All three RNA recognition motifs and the hinge region of HuC play distinct roles in the regulation of alternative splicing

Melissa N. Hinman¹, Hua-Lin Zhou¹, Alok Sharma¹ and Hua Lou¹,²,³,*

¹Department of Genetics and Genome Sciences, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA, ²Center for RNA Molecular Biology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA and ³Case Comprehensive Cancer Center, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

ABSTRACT

The four Hu [embryonic lethal abnormal vision-like (ELAVL)] protein family members regulate alternative splicing by binding to U-rich sequences surrounding target exons and affecting the interaction of the splicing machinery and/or local chromatin modifications. Each of the Hu proteins contains a divergent N-terminus, three highly conserved RNA recognition motifs (RRM1, RRM2 and RRM3) and a hinge region separating RRM2 and RRM3. The roles of each domain in splicing regulation are not well understood. Here, we investigate how HuC, a relatively poorly characterized family member, regulates three target pre-mRNAs: neurofibromatosis type I, Fas and HuD. We find that the HuC N-terminus is dispensable for splicing regulation, and the three RRM3 are required for splicing regulation of each target, whereas the hinge region contributes to regulation of only some targets. Interestingly, the regions of the hinge and RRM3 required for regulating different targets only partially overlap, implying substrate-specific mechanisms of HuC-mediated splicing regulation. We show that RRM1 and RRM2 are required for binding to target pre-mRNAs, whereas the hinge and RRM3 are required for HuC–HuC self-interaction. Finally, we find that the portions of RRM3 required for HuC–HuC interaction overlap with those required for splicing regulation of all three targets, suggesting a role of HuC–HuC interaction in splicing regulation.

INTRODUCTION

The mammalian Hu/embryonic lethal abnormal vision-like (ELAVL) family consists of four RNA-binding proteins that play important roles in several biological processes. HuR (ELAVL1) is widely expressed and plays roles in DNA damage response, negative regulation of apoptosis, response to hypoxia, carcinogenesis, inflammation and several other diseases (1–8). The other family members, HuB (ELAVL2), HuC (ELAVL3) and HuD (ELAVL4), are neuron-enriched and play roles in neuronal differentiation, neuronal maintenance, learning, memory and regulation of neuronal excitability, as well as roles outside of the nervous system, including regulation of insulin translation in pancreatic β cells by HuD (7,9–13). On a molecular level, Hu proteins bind to U-rich (AU or GU) sequences in RNA targets and regulate RNA metabolism through diverse mechanisms (7,10,13–16). These proteins are best known for their cytoplasmic functions, including the stabilization of specific mRNAs through interactions with U-rich elements in 3′-untranslated regions and the regulation of translation (14,17,18). In the nucleus, Hu proteins regulate alternative polyadenylation and alternative splicing (13,19–28).

Although the mechanisms underlying the cytoplasmic functions of Hu proteins have been studied extensively, the nuclear functions of Hu proteins, including splicing regulation, were discovered more recently and are poorly understood. Because of recent genome-wide studies involving HuR, HuC and HuD, we now appreciate that the regulation of splicing by Hu proteins is widespread, but only a handful of splicing targets of Hu proteins have been studied in detail (13,29,30). Like many splicing regulatory proteins, such as PTB, Nova and Mbnl2, Hu proteins positively regulate the inclusion of some exons and suppress the inclusion of others, although they bind to similar RNA sequences surrounding both types of exons (31–34). HuR suppresses Fas exon 6 inclusion, whereas HuB, HuC and HuD promote the neuron-specific splicing and polyadenylation of calcitonin/calcitonin gene-related peptide, suppress neurofibromatosis type I (NF1) exon 23a inclusion and, along with HuR,

*To whom correspondence should be addressed. Tel: +1 216 368 6419; Fax: +1 216 368 0491; Email: hxl47@case.edu

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promote HuD exon 6 inclusion (19,21–26). Studies so far indicate that Hu proteins suppress inclusion of target exons by binding to U-rich sequences within or surrounding the exon and blocking the action of core splicing factors. For example, the neuron-specific Hu proteins bind to U-rich sequences upstream and downstream of NF1 exon 23a and inhibit the interaction of key splicesomal components, including U1 small nuclear ribonucleoprotein (snRNP), U6 snRNP and U2AF65 with the pre-mRNA, and HuR similarly binds to an exonic U-rich sequence to prevent the interaction of U2AF65 with the Fas pre-mRNA (23,24). Hu proteins often antagonize the actions of TIA-1/TIAR, another group of splicing regulatory proteins that bind to U-rich sequences (19,21,23–25). Whether Hu proteins inhibit the activity of splicing factors by simply binding to and blocking their binding sites or through more complex mechanisms is not fully understood. An additional mode by which Hu proteins can act is through feeding back from target RNA to DNA, affecting local histone acetylation and transcriptional elongation rate to regulate exon inclusion (35).

