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

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Tissue-specific alternative pre-mRNA splicing is essential 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 *sup-12* 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 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.,

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Abbreviations used in this paper: ADF, actin depolymerizing factor; A/Q-rich, alanine- and glutamine-rich; EMSA, electophoretic mobility shift assay; RRM, RNA recognition motif.

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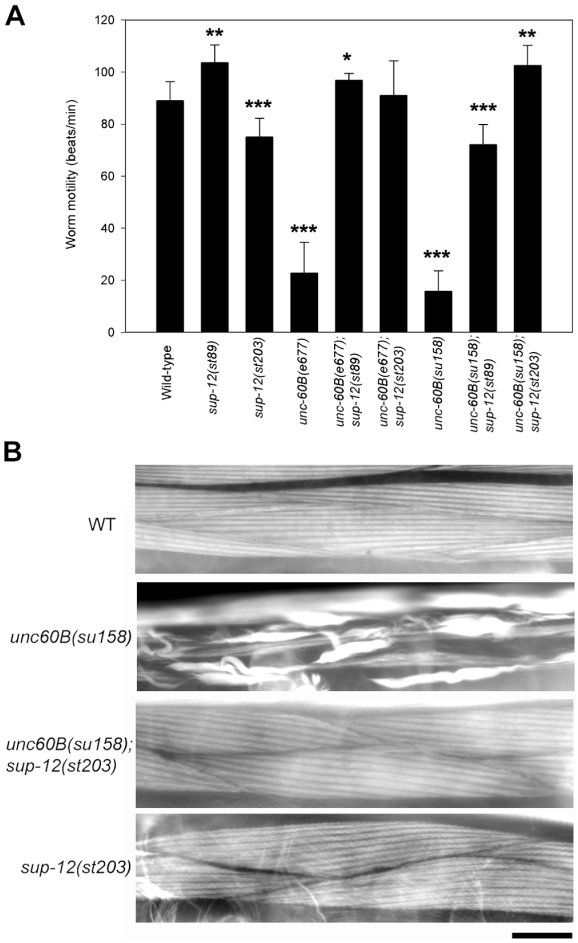

Figure 1. **Suppression of the *unc-60B* mutant phenotype by *sup-12* mutations.** (A) Worm motility was quantified as beating frequency (beats/min) as described previously (Epstein and Thomson, 1974). Data are average ± SD ($n = 10$). Differences between wild-type and various strains were statistically examined by a *t* test. *$P < 0.05$, **$P < 0.005$, and ***$P < 0.001$. (B) Actin organization in the body wall muscle was visualized by staining worms with tetramethylrhodamine-phalloidin. Bar, 20 μm.

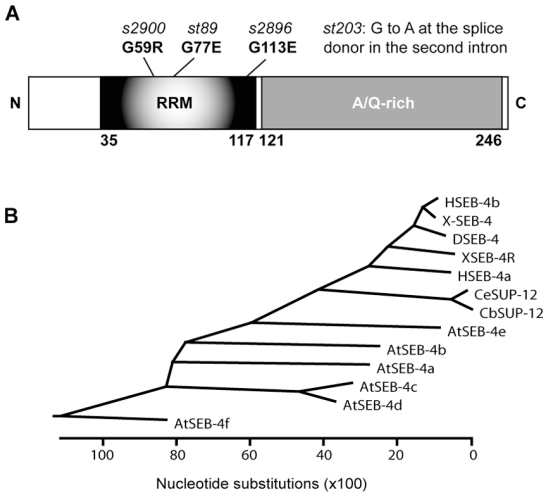

Figure 2. **Sequence of SUP-12.** (A) Domain structure of the SUP-12 protein (248 aa). Positions of the RRM domain, A/Q-rich sequence, and mutations in *sup-12* mutant alleles are shown. (B) A phylogenetic tree of SUP-12 and SEB-4–related proteins generated by a Clustal V method. The sequences used are human SEB-4a (HSEB-4a) (AK095016), human SEB-4b (HSEB-4b) (AY547318), *Xenopus* muscle-type SEB-4 (XSEB-4) (AF223427), *Xenopus* neuronal SEB-4 (XSEB-4R) (AAP42281), zebrafish SEB-4 (DSEB-4) (BAD12194), *C. elegans* SUP-12 (CeSUP-12) (NM_076273), *C. briggsae* SUP-12 (CbSUP-12) (CAE68500), *Arabidopsis* SEB-4a to f (AtSEB-4a to f) (NM_202440, NM_202229, NM_106296, NM_101941, NM_124747, and NM_115334).

