HRP-2, the *Caenorhabditis elegans* Homolog of Mammalian Heterogeneous Nuclear Ribonucleoproteins Q and R, Is an Alternative Splicing Factor That Binds to UCUAUC Splicing Regulatory Elements*

Received for publication, May 20, 2009, and in revised form, August 18, 2009. Published, JBC Papers in Press, August 24, 2009, DOI 10.1074/jbc.M109.023101

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Alternative pre-mRNA splicing is a mechanism for generating multiple mRNA isoforms from a single gene. This process can allow a gene to encode for more than one protein isoform. For some genes, it is a mechanism for regulating message stability through production of alternative mRNA isoforms that are substrates for the nonsense-mediated mRNA decay pathway (1). The majority of human genes undergo alternative splicing (2), and the process can be regulated in tissue-specific and developmental stage-specific manners. Current models propose that *cis* elements on the pre-mRNA, in exons and introns, serve as recognition sites for *trans*-acting protein factors that bind to the pre-mRNA and regulate assembly of the splicing machinery, thus regulating splice site choice (3).

In recent years, a number of groups have employed bioinformatics techniques to identify *cis* splicing regulatory elements (4). These techniques include using multiple interspecies sequence alignments to identify conserved intronic regions, identification of short sequences in exons that are bounded by weak consensus splice sites, and identification of common intronic sequences flanking similarly regulated alternative exons (5–9). These efforts have added many new sequences to the list of known and potential splicing regulators. The identification of the protein factor partners for these sequences will be important for understanding their function in alternative splicing regulation.

Experimental approaches have identified alternative splicing factors that interact with specific *cis* elements (10), but the number of *trans* factors discovered still lags behind the number of newly identified *cis* element partners. Some examples of well-characterized *cis* element/*trans*-acting factor interactions include the NOVA K homology domain splicing factor binding to the sequence UCAY (11), the FOX splicing factors binding to the sequence UGCAUG (12–14), and hnRNP F/H proteins binding to the sequence GGGG (15, 16). By using cross-linking immunoprecipitation followed by large scale sequencing, entire catalogs of RNAs that the splicing factors NOVA, SF2/ASF, and FOX2 bind to in vivo have been determined (17–19). These approaches have led to models for how the proteins binding to their *cis* regulatory elements may alter splicing. These models include a role for the relative position of a *cis* element to an alternative cassette exon in determining alternative exon inclusion or skipping (18, 19).

In a previous bioinformatics analysis of evolutionarily conserved intronic sequences flanking alternatively spliced exons, we identified the hexamer sequence UCUAUC as a novel splicing regulatory element (8). UCUAUC is found flanking both sides of alternative exon 16 of the *unc-52* gene of *Caenorhabditis elegans*. Genetic analysis of a class of viable *unc-52* mutants led to the discovery that exons 16–18 are alternative cassette exons and that every combination of skipping and inclusion of these three exons occurs (20). This splicing is regulated by the alternative splicing factor MEC-8 (21). Fig. 1A shows a schematic diagram of the alternatively spliced region of *unc-52*, with the MEC-8-enhanced alternative splicing events indicated. Using an *unc-52* splicing reporter gene containing alternative exons 15–19, we previously reported that alternative splicing is regulated by the intronic motif UCUAUC in the intron downstream of exon 16 (8). In addition we showed that

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*This work was supported, in whole or in part, by National Institutes of Health Grant RO1-GM061646 (to A. M. Z.).

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3 The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; RNAi, RNA interference; RT, reverse transcription.
HRP-2 Binds to UCUAUC and Regulates Splicing

In this study, we report the results of a biochemical identification of a protein factor from *C. elegans* that binds to the UCUAUC intronic splicing regulatory element. We transcribed different short RNA sequences containing the UCUAUC element in its native intronic context, or as part of a repeating unit, and immobilized these onto agarose beads. After passing embryo extracts across these beads, we found that the protein HRP-2, the *C. elegans* homolog of the mammalian hnRNP Q/R proteins, binds to this sequence with high affinity. By using RNAi to reduce the level of HRP-2 in worms, we observed changes in alternative splicing of *unc-52* and *lin-10*, two genes that contain UCUAUC elements in introns flanking alternative exons. We propose that HRP-2 is an alternative splicing factor that works through the UCUAUC intronic elements to regulate alternative splicing.