An unanswered question is how the domain structure of Hu proteins influences their splicing regulatory functions. The four mammalian Hu proteins are highly conserved among each other and share a similar domain structure (Figure 1A) (36). Each has three RNA recognition motifs (RRMs), referred to as RRM1, RRM2 and RRM3, that share >90% sequence identity among the Hu family members. In between RRM2 and RRM3 is a basic hinge region, and each Hu protein contains a unique short N-terminal region. Although Hu proteins contain three RRMs, it is RRM1 and RRM2 that cooperate to recognize and bind to U-rich sequences (37–41). RRM1 and RRM2 are required for binding to and regulating numerous mRNA targets, although this has not yet been shown for pre-mRNA splicing targets (38,42–46).

The hinge region, which is more divergent among the Hu family members than the RRMs, plays a role in protein localization. The HuR hinge region contains nuclear export and nuclear localization signals that allow it to shuttle between the cytoplasm and nucleus, and the HuD hinge region contains a nuclear export signal (47–49). Interestingly, multiple isoforms exist for HuB, HuC and HuD because of alternative splicing in the hinge region, but how the different isoforms differ in function is unknown. The hinge and RRM3 both function in protein–protein interactions. For example, the HuD hinge is required for its interaction with Akt1 that leads to neurite outgrowth, and HuR RRM3 is involved in the interaction with RNPC1, another RNA-binding protein involved in the regulation of mRNA stability (50,51).

Hu proteins can bind with themselves and with other Hu protein family members to form multimers on RNA targets (52–57). The RRM3 and hinge regions of HuR are implicated in homomultimerization, whereas HuC RRM3 seems to mediate the HuC–HuB heterodimerization (53,57). Hu–Hu interactions have been proposed to contribute to the granular distribution of Hu proteins in some cells (57). In addition to protein–protein interactions, HuR RRM3 contains an adenosine triphosphate-binding pocket that allows it to catalyse 3′-terminal adenosyl modification of non-polyadenylated RNA targets, but the functional consequences of this are unknown (58).

In the present study, we investigate how each domain of the Hu proteins affects splicing regulation. Although the majority of studies of Hu proteins focus on HuR or HuD, we focused our efforts on HuC, a lesser-characterized family member. In addition to shedding light on how Hu proteins influence alternative splicing, our study fills in gaps in the general knowledge about HuC as an RNA regulatory protein. Using mutants with different portions of the HuC protein deleted, we first show that all three RNA recognition motifs are critical for splicing regulation, and that the hinge region plays a lesser role in the regulation of specific targets. We then show that these changes in splicing regulation cannot be accounted for by changes in protein localization of the HuC mutants. In addition, we provide evidence that RRM1 and RRM2 are critical for binding to target pre-mRNAs, and that the RRM3 and hinge regions are important for HuC–HuC self-interaction. Also, we demonstrate that alternative splicing within the hinge region of HuC does not have a large impact on HuC splicing regulation or HuC–HuC interaction. Finally, we fine-map the regions of the RRM3 and hinge that are important for HuC–HuC self-interaction and show that the critical regions overlap with regions important for splicing regulation, suggesting that HuC–HuC interaction affects splicing regulation.

