regulates functional redundancy among ADF/cofilin isoforms.

Introduction

Pre-messenger RNA splicing is mediated by spliceosomes that contain small ribonucleoprotein particles and splicing factors (Jurica and Moore, 2003; Nilsen, 2003). Pre-mRNAs are often alternatively spliced to give rise to multiple mRNA species, which requires additional regulation of splice-site selection (Smith and Valcarcel, 2000; Maniatis and Tasic, 2002; Black, 2003). Tissue-specific alternative mRNA splicing is essential for expression of functionally different gene products from a single gene, and, thus, it is an important mechanism of cellular differentiation. Disruption of the splicing machinery is known to cause various human diseases (Nissim-Rafinia and Kerem, 2002; Stoilov et al., 2002; Faustino and Cooper, 2003). In particular, myotonic dystrophy has been linked to perturbation of the mechanism of alternative splicing in muscle (Kanadia et al., 2003; Ho et al., 2004). However, the molecular mechanism by which tissue-specific alternative splicing is regulated remains largely unknown, except for a few well-characterized examples (Smith and Valcarcel, 2000; Maniatis and Tasic, 2002; Black, 2003).

Actin depolymerizing factor (ADF)/cofilin proteins enhance actin filament dynamics by severing filaments and accelerating monomer dissociation from the pointed ends of the filaments (Bamburg, 1999; Bamburg et al., 1999; Maciver and Hussey, 2002; Ono, 2003). Essential functions of ADF/cofilin in vivo actin dynamics and cell viability have been demonstrated in several organisms (McKim et al., 1994; Gunsalus et al., 1995; Lappalainen and Drubin, 1997). In mammals, three ADF/cofilin isoforms are encoded by separate genes and expressed in different patterns of tissue distribution (Matsuzaki et al., 1988; Moriyama et al., 1990; Ono et al., 1994; Gillett et al., 1996; Thirion et al., 2001; Vartiainen et al., 2002). However, in the nematode Caenorhabditis elegans, the unc-60 gene undergoes alternative splicing and expresses two ADF/cofilin isoforms, UNC-60A and UNC-60B (McKim et al., 1994). Our previous studies have indicated that the two ADF/cofilin isoforms have different activities: UNC-60A strongly depolymerizes filaments, whereas UNC-60B binds to filaments with only weak depolymerizing activity (Ono and Benian, 1998; Ono, 1999; Mohri and Ono, 2003). More importantly, they are expressed in different tissues and required for specific actin-dependent processes: UNC-60A is expressed in nonmuscle cells and is required for embryonic cytokinesis (Ono et al., 2003), whereas UNC-60B is specifically expressed in the body wall muscle and regulates myofibril assembly (Ono et al., 2003).

spliceosome, UG repeats, splicing factor, muscle-specific, unc-60, SUP-12, ADF, cofilin, actin depolymerizing factor, A/Q-rich, alanine- and glutamine-rich, EMSA, electrophoretic mobility shift assay, RRM, RNA recognition motif.
The *unc-60* gene has nine exons and only the first exon is shared by *unc-60A* and *unc-60B* (McKim et al., 1994). Therefore, the tissue-specific expression of *unc-60A* or *unc-60B* is proposed to be determined by selection of the first splice acceptor site at the 5'-end of either exon 2A or 2B (McKim et al., 1994). However, the regulatory mechanism of tissue-specific splicing of the *unc-60* pre-mRNA is unknown.

In this work, we report identification and characterization of a putative splicing factor that regulates muscle-specific splicing of the *unc-60* pre-mRNA in *C. elegans*. We cloned SUP-12, a conserved RNA-binding protein, as a suppressor of *unc-60B*. *sup-12* mutations strongly suppress the muscle defects of *unc-60B* mutants. This suppression is likely due to alteration in expression of the *unc-60* splice variants in the muscle cells. SUP-12 localizes to the nuclei in body wall muscle and its RNA-binding domain directly binds to the *unc-60* pre-mRNA in vitro. Our data support that SUP-12 is a novel member of tissue-specific regulators of alternative splicing.