**EXPERIMENTAL PROCEDURES**

**Growth of *C. elegans* and Preparation of Embryo Protein Extract—** *C. elegans* Bristol N2 was grown on egg plates at room temperature as previously described (22). Embryos were collected from gravid adults by using a 1% sodium hypochlorite, 0.5 mM NaOH solution. The embryos were flash-frozen and ground with a mortar and pestle under liquid nitrogen. 10 g of embryo powder were added to extract buffer for a final volume of 40 ml (extract buffer, 20 mM HEPES, pH 7.5, 140 mM potassium chloride, 5 mM magnesium chloride, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). The mixture was homogenized for 10 strokes in a metal Dounce homogenizer and then stirred for 30 min at 4 °C. Crude extract was centrifuged at 27,000 g for 15 min at 4 °C, and supernatant was transferred to a new tube and centrifuged at 140,000 g for 1 h at 4 °C. Supernatant was then dialyzed with a 10,000 Mr cut-off membrane in extract buffer with 20% glycerol at 4 °C overnight. Aliquots were flash-frozen in liquid nitrogen.

**RNA Transcription and Immobilization on Agarose Beads—** Substrate RNAs were synthesized by *in vitro* transcription using T7 RNA polymerase and DNA oligonucleotide templates. RNAs were oxidized and linked to adipic acid dihydrazide-agarose beads following previously published methods (23). Beads with bound RNA were washed two times with 2 M NaCl and three times with extract buffer. Typical binding efficiency was >80% as measured by UV spectrophotometry.

**Protein Binding Assays and Analysis—** Agarose beads bound to RNA were incubated with 200 μl of embryo extract, with rotation at room temperature for 1 h. Beads were washed four times with extract buffer, and proteins that remained bound were eluted by addition of 60 μl of SDS-PAGE protein sample buffer containing 2% β-mercaptoethanol. Proteins were separated by 12% SDS-PAGE and visualized by Coomassie Blue staining, silver staining, or immunoblot analysis. For immunoblot assays, proteins from the gel were electroblotted onto BA-85 nitrocellulose membrane and probed using R64Ab. R64Ab is a polyclonal antibody raised against HRP-2 and was a generous gift from the Devaney lab (24). Secondary anti-rabbit horseradish peroxidase antibody (Amersham Biosciences) was used and visualized using the SuperSignal West chemiluminescence kit (Pierce).

**Protein Identification—** Data for protein identification were acquired at the Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University. Coomassie-stained protein bands were excised, followed by in-gel trypsin digestion. Trypsin-generated peptides were analyzed by liquid chromatography-coupled tandem mass spectrometry. Proteins were identified by comparing the mass spectrometry data against the NCBInr Protein Database through the Mascot search engine.

**hrp-2 RNAi Vector Construction and Feeding Protocol—** Genomic fragments of the *hrp-2* gene were obtained by PCR using the sequences 5'-AAATCTGCTGGAAACATCAAA (forward) and 5'-TAGCAGGGTTCTTCCCAA (reverse) and cloned into the Timmons and Fire L4440 feeding vector (25). This vector was transformed into HT115 cells for isopropyl-β-D-thiogalactopyranoside-induced expression (26). An empty version of L4440 was transformed into HT115 cells as the feeding control. N2 worms were grown on egg plates until gravid, and their embryos were harvested by sodium hypochlorite/NaOH treatment and then hatched in M9 buffer during shaking overnight. Starved L1 worms were then transferred to 150-mm-diameter RNAi plates; growth and induction of RNAi bacteria were performed as previously described (27). *C. elegans* strains were grown for 60 h at 20 °C to the young adult stage before harvest. RNA was extracted from worms as previously described (28).