1999). 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. 1 A). However, in the *unc-60B* mutant backgrounds, *sup-12* strongly suppressed the motility defects (Fig. 1 A). 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)* (Fig. 1 A), as well as other *unc-60B* loss of function alleles (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. 1 A). 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). Actin was predominantly found in the large aggregates but not in the myofibrils (Fig. 1 B). However, in the *unc-60B;sup-12* double mutants, actin was organized into the myofibrils as well as wild type (Fig. 1 B). The *sup-12* single mutants had no detectable phenotype in the myofibril organization (Fig. 1 B; Francis and Waterston, 1985). *sup-12(st203)* exhibited slightly stronger suppressor effect than *sup-12(st89)* in worm motility (Fig. 1 A) 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.

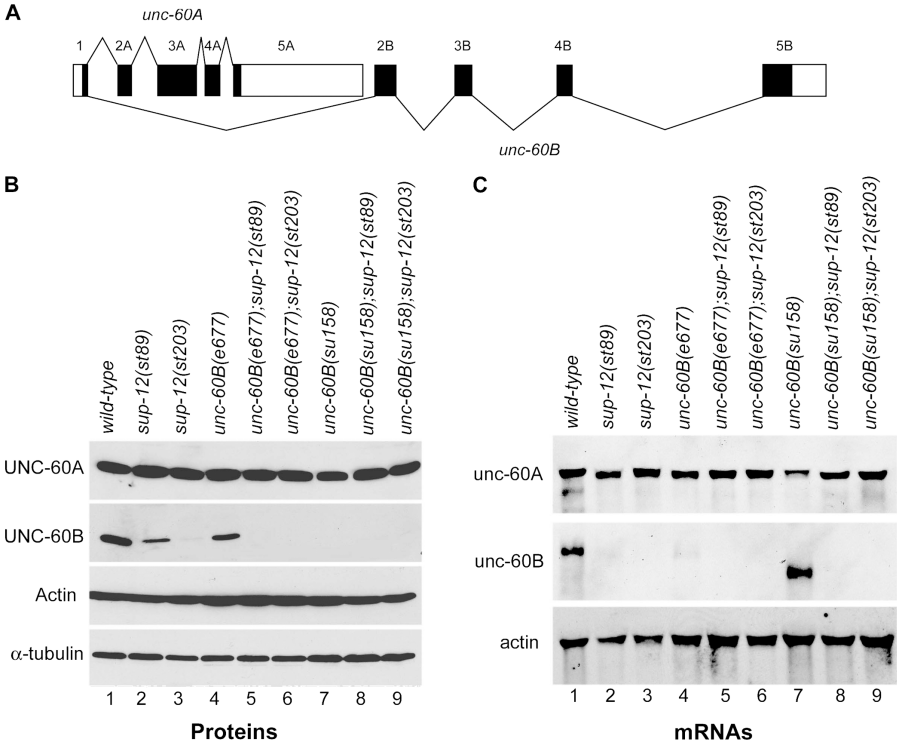

Figure 3. **Altered expression of UNC-60B in the *sup-12* mutants.** (A) Exon-intron structure of the *unc-60* gene (McKim et al., 1994). Boxes represent exons. Protein-coding regions are shown in black. (B) Western blot analysis of the protein levels of UNC-60A, UNC-60B, and SUP-12 in the total worm lysates (20 μg protein). Levels of actin and α-tubulin show nearly equal loading of the proteins. (C) Northern blot analysis of the mRNA levels of unc-60A and unc-60B in the total RNA preparations (10 μg RNA). Levels of actin (act-1) show nearly equal loading of the RNAs.

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 NH_2 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*, *st89*, and *s2896*, respectively (Fig. 2 A). *s2901* has the same mutation as *st89*, 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 suppressor phenotype of *sup-12* mutants 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 NH_2 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.