### Results

#### Identification of SUP-12 as a suppressor of *unc-60B*

In a previous effort to identify genes that functionally interact with *unc-60*, *sup-12* mutant alleles *st89* and *st203* were isolated from a screen for extragenic suppressors of *unc-60* mutants (G.R. Francis and R.H. Waterston, personal communication; Waterston, 1988). *sup-12* mutations alone have only minor effects on motility of the worms (Fig. 1A). However, in the *unc-60B* mutant backgrounds, *sup-12* strongly suppressed the motility defects (Fig. 1A). The suppression by *sup-12* was also equally strong for a strong loss of function allele *unc-60B(e677)* and a null allele *unc-60B(su158)* (McKim et al., 1988), and the motility of the *unc-60B;sup-12* double mutants was restored nearly to the level of wild-type worms (Fig. 1A). The motility defects of the *unc-60B* mutants are caused by disorganization of actin filaments in the body wall muscle (Ono et al., 1999; Ono et al., 2003). *sup-12* single mutants had no detectable phenotype in the myofibril organization (Fig. 1B). However, in the *unc-60B;sup-12* double mutants, actin was organized into the myofibrils as well as wild type (Fig. 1B). The *sup-12* single mutants had no detectable phenotype in the myofibril organization (Fig. 1B; Francis and Waterston, 1985). *sup-12(st203)* exhibited slightly stronger suppressor effect than *sup-12(st89)* in worm motility (Fig. 1A) and in actin organization in muscle (unpublished data). These results suggest that *sup-12* genetically interacts with *unc-60B* and regulates actin organization in the body wall muscle.
By positional cloning and identification of mutation sites, we cloned the sup-12 gene and identified it as T22B2.4 (GenBank/EMBL/DDBJ accession no. NM_076273) that is mapped to the left arm of the X chromosome by the C. elegans Sequencing Consortium (1998). The SUP-12 protein (248 aa) is a putative RNA-binding protein that has a single RNA recognition motif (RRM) domain (Shamoo et al., 1995) in its NH₂ terminus (residues 35–117) and alanine- and glutamine-rich (A/Q-rich) sequence in its COOH terminus (26 A and 24 Q in residues 121–246; Fig. 2 A). Missense mutations G59R, G77E, and G113E were found within the RRM domain in the sup-12 mutant alleles s2900, s169, and s2896, respectively (Fig. 2 A). s2901 has the same mutation as s169, although they were isolated in different laboratories. A mutation (G to A) was found in st203 at the splice donor site in the second intron. RT-PCR analysis showed that aberrantly spliced sup-12 mRNA was predominantly expressed in the sup-12(st203) mutants (unpublished data). RNA interference of sup-12 phenocopied the sup-12 mutant phenotype and suppressed the Unc-60B phenotype of multiple unc-60B alleles (unpublished data). Homology searches revealed that SUP-12 is an orthologue of a human protein SEB-4 (Fig. 2 B). Two human SEB-4 isoforms (GenBank/EMBL/DDBJ accession no. AK095016 and NM_153020) are found in the database, but their function is unknown. In Xenopus, two SEB-4 isoforms, XSEB-4 and XSEB-4R, are reported: XSEB-4 is expressed in muscle precursor cells (Fetka et al., 2000), whereas XSEB-4R is strongly expressed in neuronal cells and involved in neural differentiation (Boy et al., 2004). Interestingly, Arabidopsis has at least six SEB-4 orthologues (Fig. 2 B), but functional studies on these proteins are not reported. However, no SEB-4 orthologues were found in yeasts and Drosophila. Sequence alignment of these proteins showed highly conserved sequences in the RRM domain in the NH₂ termini (unpublished data). The COOH-terminal halves are not highly conserved, yet the A/Q-rich sequences are present in all these proteins. The similarity in the sequences suggests that SUP-12 and SEB-4 homologues belong to a new family of functionally conserved RNA-binding proteins.

SUP-12 alters expression patterns of the unc-60 splice variants

To understand the mechanism of suppression of the Unc-60 mutant phenotype by sup-12, expression patterns of the splice variants of the unc-60 gene products were examined. Surprisingly, the protein level of UNC-60B, the muscle-specific isoform, was greatly reduced in the sup-12 mutants both in wild-type and unc-60B(e677) backgrounds (Fig. 3 B, lanes 1–6). In contrast, the total level of UNC-60A, the nonmuscle isoform, was not significantly altered by mutations in sup-12 or unc-60B (Fig. 3 B). Similar changes were detected at the mRNA levels: the unc-60B mRNA was reduced as compared with wild type, whereas the unc-60A mRNA was not significantly altered (Fig. 3 C). In wild-type background, the unc-60B mRNA was decreased in the sup-12 mutants (Fig. 3 C, lanes 1–3). unc-60B(e677) has a missense mutation (Ono et al., 1999) and had a greatly reduced level of the unc-60B mRNA (Fig. 3 C, lanes 4–6), which correlates with the reduced protein level (Fig. 3 B, lane 4). unc-60B(su158) has a 600-bp deletion in the unc-60B region without affecting unc-60A (Ono et al., 2003) and expressed a shorter unc-60B mRNA (Fig. 3 C, lane 7) that is not translated into a protein (Fig. 3 B, lane 7). Nonetheless, its mRNA level was still reduced in the sup-12 mutants (Fig. 3 C, lanes 8 and 9), strongly suggesting that SUP-12 affects the levels of the unc-60B mRNA, but not directly of the protein.
UNC-60A is expressed in a variety of nonmuscle cells (Ono et al., 2003). Therefore, changes in the level of UNC-60A in a subset of tissues may not be detected by the Northern blot analysis. Therefore, we examined the tissue distribution of UNC-60A by immunofluorescence microscopy and found that the level of UNC-60A in body wall muscle is altered by sup-12. In wild type and the unc-60B(e677) single mutant, UNC-60A was not detectable in body wall muscle (Fig. 4, A–F, arrows). However, in the unc-60B;sup-12 double mutant and the sup-12 single mutant, UNC-60A protein was detected in the diffuse cytoplasm (Fig. 4, G–L, arrows). These results indicate that the sup-12 mutations have opposite effects on the levels of the two splice variants, unc-60A and unc-60B, in muscle cells. Although UNC-60A and UNC-60B have quantitatively different biochemical activities, both isoforms can enhance actin filament dynamics by depolymerizing actin filaments (Ono and Benian, 1998; Ono, 1999; Ono et al., 1999). Thus, the suppression of the Unc-60 phenotype by sup-12 could be explained by the up-regulation of UNC-60A in muscle, which may compensate for the function of UNC-60B.