**Confirmation of HRP-2 Knockdown—** The extent of knockdown of *hrp-2* expression levels by RNAi was determined by two assays. First, embryonic lethality was monitored. Bacteria-fed RNAi plates were monitored for 24 h after harvest. The number of eggs remaining on the plate were counted and examined for hatching. The RNAi phenotype resulted in typically >90% lethality (no hatching), whereas control plates experienced almost 100% survival. Second, RT-PCR was performed to monitor the levels of *hrp-2* mRNA. RT-PCR of *daf-15* was used as a loading control to compare relative amounts of *hrp-2* RT-PCR product from *hrp-2* (RNAi) and control samples (L4440). RNA was extracted from worms as previously described (28). cDNAs were synthesized in 20-μl reaction mixtures. The annealing mixture contained 2 μg of total RNA and 25 pmol of anchored oligo(dT)20 primer, and we followed the protocol outlined for SuperScript III (Invitrogen). 1.0 μl of the cDNA reaction mixture was used as the template in a 25-μl PCR. The reaction mixtures also contained 1X PCR buffer, 0.25 mM each of the four dNTPs, Taq DNA polymerase, and 1 pmol of each primer (HRP-2, 5'-CACAGGAAGAAGAAGAAGAAGCC and 5'-GCCATTCCTTCTTAATTCAC; DAF-15, 5'-GGAGATGCTGGAATCACA and 5'-TCAACCCGTAATCTAGGACA). Reaction mixtures were incubated for 27 cycles of 94 °C for 1.0 min, 55 °C for 1.0 min, and 72 °C for 45 s.

**RT-PCRs for Measuring unc-52 and lin-10 Alternative Splicing—** RT-PCR was performed using the SuperScript III One-Step RT-PCR kit (Invitrogen) following the manufacturer’s recommendations. PCR products were analyzed using an Agilent Bioanalyzer 2100 with the Agilent DNA 1000 kit. Cassette exon skipping proportions were calculated from the molar concentrations of each isoform as reported by the Bioanalyzer.
HRP-2 Binds to UCUAUC and Regulates Splicing

A. **unc-52**

![Diagram of splicing regulation](image)

B. **Substrate name** | **RNA sequence**
--- | ---
Control | 5'gaauuuccagauucaaugagagaacauuuccagauuucaguuccagag 3' |
Intron 16 | 5'gaacauuucuaucuccuaagauuuccuauugcaauuucuc 3' |
Intron 16ΔUCUAUC | 5'gaacauu------------cuauuagauuuccuauugcauuc 3' |
Intron 15 | 5'gaugcauugaaacuuuuccuucuauucuaucgaucaaacuuuuccuauucc 3' |
Intron 15ΔUCUAUC | 5'gaugcauugaaacuuuuccu------------cuauucuauuccuauucc 3' |
4X UCUAUC | 5'guuucuauuucuauucuauuauuccuauuccuauuccuauucc 3' |
4X UCUAGC | 5'guuucuagccuuuucagccuuuucagccuuuucagccuuuucagccuuuucagccuu 3' |

C. **Image of protein levels**

D. **Image of HRP-2 expression**

E. **Image of HRP-2 concentration**
2100 software (Agilent). The unc-52 PCR primer sequences were previously reported (21). The lin-10 primers used were 5′-AAGAATCATCAGGGAGGAGGA-3′ and 5′-GATGT-GGTGTCATCATCAGG-3′.

unc-52 In Vivo Splicing Reporters—The construction of the unc-52 splicing GFP-lacZ fusion reporter genes, for both the wild-type sequence and the intron 16 ąUCUAUC construct, was described previously (8). Adult roller worms expressing the trans gene were put onto the RNAi feeding plates for 12 h and allowed to lay eggs. The adults were removed, after 72 h 50 young adult F1 roller progeny from each plate were picked, and RNA was extracted using the Absolutely RNA micro prep kit (Stratagene). For the 3′ RT-PCR primer, we used a primer that hybridizes to the 3′ end of the lacZ gene in the reporter construct. For the 5′ primer, we used the unc-52 exon 16 primer described above for the native gene.