**MATERIALS AND METHODS**

**Plasmids**

The NF1 splicing reporter (HMT-NF1 863/499 WT) and the HuD splicing reporter (E6) contain human NF1 exon 23a or human HuD exon 6 with part of the surrounding intronic sequences cloned into the first intron of the human metallothionein 2A gene as described previously (23,26). The cloning of the four mouse HuC splice variants into pcDNA3.1 His B (Invitrogen) was described previously (23). HuCsv1 was cloned into pMAL, and maltose-binding protein (MBP)–HuCsv1 was produced using the pMAL Protein Fusion and Purification System (New England Biolabs). HuC domain deletion constructs were generated by standard cloning methods using mouse HuC as the template. HuC splice variant 2 (HuCsv2) was used as the template for all HuC domain and sub-domain deletion constructs, except for the hinge region sub-domain deletion constructs, which used mHuCsv1 as a template. A 5′ segment upstream of and a 3′ segment downstream of the deleted region were amplified by polymerase chain reaction (PCR) and joined together in a subsequent PCR reaction. Supplementary Table S1 indicates the primers used to generate the 5′ and 3′ segments for each HuC deletion construct and the amino acids that were deleted, whereas Supplementary Table S2 lists the sequences of those primers. HuC constructs were cloned into pMT-6myc (a gift from Sachiyo Kawamoto, National Institutes of Health, Bethesda, MD, USA) for generation of Myc-tagged proteins, pcDNA3.1 HisB (Invitrogen) for
Xpress-tagged proteins and pGEX-2TK (Amersham) for glutathione-S-transferase (GST) proteins.

**Cell culture and transfection**

HeLa cells were maintained in accordance with the protocol of the American Type Culture Collection (Manassas, VA, USA), and CA77 cells were maintained as described previously (23). HeLa transfections and CA77 transfections were performed using Lipofectamine 2000 (Invitrogen) or PolyJet (SigmaGen) in accordance with manufacturer recommendations.

**Measurement of exon inclusion by reverse transcription–polymerase chain reaction**

For reverse transcription (RT)–PCR experiments, 0.5–1 μg of splicing reporter plasmid (HMT-NF1 863/499 WT for NF1, E6 for HuD or no splicing reporter for Fas) and 2–4 μg of Myc-tagged HuC constructs (Figures 1, 2 and 4) or Xpress-tagged HuC splice variants (Figure 5) were co-transfected into HeLa cells or CA77 cells that were grown on 6-cm plates. RNA and protein were harvested 24–48 h post-transfection, and RT-PCR was performed as described previously (59). Primers 1 and 2 (Supplementary Table S2) and 17–20 cycles were used for HuD and NF1 splicing reporter RT–PCR. Primers 3 and 4 (Supplementary Table S2) and 22–25 cycles were used for endogenous Fas RT–PCR. Percentage exon inclusion [\(\frac{\text{exon included}}{\text{exon included} + \text{exon skipped}}\) \(\times 100\)] was measured using a Typhoon Trio Variable Mode Imager (GE Healthcare), and results were averaged using at least three independent experiments. To measure protein expression, western blot analysis was performed using 20–50 μg of total protein and anti-Myc (1:10000, Invitrogen) or anti-Xpress (1:3500, Invitrogen), as well as anti-U1 70K (1:250, a gift from Susan Berget, Baylor College of Medicine) or anti-γ-tubulin (1:10000, Sigma) as primary antibodies and goat anti-mouse IgG (1:2000, Thermo Scientific) as the secondary antibody.

**RNA gel mobility shift assay**

Recombinant GST proteins were prepared from bacteria expressing HuC proteins from the pGEX-2TK vector using the B-PER GST spin purification kit (Thermo Scientific) (Figure 7B). Gel mobility shift assays were performed as described previously (26). Briefly, for the NF1 gel mobility shift assay, 1 ng (80 fmol) of radiolabelled WT or mutant RNA oligonucleotides (Figure 3A) (Thermo Fisher Scientific Inc.) were incubated with 200 ng of GST or 10, 25 or 200 ng of GST–HuC fusion proteins in 25 μl of reaction buffer for 30 min at 30°C and run on a non-denaturing polyacrylamide gel. For the HuD gel mobility shift assay, 1 ng (80 fmol) of radiolabelled WT RNA oligonucleotide (Figure 3B) (Thermo Fisher Scientific Inc.) was incubated with 50 ng of GST or 2, 10 or 50 ng of GST-tagged recombinant HuC proteins for 30 min at 30°C in 25 μl of reaction buffer and run on a non-denaturing polyacrylamide gel. Mutant HuD RNA oligonucleotides were incubated with 50 ng of each GST protein.

**Immunofluorescence**

Myc-tagged HuC plasmids (2.5 μg) were transfected into HeLa cells in 60-mm dishes. Twenty-four hours post-transfection, HeLa cells were trypsinized and plated at low density onto glass coverslips. The next day, cells were fixed in 4% paraformaldehyde in 1× phosphate-buffered saline (PBS), then permeabilized in 0.2% Triton X-100/PBS and incubated with primary (rabbit anti-Myc 1:2000, Sigma) followed by secondary (goat anti-rabbit fluorescein isothiocyanate 1:500, Sigma) antibodies diluted in 0.5% normal goat serum/PBS. Nuclei were stained using 4,6-diamidino-2-phenylindole (DAPI) 33 ng/ml in PBS. Images were taken at 40× magnification using a Leica DM6000 microscope.