**SUP-12 is expressed in body wall muscle and localizes to the nuclei**

We next investigated expression and subcellular localization of SUP-12. An anti–SUP-12 antibody against its COOH terminus specifically recognized the SUP-12 protein by Western blot but did not detect the protein by immunofluorescence microscopy (unpublished data). The 3.1-kb promoter region of the sup-12 gene was able to drive expression of a reporter GFP in body wall muscle (Fig. 5 A, arrowheads) and pharynx (Fig. 5 A, asterisk). GFP-tagged SUP-12, which was expressed in the body wall muscle under the control of the myo-3 promoter (Okkema et al., 1993), predominantly localized to the nuclei (Fig. 5, B–D). Within the nuclei, GFP-SUP-12 was found in diffuse and often speckled patterns in the nucleoplasm but excluded from the globular region, possibly representing the nucleolus (Fig. 5 B). The speckled localization of SUP-12 in the nucleus is similar to patterns that are commonly observed for other splicing factors (Misteli, 2000; Dundr and Misteli, 2001; Lamond and Spector, 2003). Although the myo-3 promoter is active in the body wall muscle but not in the pharynx, myo-3–driven expression of GFP-SUP-12 was sufficient to rescue the Sup-12 mutant phenotype (Table I), indicating that GFP-SUP-12 is functional and expression of SUP-12 in the body wall muscle is functionally important for its interaction with unc-60. These results strongly suggest that SUP-12 is a muscle-specific regulator of pre-mRNA splicing in the nucleus.

Neither the NH2-terminal RRM domain nor the COOH-terminal A/Q-rich domain alone was able to rescue the sup-12 mutant phenotype (Table I). The RRM domain of SUP-12 (residues 1–117) localized to both nuclei and cytoplasm in a diffuse pattern (Fig. 5 E), whereas the A/Q-rich domain of SUP-12 (residues 118–248) strongly localized to the nuclei in a similar speckled pattern to the full-length protein and weakly to the cytoplasm (Fig. 5 F). These results suggest that either the RRM or the A/Q-rich domain is sufficient for nuclear localization, but both domains are required for the function of SUP-12.

**Direct binding of SUP-12 to the unc-60 pre-mRNA**

To determine whether the SUP-12 protein may be directly involved in pre-mRNA splicing of unc-60, we examined direct interaction between SUP-12 and unc-60 pre-mRNA in vitro by an electrophoretic mobility shift assay (EMSA). Recombinant GST-tagged full-length SUP-12 protein or the COOH-terminal portion (residues 118–248) of SUP-12 were poorly soluble (unpublished data) and therefore were not examined. However, the NH2-terminal portion (residues 1–117) of SUP-12 containing the RRM domain was stable and soluble as a GST-fusion protein. When purified GST-SUP-12 (RRM) was incubated with various portions of in vitro transcribed unc-60 pre-mRNA (Fig. 6 A), it caused a band shift of only the 978-nt RNA fragment that encompasses the sequence of exon 1 to a portion of exon 5A (Fig. 6, A and B). GST alone did not cause a band shift of the RNAs (Fig. 6 B). These results indicate that the RRM domain of SUP-12 is sufficient for direct RNA-binding and so could confer specificity for the 5′-region of the unc-60 pre-mRNA.

The SUP-12 binding region in the unc-60 pre-mRNA was further narrowed down by a pull-down assay with biotin-labeled RNA fragments (Fig. 6, C–G). We first tested interac-

| Domain | Residues | Rescue | Localization | RNA-binding | Solubility |
|--------|----------|--------|--------------|-------------|-----------|
| Full-length | 1–248 | Yes | Nuclei | ND | Insoluble |
| RRM | 1–117 | No | Nuclei cytoplasm | Yes | Soluble |
| A/Q-rich | 118–248 | No | Nuclei cytoplasm | ND | Insoluble |
tions between GST-SUP-12 (RRM) and four RNA fragments, A1-1, A1-2, A1-3, and A1-4, within the \textit{unc-60A} region (Fig. 6 C). GST-SUP-12 (RRM) bound to the magnetic beads in the presence of A1-1 or A1-2 (Fig. 6 E, lanes 2–5). However, in the presence of A1-3 or A1-4 (Fig. 6 E, lanes 6–9), the amounts of captured GST-SUP-12 (RRM) were not significantly different from that in the absence of RNA (Fig. 6 E, lane 1). GST alone did not bind to the beads in the presence or absence of RNA (Fig. 6 E), indicating that the RRM domain of SUP-12 mediated the interactions with RNAs.