RESULTS

Splicing Regulatory Motif UCUAUC Is Bound by HRP-2—To identify trans-acting factors that recognize the UCUAUC regulatory motif, RNA affinity chromatography was performed (15, 23). In this assay, in vitro transcribed RNAs are covalently linked to agarose beads by their 3′ ends. These bead-linked RNAs are then used to affinity purify sequence-specific factors from C. elegans embryo extracts. Proteins that remain bound to the RNA after a series of wash steps are eluted and visualized by SDS-PAGE and Coomassie or silver staining. Unique bands are then analyzed by mass spectrometry and confirmed by immunoblotting.

Short RNA substrates were designed to identify proteins that interact with UCUAUC (Fig. 1B). The short RNA “intron 16” is derived from native intronic sequence downstream of alternatively spliced unc-52 exon 16 and contains within it the previously confirmed UCUAUC regulatory element. A control version of this substrate, “intron 16 ΔUCUAUC,” is the same sequence with a deletion of the UCUAUC hexamer. Intron 15 upstream from exon 16 also contains within it an evolutionarily conserved UCUAUC sequence. Short RNA “intron 15” has the UCUAUC sequence in its native context repeated two times. “Intron 15 ΔUCUAUC” has the two UCUAUC elements. A 41-nucleotide-long RNA substrate containing four repeats of the UCUAUC motif (4× UCUAUC) and a similar sequence with four repeats of UCUAUC (4× UCUAUC) were also tested. A 53-nucleotide substrate RNA lacking any of the top 20 motifs from our compendium of intronic splicing regulatory elements (8) was also tested (control). RNAs were oxidized, covalently attached to agarose beads, and challenged with N2 embryo extract. Proteins that remained bound to the test RNA sequences after washing were then eluted and analyzed by SDS-PAGE. Coomassie staining revealed several bands that were submitted for mass spectrometry analysis (Fig. 1C).

Several protein bands were identified that had unique specificities in binding to the various substrates. A protein of ~30 kDa bound to all test sequences except the control. Mass spectrometry proteomic analysis identified this protein as HRP-1, the nematode homolog of the mammalian splicing factor hnRNP A1. A small (~20-kDa) protein was observed to bind only to the 4× UCUAUC-containing RNA and was identified as CEY-1. The cey-1 mRNA was identified originally as one of the most ubiquitous mRNA transcripts in C. elegans embryos (29). It encodes a protein of unknown function with a cold shock/Y-box domain. The binding of CEY-1 to the RNA substrate was not consistently reproducible, so the potential activity of CEY-1 in splicing regulation was not pursued.

A protein of ~70 kDa was observed to bind to the intron 16 and 4× UCUAUC substrates, but it bound with much weaker affinity to the intron 16 ΔUCUAUC substrate, the 4× UCUAGC substrate, and the control substrate. This protein appeared to potentially have the binding specificity of a UCUAUC-interacting factor. Mass spectrometry of this band identified it as HRP-2. The hrp-2 gene is essential for oogenesis and embryogenesis in the worm. It encodes a homolog of mammalian proteins hnRNPs Q and R. Similar to many known hnRNP protein factors, HRP-2 has three RNA recognition motifs and a C-terminal region enriched in RGG repeats; the protein is localized to the nucleus (24).

To confirm the specificity of HRP-2 for our RNA sequences, we used Western blot analysis to probe RNA affinity chromatography fractions. A polyclonal antibody specific for the N-terminal region of HRP-2 had been previously reported and was kindly provided to us by Eileen Devaney (24). Fig. 1D shows a silver-stained SDS-polyacrylamide gel of an RNA affinity chromatography experiment side by side with an anti-HRP-2 immunoblot of the same samples. The intensity of the polyclonal antibody staining confirms the identification of the enriched 70-kDa protein that binds to the UCUAUC-containing substrates as HRP-2. Using the HRP-2 antibody, we tested the requirement of UCUAUC for HRP-2 binding within the context of the native unc-52 intron 15 and intron 16 sequences. Deletion of UCUAUC from either of these substrates leads to a dramatic decrease in the affinity of HRP-2 for these introns (Fig. 1E).