**Co-immunoprecipitation**

HeLa cells in 60-mm dishes were co-transfected with 1.25 μg of Myc-tagged HuC and 1.25 μg of Xpress-tagged HuC plasmids. Co-immunoprecipitation (Co-IP) was performed 48 h post-transfection. Cells were lysed in buffer containing 50 mM Tris–HCl, pH 8.0, 120 mM NaCl and 0.5% nonidet P40. Lysates were incubated overnight at 4°C with anti-c-Myc agarose affinity gel (Sigma) or with GammaBind G Sepharose beads (Amersham) coated with human IgG (non-specific control). Beads were washed six times for 5 min each at 4°C with NETN buffer (100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 50 mM Tris–HCl and 0.5% nonidet P40) with 50 μg/ml of RNase A (Sigma) added during the final wash when indicated. Proteins were eluted from beads, and western blot analysis was performed using anti-Xpress (1:3500, Invitrogen) primary antibody and goat anti-mouse (1:1250, Thermo Scientific) secondary antibody. Verification of expression of Myc-tagged proteins was done by western blot analysis using 20 μg of cell lysate with anti-Myc primary antibody (1:10000, Invitrogen) and goat anti-mouse secondary antibody (1:1250, Thermo Scientific).

**GST pull-down**

MBP–HuCsv1 (2 μg), GST-tagged HuCsv2 proteins or GST alone (2 μg) and 30 μl of glutathione sepharose 4B beads (GE Healthcare) were co-incubated in 1 ml of GST lysis buffer (150 mM NaCl, 20 mM Tris–HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid and 0.5% nonidet P40) for 1 h at 4°C. Beads were washed three times in GST lysis buffer, and proteins were eluted from them. Western blot analysis was performed using anti-MBP primary antibody (1:5000, New England Biolabs) and goat anti-rabbit secondary antibody (1:1250, Pierce). The recombinant proteins (2 μg) were also run on a protein gel and stained with GelCode Blue Stain Reagent (Thermo Scientific) (Figure 7B).

**RESULTS**

All three RNA recognition motifs and the hinge region of HuC are important for splicing regulation

To better understand the mechanisms by which Hu proteins regulate splicing, we investigated which domains of HuC, a representative Hu family member, were
required. We generated HuC constructs in which one domain (N-terminus, RRM1, RRM2, hinge or RRM3) was deleted, whereas the remainder of the protein was left intact (Figure 1A) (35). The splice variant 2 isoform of HuC (HuCsv2), to be discussed in Figure 5, was used for these experiments. We expressed the HuC domain deletion mutants in HeLa cells where HuC is not endogenously expressed, and we used RT–PCR to measure the inclusion of three different alternative exons whose inclusion is known to be regulated by Hu proteins (23,24,26).

We first investigated the regulation of NF1 exon 23a inclusion by the HuC domain deletion mutants. Full-length HuC expression suppresses the inclusion of NF1 exon 23a both from the endogenous locus and from a splicing reporter (Figure 1B) (23), whereas knockdown of endogenous HuC in CA77, a neuron-like cell line, increases reporter NF1 exon 23a inclusion (23). Deletion of any of the HuC RRMs completely abolished HuC-mediated suppression of reporter NF1 exon 23a inclusion in HeLa cells, whereas deletion of the hinge region decreased HuC-mediated suppression of exon 23a inclusion (Figure 1B). Deletion of the HuC N-terminus had no effect on the HuC-mediated suppression of reporter exon 23a inclusion (Figure 1B).

We then investigated whether the same domains of HuC were required to suppress the inclusion of another HuC target alternative exon. HuR, another Hu protein family member, suppresses Fas exon 6 inclusion, and we found that HuC is also capable of suppressing Fas exon 6 inclusion when expressed in HeLa cells (Figure 1C) (24,35). HuC domain deletion mutants were exogenously expressed in HeLa cells, and endogenous Fas exon 6 inclusion was measured by RT–PCR, whereas protein expression was confirmed by western blot analysis. Although overall suppression of endogenous Fas exon 6 inclusion by HuC expression was not as robust as the suppression of reporter NF1 exon 23a inclusion (Figure 1C), possibly because of moderate transfection efficiency, a similar trend was observed in which all three RRMs of HuC were required for optimal suppression of exon 6 inclusion, and the N-terminus was not required for regulation (Figure 1C). However, in contrast to the regulation of NF1 exon 23a, the HuC hinge region was dispensable for regulation of Fas exon 6 inclusion (Figure 1C).