We then used shorter RNA fragments of 10–152 bases in the pull-down assay with GST-SUP-12 (RRM) (Fig. 6, C and D) and characterized the interactions in a quantitative manner (Fig. 6, F and G). GST-SUP-12 (RRM) showed relatively strong binding with exon 1 (A1-1-1), the first intron (A1-1-2), and the second intron (A1-2-2) but did not significantly interact with exon 2A (A1-2-1; Fig. 6 F). Interestingly, an 18-nt truncation of A1-1-2 at the 3'-end (A1-1-2-ΔUG; Fig. 6, C and D) weakened the interaction with GST-SUP-12 (RRM; Fig. 6, F and G). The truncated region contains repeats of UG that have been reported to bind to several RNA-binding proteins (Mittag, 1996; Takahashi et al., 2000; Buratti et al., 2004). The RNA oligonucleotide UG (5'-UGUGUGCCUG-3') strongly interacted with GST-SUP-12 (RRM; Fig. 6 E), whereas the oligonucleotide UC (5'-UCUCUCCUC-3') showed nearly insignificant interaction (Fig. 6 E). Densitometric quantification of the results in Fig. 6 E indicates that only UG exhibited strong saturable binding with GST-SUP-12 (RRM; Fig. 6 F). Binding of GST-SUP-12 (RRM) to UG was saturated at a molar ratio of 1:1:1.0 with a dissociation constant of 0.31 μM, suggesting that they form a stoichiometric 1:1 complex with physiologically strong affinity. Removal of the UG repeats from A1-1-2 did not completely abolish the interaction of GST-SUP-12 (RRM) with A1-1-2-ΔUG (Fig. 6, F and G), suggesting that the UG repeats are sufficient but not necessary for this interaction. Binding of GST-SUP-12 (RRM) to A1-1-1, A1-1-2, or A1-2-2 did not reach saturation within the conditions used in this work (Fig. 6 G), so we were not able to determine stoichiometry and affinity. These results demonstrate that the RRM domain of SUP-12 directly interacts with the \textit{unc-60} pre-mRNA at multiple sites within exon 1 and the first and second introns. In particular, strong interaction of the SUP-12 RRM domain with the UG repeats near the 3'-splice site in the first intron supports that SUP-12 may function as a regulator of pre-mRNA splicing.

**Discussion**

In this work, we identified SUP-12 as a critical regulator of the muscle-specific pre-mRNA splicing of \textit{unc-60}. \textit{sup-12} mutations suppressed muscle-specific defects in actin organization which was caused by \textit{unc-60B} mutations. This suppression involved switching of the \textit{unc-60} isoforms in the muscle cells: \textit{sup-12} mutations enhanced muscle expression of UNC-60A, the nonmuscle ADF/cofilin isoforms, but reduced expression of UNC-60B, the muscle isoforms, thus allowing UNC-60A to compensate for the function of UNC-60B in the muscle cells. SUP-12 localized to the muscle nuclei and bound directly to exon 1 and the first and second introns of the \textit{unc-60} pre-mRNA in vitro, suggesting that SUP-12 is directly involved in muscle-specific splicing of the \textit{unc-60} pre-mRNA.

Our data indicate that, in muscle cells, SUP-12 normally inhibits production of the unc-60A mRNA, but it enhances expression of the unc-60B mRNA. Several possibilities for the
mechanism by which SUP-12 regulates expression of unc-60A and unc-60B could be considered. The most probable model is that the general splicing machinery may preferentially induce the splicing between exons 1 and 2A to produce unc-60A, whereas SUP-12 likely acts as an inhibitor of this splicing event. This model is strongly supported by the presence of UG repeats near the 3'-end of the first intron, which strongly interacts with the RRM domain of SUP-12. In the human cystic fi-

Figure 6. Direct interaction of the SUP-12 RRM domain with the unc-60 pre-mRNA. Interactions between the SUP-12 RRM domain and fragments of the unc-60 pre-mRNA were examined by EMSA (A and B) or a biotin-RNA pull-down assay (C–G). A Four synthetic RNA fragments A1, A2, A1, and B2, were transcribed in vitro and used for EMSA with GST or GST-SUP-12 (RRM domain). B 32P-labeled RNAs were incubated with buffer alone (lanes 1, 4, 7, and 10), GST (lanes 2, 5, 8, and 11), or GST-SUP-12 (RRM) (lanes 3, 6, 9, and 12) and separated by agarose-gel electrophoresis. Arrow indicates unbound RNAs. Band shift (asterisk) was observed only in a mixture of A1 and GST-SUP-12 (RRM) (lane 3). C Schematic representation of RNA fragments used in the biotin-RNA pull-down assays. D Sequence of the unc-60 pre-mRNA near the splice site at the 5'-end of exon 2A. Intron sequence is shown in small letters, exon sequence in capital letters. The UG-repeat sequence used in the oligonucleotide UG is in bold. UC is a control oligonucleotide that has UC repeats instead of UG repeats. E GST-SUP-12 (RRM) or GST (0.5 or 5.0 μM) was incubated with a biotin-labeled RNA fragment (80 nM), and the protein–RNA complex was captured by streptavidin magnetic particles and analyzed by SDS-PAGE and Coomassie staining. GST-SUP-12 (RRM), but not GST, showed significant interactions with A1-1 and A1-2 (lanes 2 and 3) and A1-2 (lanes 4 and 5). F Interactions between GST-SUP-12 (RRM) at varied concentrations (0.2–5.0 μM) and various RNA fragments at 0.1 μM (total 20 pmol) were examined by the biotin-RNA pull-down assay in a final volume of 200 μl. Known amounts (10, 25, or 50 pmol) of GST-SUP-12 (RRM) were applied to each gel as standards for densitometric quantification. G Densitometric quantification of GST-SUP-12 (RRM) that was bound to biotin-RNA.
brosis transmembrane conductance regulator pre-mRNA, the nuclear RRM protein TDP-43 binds to the UG repeats at the 3'-end of intron 8 and causes exon skipping (Niksic et al., 1999; Pagani et al., 2000; Buratti et al., 2004). Thus, SUP-12 and TDP-43 may negatively regulate splicing in a similar manner by binding to UG repeats and directly competing with the U2 auxiliary factor, which is an essential splicing factor that binds to 3'-splice sites (Merendino et al., 1999; Wu et al., 1999; Zorio and Blumenthal, 1999).