Specificity of HRP-2 Binding to UCUAUC Sequences in RNA—As demonstrated in Fig. 1C, binding efficiency of the HRP-2 protein for the UCUAUC hexamer can be reduced with the alteration of just 1 base in the sequence. In an attempt to better understand the importance of each base of the hexamer in its recognition by HRP-2, an RNA affinity chromatography experiment was performed with C. elegans embryo extracts and RNA substrates containing alterations at each base position of the UCUAUC hexamer (Fig. 2). Anti-
HRP-2 binds to UCUAUC and regulates splicing

A.

| Substrate name | RNA Sequence (5' to 3') |
|----------------|-------------------------|
| Control       | gauausccgagugugagugacauacgccagauacauauauauauauauaguag |
| UCUAUC        | guauausccgagugugagugacauacgccagauacauauauauauauauaguag |
| GCUAUC        | guauausccgagugugagugacauacgccagauacauauauauauauauaguag |
| UUCGAC        | guauausccgagugugagugacauacgccagauacauauauauauauauaguag |
| UCUAUA        | guauausccgagugugagugacauacgccagauacauauauauauauauaguag |
| UCUAGC        | guauausccgagugugagugacauacgccagauacauauauauauauauaguag |
| UCUAGC        | guauausccgagugugagugacauacgccagauacauauauauauauauaguag |
| UCUAUA        | guauausccgagugugagugacauacgccagauacauauauauauauauaguag |
| UCUAGC        | guauausccgagugugagugacauacgccagauacauauauauauauauaguag |

HRP-2 immunoblots of two representative independent RNA affinity chromatography experiments are shown.

B.

![HRP-2 immunoblot](image)

**FIGURE 2. Analysis of the sequence specificity of HRP-2 binding to RNA.** A, the table shows the sequences of the different immobilized RNA substrates tested. B, HRP-2 immunoblot of proteins bound to RNA substrates. Embryo extracts were incubated with the indicated immobilized RNAs. Proteins bound to the RNAs were separated by SDS-PAGE and immunoblotted with anti-HRP-2 polyclonal antibody. Immunoblots of two representative independent RNA affinity chromatography experiments are shown.

HRP-2 binds to UCUAUC and regulates splicing. The preferential binding of HRP-2 to the consensus alternative splicing cis regulatory element UCUAUC suggests a potential role for HRP-2 in alternative splicing regulation. Also suggestive of this is the recent report that human hnRNP Q is involved in the splicing regulation of SMN2 (30). Testing a functional role for HRP-2 in *C. elegans* in vivo is challenging because this protein is essential for oocyte formation and embryogenesis in this organism (24). To address this problem, we performed RNAi by feeding worms bacteria that produce double-stranded RNA (26). To avoid the embryonic lethality of *hrp-2(RNAi)*, we started with wild-type *C. elegans* embryos and allowed them to hatch overnight in liquid medium without food, which stopped their development at the L1 stage. We took these synchronized L1 worms and put them on plates with bacterial food that expressed double-stranded RNA for *hrp-2*, and in parallel we grew worms on control plates containing bacterial food harboring the same plasmid vector (L4440) with no gene insert. Worms were allowed to develop to young adults, the stage at which they begin to produce mature oocytes and embryos, at which time they were harvested and RNA was extracted.