In addition, we were interested in whether the same domains of HuC were required for promoting exon inclusion as were required for suppressing inclusion. We examined the splicing of HuD exon 6, the inclusion of which, in contrast to NF1 exon 23a and Fas exon 6, is promoted by HuC expression both from the endogenous locus and a splicing reporter (Figure 1D) (26). Knockdown of endogenous HuC in CA77 cells leads to decreased inclusion of HuD exon 6 from a splicing reporter (26). All three HuC RRMs are critical for the regulation of reporter HuD exon 6 inclusion, which is like the other two alternative exons even though the regulation is in the opposite direction (Figure 1D). Deletion of the HuC hinge region resulted in slightly decreased regulation of HuD exon 6 inclusion (Figure 1D).

In CA77 cells, HuC expression led to decreased reporter NF1 exon 23a inclusion and increased reporter HuD exon 6 inclusion (Figure 2A and B). However, the overall change in exon inclusion was smaller than in HeLa cells,

Figure 1. The three HuC RNA recognition motifs and the hinge region are all important for splicing regulation in HeLa cells. (A) Schematic of HuC domain deletion mutants (35). Dotted lines indicate the deleted portion of the protein. (B–D) Reporter NF1 exon 23a (23) (B), endogenous Fas exon 6 (C) and reporter HuD exon 6 (26) (D) inclusion in HeLa cells after expression of HuC proteins as measured by RT–PCR. Western blot analysis using anti-Myc antibody to detect Myc-tagged HuC domain deletion constructs. U1 70K and γ-tubulin are loading controls. Arrows indicate locations of RT–PCR primers. Error bars represent 1 SD. N > 3.
possibly because endogenous HuC is already present in this cell type. Similar to in HeLa cells, deletion of the HuC N-terminus did not prevent the regulation of either target exon, whereas deletion of any of the three RRMs greatly decreased regulation of both exons (Figure 2A and B). In the context of CA77 cells, the deletion of the HuC hinge diminished the regulation of the inclusion of both reporter HuD exon 6 and reporter NF1 exon 23a (Figure 2A and B). None of the HuC domain deletion proteins seem to act as dominant negatives that inhibit the splicing regulatory activity of endogenous HuC, as we did not observe a decrease in reporter HuD exon 6 inclusion or increase in NF1 exon 23a inclusion on expression of these proteins.

Taken together, these results indicate that all three RRMs and, to a lesser extent, the hinge region of HuC play roles in both the suppression and promotion of exon inclusion by HuC, whereas the N-terminus is not required. The involvement of multiple domains of HuC suggests complex mechanisms for splicing regulation.

**HuC domain deletions do not alter protein localization**

We sought to understand why each domain of HuC is required for the regulation of exon inclusion by HuC. Another member of the Hu protein family, HuR, contains both nuclear export and nuclear localization signals within the hinge region, allowing it to shuttle between the nucleus and cytoplasm, although it remains to be determined how HuC protein localization is regulated (47). If HuC contains similar signals and one or more of the deletion mutants disrupts them resulting in exclusion of HuC from the nucleus, this could explain a failure to regulate splicing, as splicing is a nuclear event. To investigate this possibility, we expressed the HuC domain deletion mutants in HeLa cells and examined their cellular localization using immunofluorescence. Full-length HuC was present in both the nucleus and cytoplasm of most cells at steady-state (Supplementary Figure S1). Likewise, all of the HuC domain deletion mutants were present in both the cytoplasm and nucleus of most cells (Supplementary Figure S1). Thus, our mutations do not seem to interfere with the nuclear localization of HuC, and the inability of some mutants to regulate exon inclusion is not likely a simple consequence of failure to localize to the site of splicing regulation.