An alternative model is that SUP-12 may indirectly inhibit 3'-end processing of the unc-60A pre-mRNA. Because the SUP-12–binding sites on the unc-60 pre-mRNA are not close to the polyadenylation site of unc-60A, SUP-12 may have to interact with 3'-end processing factors to influence this process. The last exon (5A) for unc-60A that contains the 3'-untranslated region resides upstream of exon 2B (Fig. 3 A). In nonmuscle cells, the mRNA 3'-end processing factors may cleave the pre-mRNA and polyadenylate after exon 5A but before exon 2B is transcribed. Therefore, in muscle cells, SUP-12 may inhibit 3'-end processing at exon 5A and promote elongation of the pre-mRNA. Indeed, in case of polycistronic genes in C. elegans, an interaction between a 3'-end processing factor and a factor for trans-splicing is reported (Evans et al., 2001). Also, it is possible that SUP-12 affects RNA stability or interacts with transcription factors and regulates transcription and pre-mRNA processing because splicing factors and transcription factors functionally interact and regulate pre-mRNA splicing in many instances (Bentley, 2002). In addition, we cannot exclude the possibility that SUP-12 may regulate relative stability of the two mRNAs.

The RRM domain of SUP-12 had activity to bind to the 5'-region of the unc-60 pre-mRNA but was unable to rescue the sup-12 mutant phenotype. This suggests that the COOH-terminal A/Q-rich sequence plays an important function. Although the function of the A/Q-rich sequence is unknown, it is intriguing that MEC-8, which regulates alternative splicing of unc-52 in the hypodermis in C. elegans (Lundquist et al., 1996; Spike et al., 2002), also contains an A/Q-rich region in addition to two RRM domains. The A/Q-rich sequence might have a regulatory function for a splicing factor or mediate interactions with other splicing factors, transcription factors, or 3'-end processing factors. We showed that the A/Q-rich region of SUP-12 is necessary and sufficient for speckled localization in the nuclei, suggesting that this region is important for SUP-12 to localize to speckles. Also, we noted that the bacterially expressed full-length SUP-12 protein was not only insoluble but also very susceptible for proteolysis (unpublished data), suggesting that the A/Q-rich sequence may regulate protein stability.

Furthermore, this work suggests that functional redundancy of the two ADF/cofilin isoforms in muscle is normally masked by tight regulation of tissue-specific splicing by sup-12. Thus, sup-12 mutations unmask the redundancy and allow UNC-60A to compensate for mutated UNC-60B in unc-60B mutants. From our previous work, it seemed logical to hypothesize that UNC-60B with weaker depolymerizing activity might be more suitable in muscle cells than UNC-60A, where less dynamic actin reorganization is needed than nonmuscle cells. Therefore, it is somewhat surprising that UNC-60A can substitute for UNC-60B in muscle. However, it is possible that, although the sup-12 mutants have apparently normal myofibrils, their muscle may exhibit different physiological properties from that of wild type under specific conditions. A number of human diseases are caused by alterations in the pre-mRNA splicing (Nissim-Rafinia and Kerem, 2002; Stoilov et al., 2002; Faustino and Cooper, 2003). However, our results suggest that, the splicing machinery is a potential therapeutic target for certain genetic diseases in which manipulation of tissue-specific splicing machinery may reveal hidden functional redundancy among splice variants to compensate for a disease gene. We propose that SUP-12– and SEB-4–related proteins are a new family of tissue-specific splicing factors in multicellular organisms.

Materials and methods

Nematode strains

Wild-type N2 and unc-60B(e677) [Waterston et al., 1980] were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN). sup-12(sib9) and sup-12(p203) were obtained from G.R. Francis (Exelixis, Inc., South San Francisco, CA) and R. Waterston (University of Washington, Seattle, WA). sup-12(s2900) and sup-12(s2901) were isolated by a screen for suppressors of unc-60B. In brief, unc-60B(e677) homozygotes were mutagenized by ethyl methanesulfonate and F2 worms with improved motility were isolated. unc-60B(su158) (Zengel and Epstein, 1980) was originally obtained from H.F. Epstein (University of Texas Medical Branch at Galveston, Galveston, TX) and characterized previously (Ono et al., 2003).