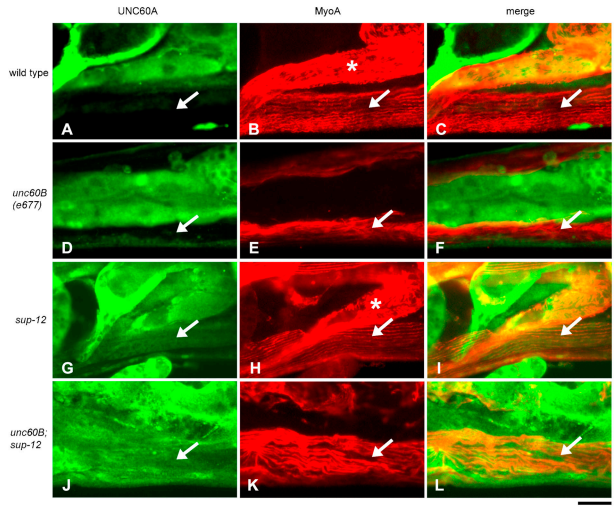

Figure 4. **Altered expression of UNC-60A in the body wall muscle of the *sup-12* mutants.** Wild-type (A–C), *unc-60B(e677)* (D–F), *sup-12(st203)* (G–I), or *unc-60B(e677);sup-12(st203)* (J–L) worms were stained for UNC-60A (A, D, G, and J) and myoA, the muscle-specific myosin heavy chain (B, E, H, and K). Merged images are shown in C, F, I, and L. MyoA is a marker for the body wall muscle (arrows), but it is also expressed in the myoepithelial sheath of the ovary (asterisks). UNC-60A is widely expressed in nonmuscle tissues, but its expression in the body wall muscle is increased in the *sup-12* mutants (arrows). Bar, 20 μm.

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.

Table I. **Dissection of the domains of SUP-12**

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

Neither the NH_2-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 electophoretic 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 NH_2-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-

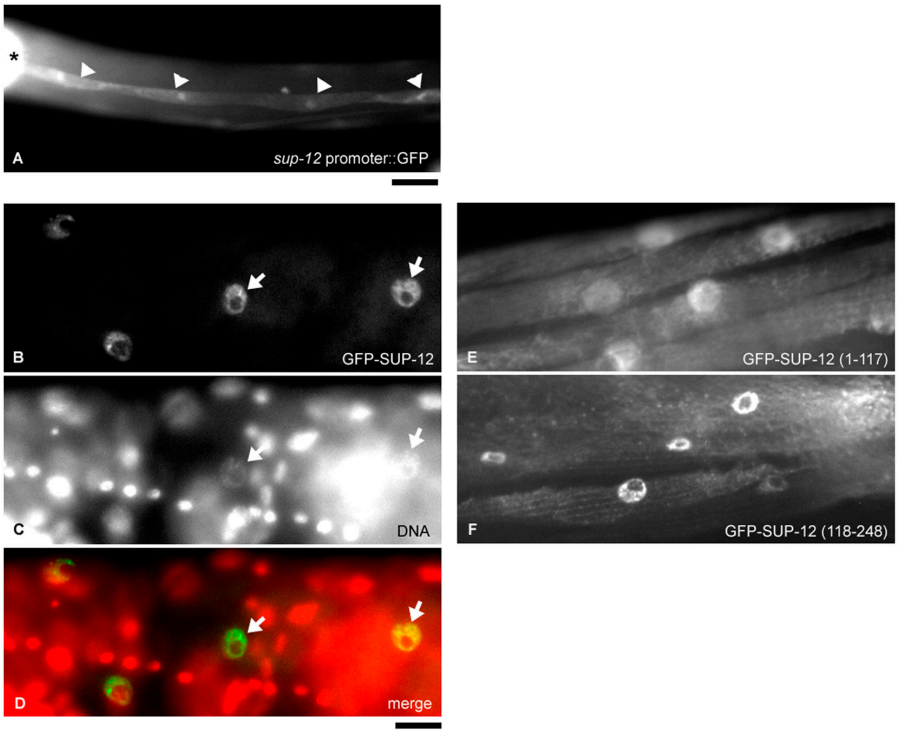

Figure 5. **Expression and subcellular localization of SUP-12.** (A) A 3.1-kb promoter region of *sup-12* drove expression of the GFP reporter in the body wall muscle (arrowheads). The promoter activity was also strong in the pharynx (asterisk). A representative L3 larva expressing GFP is shown. Bar, 20 μm. (B–D) Nuclear localization of the GFP-SUP-12 fusion protein driven by the body wall muscle-specific *myo-3* promoter. Staining of nuclei by DAPI (C) revealed colocalization of some of the muscle nuclei (arrows) with GFP-SUP-12 (D). (E and F) Localization of GFP-SUP-12 (1–117) (RRM domain) (E) or GFP-SUP-12 (118–248) (A/Q-rich) (F). Bar, 10 μm.

tions between GST-SUP-12 (RRM) and four RNA fragments, A1-1, A1-2, A1-3, and A1-4, within the *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′-UCUCUCCCUC-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 *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 *unc-60*. *sup-12* mutations suppressed muscle-specific defects in actin organization which was caused by *unc-60B* mutations. This suppression involved switching of the *unc-60* isoforms in the muscle cells: *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 *unc-60* pre-mRNA in vitro, suggesting that SUP-12 is directly involved in muscle-specific splicing of the *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