**HuC RRM1 and RRM2 are required for binding to the NF1 and HuD pre-mRNAs**

One reason why a mutant form of HuC might not regulate exon inclusion is if it were to have a reduced or abolished ability to bind to the pre-mRNA. The regulation of splicing by Hu proteins is mediated through their interactions with U-rich sequences within or surrounding their target exons (23–26,59). We asked which domains of HuC were required for binding to its splicing target pre-mRNAs. Gel mobility shift assays were performed using recombinant GST–HuC fusion proteins and 32P-labelled RNA oligonucleotides containing U-rich sequences previously shown to be important for HuC-mediated suppression of NF1 exon 23a inclusion (Figure 3A) (23) and promotion of HuD exon 6 inclusion (Figure 3B) (26). Addition of GST–HuC caused shifts in the mobility of both the NF1 and HuD RNA sequences, indicative of protein–RNA complex formation, that were greatly reduced when the U-rich sequences were mutated, suggesting sequence specificity of the interaction (Figure 3A and B). Deletion of HuC RRM1 or RRM2, but not of the other HuC domains, abolished the shift in mobility of both target RNAs (Figure 3A and B). This indicates that RRM1 and RRM2 are critical for the interaction of HuC with the NF1 and HuD pre-mRNAs, despite the fact that HuC suppresses the inclusion of one exon and promotes the inclusion of the other. This is consistent with other studies of HuC–mRNA interaction (37,38,43,44). Thus, the deletion of HuC RRM1 or RRM2 abolishes splicing regulation most likely because of failure of the protein to bind to the target pre-mRNA.

**The 5' portion of HuC RRM3 is important for alternative splicing regulation**

After determining that RNA binding is likely the major role for HuC RRM1 and RRM2 in regulating alternative splicing, we focused our attention on understanding the roles that the HuC hinge and RRM3 play in regulating
Figure 3. RRM1 and RRM2 of HuC are important for binding to the NF1 and HuD pre-mRNAs. (A) RNA gel mobility shift assay. Radioactively labelled RNA oligonucleotides (1 ng) corresponding to wild-type or mutant Hu regulatory sequences upstream of NF1 exon 23a (23) were incubated with 200 ng of GST or increasing amounts (10, 25 or 200 ng) of GST–HuC mutants and run on a non-denaturing polyacrylamide gel. At the bottom are sequences of the wild-type and mutant NF1 oligonucleotides with mutated nucleotides underlined. (B) RNA gel mobility shift assay. Radioactively labelled RNA oligonucleotides (1 ng) corresponding to wild-type Hu regulatory sequences upstream of HuD exon 6 (26) were incubated with 50 ng of GST or increasing amounts (2, 10 or 50 ng) of GST–HuC mutants and run on a non-denaturing polyacrylamide gel. Mutant RNA oligonucleotides were incubated with 50 ng of each GST protein. At the bottom are sequences of the wild-type and mutant HuD oligonucleotides with mutated nucleotides underlined.
We mapped the parts of these domains that were required for this activity. To accomplish this, we created constructs in which specific portions of the hinge or RRM3 were deleted, referred to as the hinge sub mutants (Figure 4A). These constructs were transfected into HeLa cells to test their ability to regulate splicing.

Consistent with the small changes in splicing regulation of Fas exon 6 and HuD reporter exon 6 on deletion of the entire hinge region (Figure 1C and D), none of the HuC hinge sub mutants showed strong decreases compared with wild-type HuC in the ability to regulate the inclusion of these two exons (Figure 4C and D). With NF1, where deletion of the entire HuC hinge region strongly decreases splicing regulation, we were able to identify specific regions of the hinge important for splicing regulation. The HuC hinge sub2 and hinge sub3 mutants, in which the middle portions of the hinge were deleted, showed reduced and abolished abilities, respectively, to regulate NF1 reporter exon 23a inclusion as compared with wild-type HuC (Figure 4B). The 5' and 3' portions of the hinge region, deleted in the hinge sub1 and hinge sub4 mutants, however, are dispensable for regulation of reporter NF1 exon 23a inclusion (Figure 4B).

With HuC RRM3, the 5' portion seems to be especially important for splicing regulation, as the RRM3 sub1, sub2 and sub3 deletion mutants all led to completely abolished regulation of Fas exon 6 and HuD exon 6 inclusion and greatly reduced regulation of reporter NF1 exon 23a inclusion (Figure 4C and D). In contrast, the 3'end of RRM3 that was deleted in the RRM3 sub4 mutant was dispensable for regulation of Fas exon 6 and HuD reporter exon 6 inclusion (Figure 4B and D). The 3'end of RRM3 may play a role specific to NF1 splicing regulation, as the RRM3 sub4 mutant showed greatly decreased suppression of reporter exon 23a inclusion as compared with wild-type HuC (Figure 4B).