Cloning of sup-12

sup-12 was mapped to the left arm of the X chromosome that was included in the duplication mDp33 (Zengel and Epstein, personal communication). We further narrowed down sup-12 by the snip-SNP mapping with polymorphisms in CB4856 (Wicks et al., 2001) to an interval between cosmid clones ZC64 and T06F4 that contained ~20 genes. We performed feeding RNA interference of 10 genes in wild type and unc-60B(su158) and examined for a suppressor phenotype for unc-60B(su158). We found that T22B2.4(RNAi) suppressed the motility defect of unc-60B(su158) but did not affect motility of wild type. To confirm that T22B2.4 is sup-12, we sequenced the T22B2.4 gene in the sup-12 mutants and identified mutations in multiple sup-12 alleles (Results and Fig. 2 A).

Fluorescence microscopy

Actin filaments were visualized by staining adult worms with tetramethylrhodamine-phallolidin (Sigma-Aldrich) as described previously (Ono, 2001). Immunofluorescent staining was performed on adult worms that were permeabilized with a freeze-crack method (Epstein et al., 1993) and fixed with methanol for 5 min at –20°C. Primary antibodies used were anti-UNC-60A [Ono et al., 1999] and anti-myosin (mAb 5.6, obtained from H.F. Epstein; Miller et al., 1983). Secondary antibodies were Alexa488-labeled goat anti-rabbit IgG and Alexa647-labeled goat anti-mouse IgG [Molecular Probes]. To visualize nuclei, worms were fixed with 4% formaldehyde in PBS for 30 min at RT, permeabilized with acetone at –20°C for 5 min, and stained with DAPI [Sigma-Aldrich] at 1 μg/ml in PBS containing 0.5% Triton X-100, 1 mM EDTA, and 0.05% sodium azide for 15 min. Fluorescent samples were mounted with the Prolong antifading reagent [Molecular Probes] and viewed by epifluorescence using an inverted microscope (model Eclipse TE2000; Nikon) with a 40× CFI Plan Fluor objective (dry; NA 1.4). Images were captured by a SPOT RT Monochrome CCD camera (Diagnostic Instruments) and processed by the IP Lab imaging software (Scanalytics, Inc.) and Adobe Photoshop 6.0.

Northern and Western blots

Total nematode RNA was isolated using a TRI reagent (Sigma-Aldrich). RNA samples (10 μg) were subjected to formaldehyde-agarose gel electrophoresis, transferred to positively charged nylon membrane (Millipore), and fixed by ultraviolet irradiation. cDNAs for unc-60A (503 bp), unc-60B (660 bp), and act (act-1) (1.1 kb) were amplified by PCR, and the PCR products were subcloned into pCR2.1 and sequenced. C. elegans pre-mRNA was reamplified as an internal control.
beled with digoxigenin with random priming, and used as probes for Northern blotting using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science). Western blot was performed as described previously (Ono and Ono, 2002) using the following primary antibodies: anti-UNC-60A (Ono et al., 1999), anti-UNC-60B (Ono et al., 1999), anti-actin monoclonal C4 (ICN Biomedicals), and anti-α-tubulin (Amersham Biosciences).

**Transgenic expression of GFP and GST-SUP-12**

To determine the promoter activity of sup-12, a 3,418-bp genomic fragment containing the 3,087-bp upstream region, exon 1, intron 1, and 358-bp of exon 2, was amplified by PCR using ExTaq DNA polymerase (Takara) and cloned into the gfp expression vector pPD95.67 (obtained from A. Fire, Stanford University, Stanford, CA) at the 5′-end of the gfp coding region. For expression of GFP-SUP-12 in body wall muscle, the sup-12 cDNA (ykl1125e08, obtained from Y. Kohara, National Institute of Genetics, Mishima, Japan) was ligated with the 3′-end of gfp in pPD118.20 (obtained from A. Fire) that has the myo-3 promoter. For expression of fragments of SUP-12 as GFP-fusion proteins, fragments of the sup-12 cDNA encoding residues 1–117 or 118–248 were amplified by PCR and ligated in-frame with the 3′-end of gfp in pPD118.20. A synthetic stop codon was added for expression of residues 1–117. The plasmids were injected into hermaphroditic gonads of wild type or strains, A. Fire for expression vectors, H.F. Epstein for anti-myosin antibody and a strain, and K. Bhat and D. Kalman for critical comments.

Some preliminary data were obtained by S. Ono while he was a post-doctoral fellow in the laboratory of Guy Benian, which was supported by a grant from the National Science Foundation (MCB-9728762). Some worm strains were provided by the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources. This work was supported by grants from the NSERC Canada to R.C. Johnson and D.L. Baillie, the American Heart Association Southeast Affiliate, and the National Institutes of Health (RO1 AR048615) to S. Ono.

Submitted: 13 July 2004
Accepted: 12 October 2004

**References**

Bamburg, J.R. 1999. Proteins of the ADF/cofilin family: essential regulators of actin dynamics. Ann. Rev. Cell Dev. Biol. 15:185–230.

Bamburg, J.R., A. McGough, and S. Ono. 1999. Putting a new twist on actin: ADF/cofilins modulate actin dynamics. Trends Cell Biol. 9:364–370.

Bentley, D. 2002. The mRNA assembly line: transcription and processing machines in the same factory.Curr. Opin. Cell Biol. 14:336–342.

Black, D.L. 2003. Mechanisms of alternative pre-messenger RNA splicing. Annu. Rev. Biochem. 72:291–336.