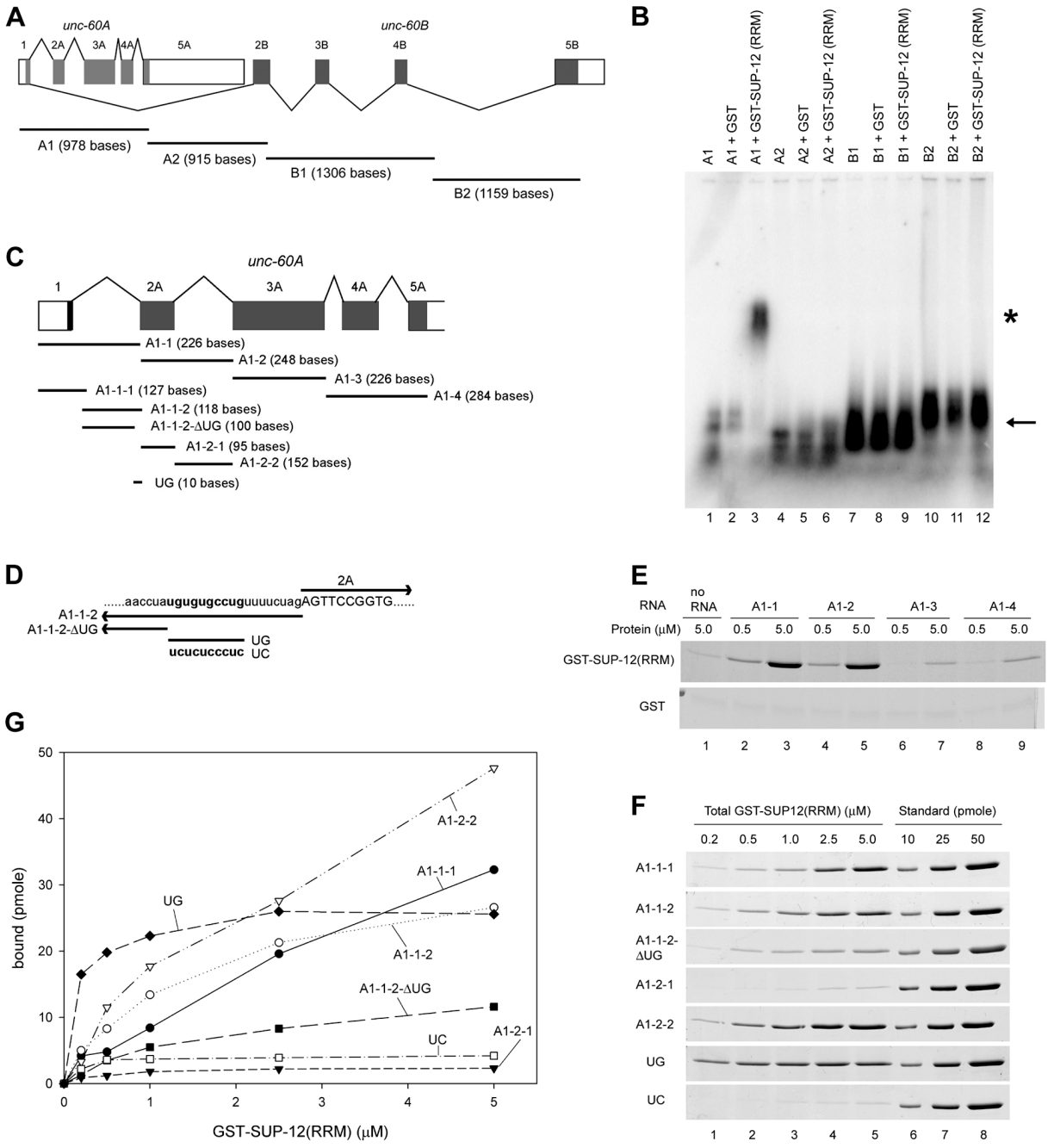

Figure 6. **Direct interaction of the SUP-12 RRM domain with the *unc-60* pre-mRNA.** Interactions between the SUP-12 RRM domain and fragments of the *unc-60* pre-mRNA were examined by EMSA (A and B) or a biotin-RNA pull-down assay (C–G). (A) Four synthetic RNA fragments A1, A2, B1, and B2, were transcribed in vitro and used for EMSA with GST or GST-SUP-12 (RRM domain). (B) ^{32}P-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 (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.