We first tested whether *hrp-2(RNAi)* had an effect on the alternative splicing of *unc-52*. Lundquist et al. (21) reported an RT-PCR assay in which amplification of a region between exons 16 and 19 of *unc-52* showed an easy-to-measure splicing difference between the wild type and mec-8 mutants (Fig. 3A). Using this approach, we tested for differences in splicing of *unc-52* between young adult N2 worms fed bacteria harboring a control RNAi vector, young adult N2 worms receiving *hrp-2(RNAi)*, and young adult mec-8(e398) mutant worms. As shown in Fig. 3A, *hrp-2(RNAi)* causes an increase in the skipping of exons 17 and 18 (from 78 to 91%), whereas the mec-8 mutant has the opposite effect, causing a dramatic decrease to 5% skipping of exons 17 and 18. We take these results as a confirmation that HRP-2 is an alternative splicing regulator and that it functions in alternative splicing of a gene for which UCUAUC is known to be a regulator. It is interesting that every intron in this region for *C. elegans* has a UCUAUC element. These intronic UCUAUC elements, with the exception of the one in intron 17, are conserved in *Caenorhabditis briggsae* as well.

We next tested RNA from these same worms to identify other genes whose alternative splicing is potentially regulated by HRP-2. *lin-10* encodes a PDZ and phosphotyrosine-binding domain-containing protein that is required for polarized protein localization in the plasma membrane (31). This gene contains a conserved UCUAUC intronic element upstream of alternatively spliced cassette exon 6. Data from our lab show that alternative splicing of the *lin-10* gene is regulated by MEC-8 in embryos. Because *lin-10* has both a conserved intronic UCUAUC and MEC-8 regulation in common with *unc-52*, we decided to test whether *lin-10* splicing is regulated by HRP-2 as well. We found that the alternative splicing of the HRP-2 is an alternative splicing factor...
lin-10 gene in young adults changes under hrp-2(RNAi) (Fig. 3B). For the young adult N2 worms that were fed bacteria containing the control L4440 plasmid, exon 6 of lin-10 was skipped in 27% of the steady-state messages, whereas for the hrp-2(RNAi) young adults, 46% of the lin-10 mRNAs skipped exon 6. Therefore, HRP-2 is required to promote inclusion of exon 6, and its absence in an RNAi experiment leads to an increase in exon skipping. In synchronized mec-8(e398) young adults, there was a reproducible decrease in skipping of lin-10 exon 6 compared with N2: 27% skipping in N2 versus 22% skipping in mec-8(e398) (Fig. 3B). Therefore, lin-10 alternative splicing is regulated by both HRP-2 and MEC-8. The intron upstream of alternative cassette exon 6 contains an evolutionarily conserved UCUAUC element, consistent with potential HRP-2 binding, and the intron downstream of exon 6 contains an evolutionarily conserved GCAUG element. This is interesting in light of the fact that these two elements are both found flanking alternative cassette exons in unc-52. Knockdown of HRP-2 by RNAi does not generally lead to a change in all alternative splicing events. Other alternatively spliced genes that we tested, for example, nca-1 and Y69H2.3, whose alternative cassette exons are not flanked by UCUAUC intronic elements, showed no differences in alternative splicing in young adults between the L4440 control and hrp-2(RNAi) worms (data not shown).

We next tested unc-52 alternative splicing in worms undergoing a simultaneous loss of mec-8 and hrp-2 function (Fig. 3C). To do this, we employed an unc-52 in vivo reporter system that we had previously developed (8). For a reporter with the wild-type sequence of the unc-52 alternatively spliced region, hrp-2(RNAi) led to an increase in the relative steady-state level of exon 16–19 spliced messages relative to other exon 16-containing isoforms (from 63 to 86%), consistent with the changes detected
in the native gene. The same reporter in a mec-8(e398) mutant background showed a decrease to 21\% exon 16–19 steady-state splicing levels, also consistent with the changes of unc-52 alternative splicing detected in the native gene in an e398 background. RNAi of hrp-2 in a mec-8(e398) mutant background led to an increase of exon 16–19 splicing of the reporter to 30\%. These data confirm that MEC-8 and HRP-2 have opposing functions in unc-52 alternative splicing. Because hrp-2(RNAi) still affects unc-52 alternative splicing in a mec-8 mutant background by changing the splicing in the direction predicted for loss of hrp-2, these results indicate that these two factors are likely to work independently to affect the alternative splicing process.