In summary, the 5' portion of HuC RRM3 is important for the regulation of all three splicing targets studied, whereas the middle portion of the hinge and 3' portion
of RRM3 are also required for optimal regulation of NF1 exon 23a inclusion. The differences in the specific sequences of HuC required for regulation of different targets suggest that the mechanisms of HuC-mediated splicing regulation are fine-tuned to individual target exons. This seems to be a common trend among splicing regulators. For example, distinct portions of the divergent domain between RRM2 and RRM3 of the splicing regulator CELF4 are required for regulation of different target exons (60).

**HuC splice variants show similar abilities to regulate exon inclusion**

HuC exists in four alternatively spliced isoforms, HuCsv1, HuCsv2, HuCsv3 and HuCsv4, that differ based on whether two alternative sequences are included in the middle of the hinge region (Figure 5A). The first of these sequences is alternative cassette exon 6, which is 39 nt in length. Immediately downstream of cassette exon 6 is exon 7, which has two alternative 3' splice sites. We referred to the 21-nt sequence included by usage of the upstream 3' splice site as 7a (Figure 5A). The HuC hinge sub mutants used in Figure 4 were generated using HuCsv1, which contains both alternative sequences, as a template (Figure 5A). The HuC hinge sub2 and hinge sub3 deletion mutants that showed decreased ability to suppress reporter NF1 exon 23a inclusion both have portions of the alternative sequences deleted in addition to surrounding sequences (Figures 4B and 5A). We, therefore, asked whether the alternative sequences in the hinge region were important for splicing regulation. The four HuC splice variants were transfected into HeLa cells. For NF1 reporter, Fas exon 6 and HuD reporter (previously published) (26), all four splice variants showed robust regulation of exon inclusion, although with some small differences between splice variants (Figure 5B and C) (26). HuCsv1, containing both alternative sequences, showed slightly decreased regulation of Fas exon 6 and HuD exon 6 inclusion compared with the other isoforms, whereas HuCsv4, lacking both alternative sequences, had a decreased ability to suppress reporter NF1 exon 23a inclusion (Figure 5B and C) (26). Thus, the regulation of inclusion of alternative sequences within the HuC hinge may play a minor role in the regulation of specific alternative exons by HuC. When comparing the HuC splice variants and the hinge sub deletion mutants, it is of note that HuCsv4 and the hinge sub3 mutant have largely overlapping portions of the full-length hinge region missing, yet the hinge sub3 mutant has a much larger defect in ability to suppress reporter NF1 exon 23a inclusion (Figures 4B and 5A, B). This suggests that the four additional amino acids deleted downstream of the alternatively spliced region in the hinge sub3 mutant are particularly important for the regulation of NF1 exon 23a inclusion.

**The HuC hinge and RRM3 regions are important for HuC–HuC interaction**

Hu proteins are known to interact with themselves (52–57). We sought to understand which portions of HuC are important for binding to itself, and whether these same portions of HuC are required for splicing regulation. IP experiments were performed in which Xpress- and Myc-tagged HuC mutants were both expressed in HeLa cells. Expression of Xpress-tagged HuC proteins was confirmed by western blot analysis.
(Figure 6A and B, top portion, input lanes), as was the expression of Myc-tagged proteins (Figure 6A and B, bottom portion). Myc-tagged HuC proteins were pulled down using beads coated with anti-Myc antibody, and western blot analysis was performed with anti-Xpress antibody to detect co-immunoprecipitated Xpress-tagged HuC proteins (Figure 6A and B, top portion, α-Myc lanes). Pull-downs using IgG-coated beads were performed as negative controls (Figure 6A and B, top portion, IgG lanes).

Full-length HuCsv2 co-immunoprecipitated with itself strongly, although slightly less robustly in the presence of RNase, suggesting that RNA is not required for the interaction but may enhance it (Figure 6A, transfection number 1). This interaction was not disturbed by deletion of the HuC N-terminus, RRM1 or RRM2 (Figure 6A, transfection numbers 2, 3, 4, 8, 9 and 10). Deletion of the HuC hinge region resulted in HuC–HuC interaction that was RNA-dependent, whereas deletion of the HuC RRM3 completely abolished HuC–HuC interaction (Figure 6A, transfection numbers 5, 6, 11 and 12). These results show that both the HuC hinge and RRM3 are required for RNA-independent interaction of HuC with itself in cell lysates.