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-

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(st89)* and *sup-12(st203)* were obtained from G.R. Francis (Exelixis, Inc., South San Francisco, CA) and R. Waterston (University of Washington, Seattle, WA). *sup-12(s2896)*, *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 *mnDp33* (G.R. Francis and R.H. Waterston, 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-phalloidin (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-myoA (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 IPLab 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 membranes (Millipore), and fixed by ultraviolet irradiation. cDNAs for *unc-60A* (505 bp), *unc-60B* (460 bp), and actin (*act-1*) (1.1 kb) were amplified by PCR, la-

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 GFP-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 58-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 (yk1125e08, obtained from Y. Kohara, National Institute of Genetics, Mishima, Japan) was ligated in-frame 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 *unc-60B(su158); sup-12(st89)* at 10 μg/ml together with a dominant marker pRF4(*rol-6(su1006)*) at 90 μg/ml, and transgenic worms were selected by their roller phenotype or expression of GFP.

Electrophoretic mobility shift assay

A cDNA fragment encoding residues 1–117 of SUP-12 and a synthetic stop codon was amplified by PCR and cloned into the SmaI–EcoRI cloning site of a GST expression vector pGEX-2T (Amersham Biosciences). The insert was sequenced to confirm that no mutations were introduced by PCR. GST alone or GST-SUP-12 (RRM) was expressed in *Escherichia coli* BL21 (DE3) by induction with 0.1 mM IPTG for 3 h and purified with Glutathione-Uniflow (BD Biosciences Clontech) following manufacturer's instruction.

Template DNA fragments used in the in vitro transcription reactions were amplified from *C. elegans* genomic DNA using the primers listed in Tables S1 and S2 (available at http://www.jcb.org/cgi/content/full/jcb.200407085/DC1). The sense primers contain the SP6 promoter sequence. Each RNA was in vitro transcribed using the MAXIscript kit (Ambion) in the presence of 50 μCi of α-[^{32}P]CTP (Amersham Biosciences) and purified with a Sephadex G-25 spin column (Amersham Biosciences). RNAs (5×10^5 cpm) were denatured in water at 95°C for 2 min, chilled on ice for 2 min, and incubated with either buffer, or buffer and 1 μM GST or buffer and 1 μM GST-SUP-12 (RRM) for 30 min at RT. The buffer contained 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 40 mM DTT, 0.1 mg/ml yeast tRNA, and 10 mM Hepes, pH 7.4). The samples were resolved on a 1% agarose gel in 1 × TAE (40 mM Tris-acetate, 2 mM EDTA, pH 8.3). The gels were dried and analyzed by the PhosphorImager (Molecular Dynamics).

Biotin-RNA pull-down assay

Biotin-RNA pull-down assay (Lee and Schedl, 2001) was performed with the following modifications. Template DNAs for in vitro transcription of RNAs (95–284 bases) were amplified from *C. elegans* genomic DNA using the primers listed in Tables S1 and S2. The sense primers contain the T7 promoter sequence. Biotin-labeled RNAs were in vitro transcribed with T7 RNA polymerase (Invitrogen) in the presence of Biotin RNA Labeling Mix (Roche Applied Science) at 37°C for 2 h. The template DNAs were digested by RNase-free DNase I (Roche Applied Science) and the labeled RNAs purified with SigmaSpin Post-Reaction Clean-Up Columns (Sigma-Aldrich). RNA oligonucleotides UG (5′-UGUGUGCCUG-3′) and UC (5′-UCUCUCCCUC-3′) were synthesized and chemically labeled by biotin at the 5′-ends by Integrated DNA Technologies.

Biotin-labeled RNA at 0.1 μM was incubated with GST or GST-SUP-12 (RRM) in RP buffer (50 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes-NaOH, pH 7.5) containing 10 mM DTT and 100 μg/ml yeast tRNA (Ambion) in a final volume of 200 μl at RT for 30 min. The mixtures were incubated with 0.1 mg of streptavidin MagneSphere Paramagnetic Particle (Promega) at RT for 20 min. The magnetic particles were isolated with a magnetic separation stand, washed three times with RP buffer, suspended in 20 μl of SDS sample buffer (2% SDS, 80 mM Tris-HCl, 5% β-mercaptoethanol, 15% glycerol, 0.05% bromophenol blue, pH 6.8), and incubated at 97°C for 2 min. Bound proteins were analyzed by SDS-PAGE (12% acrylamide gel) and staining with Coomassie Brilliant blue R-250 (National Diagnostics). The Coomassie-stained gels were scanned by a UMAX PowerLook III scanner at 300 dots per inch and the band intensity was quantified with Scion Image Beta 4.02 (Scion Corporation) by comparing with the intensities of known amounts of GST-SUP-12 (RRM).

Online supplemental material

Table S1 contains a list of PCR primers for amplifying template DNAs for in vitro transcription. Table S2 contains a list of primer combinations for PCR amplification of the template DNAs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200407085/DC1.

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