We previously demonstrated that the UCUAUC hexamer in intron 16 of unc-52 is a cis splicing regulatory element (8). To determine whether this sequence is responsive to HRP-2, we compared the splicing of an intron 16 UCUAUC deletion splicing reporter (\(\Delta\)UCUAUC) with that of the wild-type sequence reporter. As can be seen in Fig. 3C, deletion of this hexamer increased the steady-state level of exon 16–19 spliced isoforms from 63\% for the wild-type reporter to 71\% for the \(\Delta\)UCUAUC reporter. This is the direction of change predicted if the role of the hexamer is to recruit HRP-2. The \(\Delta\)UCUAUC reporter is also less responsive to hrp-2(RNAi). It showed only a small change in steady-state levels of the 16–19 isoform, from 71 up to 75\%. Overlapping error bars from the RNAi control indicate that this is not a significant change. This lowered responsiveness to loss of HRP-2 is a result that might be expected if the UCUAUC sequence were required for HRP-2 function. These results are consistent with the UCUAUC element in intron 16 being important for HRP-2 activity in the alternative splicing of the gene. We also tested a larger deletion of intron 16 that contained the UCUAUC hexamer along with 22 additional downstream bases, including another highly conserved element (deletion B in Ref. 8) (data not shown). That larger deletion in the reporter construct did not change the basal level of exon 16–19 splicing from the wild-type sequence reporter, even though it removed the UCUAUC hexamer. The larger deletion may have also removed an enhancer of alternative splicing in addition to the UCUAUC element (8), which may have balanced any changes from loss of UCUAUC alone.

**DISCUSSION**

In recent years, many groups have focused on the importance of identifying cis regulators of alternative splicing in an effort to crack the “splicing code” (reviewed in Ref. 4). In previous work, we identified several conserved nematode splicing regulatory elements, including the novel hexamer sequence UCUAUC (8). We found it to be 6.96 times more likely to be found in evolutionarily conserved regions in introns flanking alternatively spliced exons than in total introns. This sequence is 2.55 times more likely to be found in an intron flanking an alternatively spliced exon than in introns flanking constitutive exons, and UCUAUC is more likely to appear in an intron upstream of an alternative cassette exon than in the downstream intron. We also showed that an intronic UCUAUC has a regulatory role in unc-52 alternative splicing. In the current work, we demonstrate that the protein HRP-2 preferentially binds to RNAs containing this sequence. Our analysis of different variations of this hexamer sequence indicates that the three U residues at the first, third, and fifth positions of the hexamer are important determinants for HRP-2 recognition of this sequence. hrp-2(RNAi) experiments demonstrated that HRP-2 regulates the alternative splicing of unc-52 and lin-10, two genes whose alternative cassette exons have UCUAUC elements in a flanking intron. Both genes have been previously demonstrated to have similar developmental regulation of alternative splicing that has been conserved between C. elegans and C. briggsae, consistent with the evolutionary conservation of their UCUAUC and UGCAUG intronic flanking elements (32).

HRP-2 is the nematode homolog of the mammalian hnRNQ and R proteins, which both share almost 50\% amino acid identity in their three RNA-binding domains with HRP-2 (24). Both mammalian proteins have been shown through biochemical assays to interact and colocalize with the splicing-associated SMN protein complex; depletion of either protein reduces the efficiency of splicing in in vitro assays (33, 34). Mammalian hnRNQs Q and R were both identified by mass spectrometry as being associated with the splicingosome (35). hnRNQ Q1 in mammals is a regulator of exon 7 splicing of the SMN2 gene; it was shown to bind to the exon in a manner that was dependent on the C-terminal RGG repeat region (30). Two alternatively spliced isoforms of hnRNQ Q, Q2 and Q3, failed to bind to exon 7 of SMN2; interestingly, Q3 has the same three RNA recognition motifs as Q1 but differs in that it has a longer C-terminal domain. There were no UCUAUC sequences in the exon 7 region of SMN2 tested in that study (30), so we are unable to comment on whether hnRNQ Q and HRP-2 have similar sequence specificities for RNA binding. It is clear from the SMN2 study that regions of the hnRNQ Q protein outside of the RNA recognition motifs have a role in hnRNQ Q sequence-specific RNA-binding activity. In a recent systematic study, RNAi of 14 human hnRNPs was used to look for effects on 56 alternative splicing events associated with apoptosis (36). Knockdown of hnRNQ Q had significant effects on four different alternative splicing events, and knockdown of hnRNQ R had significant effects on three com-