Boy, S., J. Souppou, M.A. Amato, M. Wegnez, T. Pieler, and M. Perron. 2004. XSEBR4, a novel RNA-binding protein involved in retinal cell differentiation downstream of DMEHL proneural genes. Development. 131:851–862.

Buratti, E., A. Brindisi, F. Pagani, and F.E. Baralle. 2004. Nuclear factor TDP-43 binds to the polymorphic TG repeats in CFTR intron 8 and causes skipping of exon 9: a functional link with disease penetrance. Am. J. Hum. Genet. 74:1322–1325.

C. elegans Sequencing Consortium. 1998. Genome sequence of the nematode C. elegans: a platform for investigating biology. Science. 282:2012–2018.

Dundr, M., and T. Misteli. 2001. Functional architecture in the cell nucleus. Biochem. J. 356:297–310.

Epstein, H.F., and J.N. Thomson. 1974. Temperature-sensitive mutation affecting myofilament assembly in Caenorhabditis elegans. Nature. 250:579–580.

Epstein, H.F., D.L. Casey, and I. Ortiz. 1993. Myosin and paramyosin of Caenorhabditis elegans embryo assemble into nascent structures distinct from thick filaments and multi-filament assemblies. J. Cell Biol. 122:845–858.

Evans, D., I. Perez, M. MacMorris, D. Leake, C.J. Wilusz, and T. Blumenthal. 2001. A complex containing CstF-64 and the SL2 snRNP connects mRNA 3′ end formation and trans-splicing in C. elegans oocytes. Genes Dev. 15:2562–2571.

Faustino, N.A., and T.A. Cooper. 2003. Pre-mRNA splicing and human disease. Genes Dev. 17:419–437.

Fetka, I., A. Radeghieri, and T. Bouwmeester. 2000. Expression of the RNA recognition motif-containing protein CBP-4 during Xenopus embryonic development. Mech. Dev. 94:283–286.

Francis, G.R., and R.H. Waterston. 1985. Muscle organization in C. elegans. J. Cell Biol. 101:1532–1549.

Gilliet, G.T., M.F. Fox, P.S. Rowe, C.M. Casimir, and S. Povey. 1996. Mapping of human non-muscle type cofilin (CFL1) to chromosome 11q13 and muscle-type cofilin (CFL2) to chromosome 14. Ann. Hum. Genet. 60:125–131.

Gunsalus, K.C., S. Bonaccorsi, E. Williams, F. Venn, M. Gatti, and M.L. Goldberg. 1995. Mutations in twinstar, a Drosophila gene encoding a cofilin/ADF homologue, result in defects in centrosome migration and cytokinesis. J. Cell Biol. 131:1243–1259.