**FIGURE 3. HRP-2 is an alternative splicing factor.** A, unc-52 alternative splicing. The diagram indicates the locations of RT-PCR primers in the alternatively spliced region of unc-52. N2 embryos were hatched and grown to young adults on hrp-2(RNAi) feeding bacteria or L4440 control vector bacteria, and RNA was extracted. RNA was also extracted from young adult mec-8(e398) worms synchronized under the same conditions. RT-PCR was performed with the indicated primers, the products were separated on an Agilent Bioanalyzer, and the relative levels of the different products were calculated. The image shows a pseudogel generated from the Bioanalyzer results. The percent exon skipping and standard deviations (STDEV) were calculated from at least two independent experiments. B, lin-10 alternative splicing. The diagram indicates the locations of RT-PCR primers in the alternatively spliced region of lin-10. RNA prepared from strains described in A of the figure was subjected to RT-PCR with these lin-10 primers and analyzed on an Agilent Bioanalyzer as described above. C, HRP-2 function in splicing is dependent on UCUAUC. The top part is a schematic representation of the unspliced mRNA. Arrows above exon 16 and the 3’ end of lacZ indicate the positions of the 5’ and 3’ RT-PCR primers, respectively. The site of the UCUAUC sequence deleted in the ΔUCUAUC reporter is indicated below. Bar graphs indicate the fraction of synchronized young adult steady-state messages derived from the construct that splices exons 16–19. The use of empty vector RNAi versus hrp-2(RNAi), the identity of the reporter genes with and without the intron 16 UCUAUC sequence, and the mec-8 genetic background in each experiment are indicated. NLS, nuclear localization sequence; GFP, green fluorescent protein; wt, wild-type.
completely different alternative splicing events. The study confirmed a role in alternative splicing for these two proteins, yet it is interesting that these two highly related proteins had no common targets. It remains to be determined which cis elements on these hnRNP Q and R targets interact with these mammalian proteins.

HRP-2 is an alternative splicing regulator for at least two C. elegans genes during the young adult stage of development. unc-52 and lin-10 both show increases in cassette exon skipping under hrp-2(RNAi), and both contain intrinsic UCUAUC sequences, consistent with potential regulation by HRP-2. They also both contain the consensus FOX-1-binding sequence UGCAUG in a flanking intron and are both regulated by the MEC-8 protein in a direction that appears opposite to HRP-2 splicing regulation (MEC-8 promotes exon skipping and HRP-2 promotes exon inclusion). The idea that HRP-2 regulation is balanced by another splicing factor may help explain the observation that HRP-2 is constitutively expressed and therefore not likely to be a tissue-specific alternative splicing regulator (24). There is evidence that other members of the hnRNP family can be ubiquitously expressed yet still have a role in sequence-specific promotion of alternative splicing. For example, the hnRNP A/B proteins bind with high affinity to specific sequences to promote alternative splicing of specific messages yet also function in a general way in pre-mRNA processing and export (23). The alternative splicing control by these factors occurs through a balance with another factor with opposite activity, such as an SR protein (37). MEC-8, which shows tissue-specific expression (38), may serve as the regulator with opposite activity, such as an SR protein (37). MEC-8 may serve as a tissue-specific regulator that changes their native splicing. However, when knocked down in coordination with mutation of the mammalian FOX2 homolog, asd-1, changes in the alternative splicing of the egl-15 gene were observed (39).

Acknowledgments—We are grateful to Eileen Devaney of the University of Glasgow for advice and for kindly providing the anti-HRP-2 polyclonal antibody. We thank James Williams, Nicole Lambert, Melissa Jurica, Manny Ares, and Benoit Chabot for helpful discussions.

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