Ho, T.H., B.N. Charlet, M.G. Poulos, G. Singh, M.S. Swanson, and T.A. Cooper. 2004. Muscleblind proteins regulate alternative splicing. EMBO J.
Kanadia, R.N., K.A. Johnstone, A. Mankodi, C. Lungu, C.A. Thornton, D. Es-
Jurica, M.S., and M.J. Moore. 2003. Pre-mRNA splicing: awash in a sea of pro-
Lamond, A.L., and D.L. Spector. 2003. Nuclear speckles: a model for nuclear or-
Lundquist, E.A., R.K. Herman, T.M. Rogalski, G.P. Mullen, D.G. Moerman,
Lee, M.H., and T. Schedl. 2001. Identification of UNC-52 transcripts in C. elege-
Lappalainen, P., and D.G. Drubin. 1997. Cofilin promotes rapid actin filament
Lundquist, E., R.K. Herman, T.M. Rogalski, G.P. Mullen, D.G. Moerman,
Lee, M.H., and T. Schedl. 2001. Identification of msl-2 splicing by Sex-lethal reveals interaction between U2AF35
McKim, K.S., M.F. Heschl, R.E. Rosenbluth, and D.L. Baillie. 1988. Genetic
McKim, K.S., C. Matheson, M.A. Marra, M.F. Wakarchuk, and D.L. Baillie. 1994. The Caenorhabditis elegans unc-60 gene encodes proteins homolo-
Merendino, L., S. Guth, D. Bilbao, C. Martinez, and J. Valcarcel. 1999. Inhibi-
Misteli, T. 2000. Cell biology of transcription and pre-mRNA splicing: nuclear architecture meets nuclear function. J. Cell Sci. 113:1841–1849.
Mittag, M. 1996. Conserved circadian elements in phylogenetically diverse al-
Moriyama, K., E. Nishida, N. Yonezawa, H. Sakai, S. Matsumoto, K. Iida, and I. Yahara. 1990. Destrin, a mammalian actin-depolymerizing protein, is close-
Mrri, S., and S. Ono. 2000. Actin filament disassembling activity of Caenorhabditis elegans actin-interacting protein 1 (UNC-78) is dependent on filament binding by a specific ADF/cofilin isoform. J. Cell Sci. 116: 4107–4118.
Moriyama, K., E. Nishida, N. Yonezawa, H. Sakai, S. Matsumoto, K. Iida, and I. Yahara. 1990. Destrin, a mammalian actin-depolymerizing protein, is closely related to cofilin. Cloning and expression of porcine brain destrin cDNA. J. Biol. Chem. 265:5768–5773.
Mohri, K., and S. Ono. 2003. Actin filament disassembling activity of Caenorhabditis elegans actin-interacting protein 1 (UNC-78) is dependent on filament binding by a specific ADF/cofilin isoform. J. Cell Sci. 116: 4107–4118.
Nissim-Rafinia, M., and B. Kerem. 2002. Splicing regulation as a potential gene-
Nissim-Rafinia, M., and B. Kerem. 2002. Splicing regulation as a potential gene-
Oko, P.G., S.W. Harrison, V. Plunger, A. Aryan, and A. Fire. 1993. Sequence requirements for myosin gene expression and regulation in Caenorhabditis elegans. Genetics. 135:385–404.
Ono, S. 1999. Purification and biochemical characterization of actin from Caenorhabditis elegans: its difference from rabbit muscle actin in the interaction with nematode ADF/cofilin. Cell Motil. Cytoskeleton. 43:128–136.
Ono, S. 2000. The Caenorhabditis elegans unc-78 gene encodes a homologue of actin-interacting protein 1 required for organized assembly of muscle ac-
Ono, S., and D.L. Baillie. 1996. UNC-60B, an ADF/cofilin family protein, which is required for proper assembly of actin into myofilbrils in Caenorhabditis elegans body wall muscle. J. Cell Biol. 136:487–501.
Ono, K., M. Parast, C. Alberico, G.M. Benian, and S. Ono. 2003. Specific require-
Ono, S., D.L. Baillie, and G.M. Benian. 1999. UNC-60B, an ADF/cofilin family protein, is required for proper assembly of actin into myofilbrils in Caenorhabditis elegans body wall muscle. J. Cell Biol. 145:491–502.
Ono, S., F. Buratti, C. Stuani, M. Romano, E. Zuccato, M. Niksic, L. Giglio, D. Faraguna, and F.E. Baralle. 2000. Splicing factors induce cytosolic fibrin gels. Nature. 218:339–340.
Ono, S., D.L. Baillie, and G.M. Benian. 1999. UNC-60B, an ADF/cofilin family protein, is required for proper assembly of actin into myofilbrils in Caenorhabditis elegans body wall muscle. J. Cell Biol. 136:487–501.
Shamoo, Y., N. Abdul-Manan, and K.R. Williams. 1995. Multiple RNA binding domains (RBDs) just don’t add up. Nucleic Acids Res. 23:725–728.
Smith, C.W., and J. Valcarcel. 2000. Alternative pre-mRNA splicing: the logic of combinatorial control. Trends Biochem. Sci. 25:381–388.
Spike, C.A., A.G. Davies, J.E. Shaw, and R.K. Herman. 2002. MECL-8 regulates alternative splicing of unc-52 transcripts in C. elegans hypodermal cells. Development. 129:4999–5008.
Stoilov, P., E. Mesheror, M. Gancheva, D. Glick, H. Soroeq, and S. Stam. 2002. Defects in pre-mRNA processing as causes of and predisposition to diseases. DNA Cell Biol. 21:803–818.
Takahashi, N., N. Sasagawa, K. Suzuki, and S. Ishiura. 2000. The CUG-binding protein binds specifically to UG dinucleotide repeats in a yeast three-
hybrid system. Biochem. Biophys. Res. Commun. 277:518–523.
Thirion, C., R. Stucka, B. Mendel, A. Gruhler, M. Jaksch, K.J. Nowak, N. Binz, N.G. Laing, and H. Lochmüller. 2001. Characterization of human muscle type cofilin (CFL2) in normal and regenerating muscle. Eur. J. Bioch-
Thirion, C., R. Stucka, B. Mendel, A. Gruhler, M. Jaksch, K.J. Nowak, N. Binz, N.G. Laing, and H. Lochmüller. 2001. Characterization of human muscle type cofilin (CFL2) in normal and regenerating muscle. Eur. J. Bioch-
Walter, R.H. 1988. Muscle. In The Nematode C. elegans, W.B. Wood, edi-
tor. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 281–335.
Walter, R.H., J.N. Thomson, and S. Brenner. 1980. Mutants with altered muscle structure of Caenorhabditis elegans. Dev. Biol. 77:271–302.
Wicks, S.R., R.T. Yeh, W.R. Gish, H.R. Waterston, and R.H. Plasterk. 2001. Rapid gene mapping in Caenorhabditis elegans using a high density polymorphism map. Nat. Genet. 28:160–164.
Wu, S., C.M. Romfo, T.W. Nilsen, and M.R. Green. 1999. Functional recognition of the 3’ splice site AG by the splicing factor U2AF35. Nature. 402: 832–835.
Zengel, J.M., and H.F. Epstein. 1980. Identification of genetic elements associ-
ed with muscle structure in the nematode Caenorhabditis elegans. Cell Motil. 1:77–97.
Zorio, D.A., and T. Blumenthal. 1999. Both subunits of U2AF recognize the 3’ splice site in Caenorhabditis elegans. Nature. 402:835–838.

A NOVEL MUSCLE-SPECIFIC SPlicing FACTOR • ANYANFUL ET AL. 647

A NOVEL MUSCLE-SPECIFIC SPlicing FACTOR • ANYANFUL ET AL. 647