We further mapped the regions of HuC required for self-interaction using the HuC hinge and RRM3 sub mutants (Figure 4A). The sub3 portion of the hinge region was required for RNA-independent HuC–HuC interaction, whereas the sub1 and sub2 portions of RRM3 were required for HuC–HuC interaction, even in the presence of RNA (Figure 6B, transfection numbers 5, 8 and 9). By comparing the IP data in Figure 6 with the RT–PCR data in Figure 4, we conclude that the first 46 amino acids of RRM3 are important for both optimal splicing regulation and for HuC–HuC interaction, which suggests a role for HuC self-interaction in splicing regulation.

In addition, we studied the ability of the HuC hinge region splice variants to form HuC–HuC interactions (Figure 6B, transfection numbers 12–15). All four HuC hinge region splice variants were able to form robust HuC–HuC interactions, indicating that the alternatively spliced region is not important for dimerization. When comparing data for the HuC splice variants with that of the hinge sub mutants, we found that HuCsv4 and the hinge sub3 mutant, which have similar regions missing (Figure 5A), show divergent phenotypes. HuCsv4 forms an RNA-independent HuC–HuC interaction, whereas hinge sub3 does not (Figure 6B, transfection numbers 5 and 15). This suggests that the four amino acids downstream of the alternatively spliced region that are missing

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**Figure 6.** The hinge and RRM3 domains of HuC are important for HuC–HuC interaction in cell lysates. (A and B) Myc- and Xpress-tagged HuC domain deletion mutants (A), HuC partial domain deletion mutants (B) and HuC splice variants (B) were co-transfected into HeLa cells. Top of A and B: IP of cell lysates was carried out using either IgG or Myc beads, with or without RNase treatment, followed by western blot analysis with anti-Xpress antibody. To the right of the figure is a chart summarizing the results of the co-IP experiment, with + indicating an interaction between the two exogenously expressed proteins, and – indicating a lack of interaction. Bottom of A and B: western blot analysis using 10% input with anti-Myc antibody to confirm that Myc-tagged proteins were expressed in each of the experiments shown in the top portion of the figure. Transfection numbers refer to the same experiment for the top and bottom portions of the figure.
in the hinge sub3 mutant are important not only for NF1 splicing regulation but also for RNA-independent HuC–HuC interaction. This result provides additional support to the idea that HuC–HuC interaction plays a role in NF1 splicing regulation.

To provide more evidence for the HuC domains that are required for HuC–HuC interaction, we performed an in vitro experiment in which wild-type or mutant GST–HuC was used to pull-down MBP–HuC (Figure 7). GST–HuC and each mutant GST–HuC protein, except for the one in which RRM3 was deleted, were able to pull-down MBP–HuC (Figure 7A). This suggests that HuC RRM3 is required for direct HuC–HuC interaction, whereas HuC hinge may enhance the interaction in the context of cells.

**DISCUSSION**

In summary, we carried out the first comprehensive domain analysis of HuC, a member of the Hu protein family, and found that RRM1, RRM2 and RRM3 all play critical roles in the splicing regulation of at least three target pre-mRNAs, whereas the hinge region is important for splicing regulation of some targets and not others, and the N-terminus is dispensable for splicing regulation. The first two HuC RRM3s function in splicing regulation at least in part by conferring the ability to bind to target pre-mRNAs, whereas the hinge and RRM3 play roles in HuC–HuC interaction, which may be important for splicing regulation.

We found that the N-terminal region of HuC, which is poorly conserved among the Hu protein family members, is dispensable for splicing regulation (Figures 1 and 2). This was not unexpected given the redundant function of Hu protein family members in regulating at least some of their target exons (23,26). The sequences critical for splicing regulation are likely to lie in more conserved regions of those proteins.

HuC RRM1 and RRM2 are critical for regulation of the splicing of all targets that we studied regardless of whether HuC promotes or suppresses exon inclusion (Figures 1 and 2), and they are required for binding to the U-rich regulatory sequences surrounding Hu splicing targets (Figure 3). Based on these data, we assert that RRM1 and RRM2 are critical for HuC-mediated splicing regulation because they allow HuC to recognize and bind to splicing target pre-mRNAs. This is consistent with previous studies of Hu proteins in binding to cytoplasmic target RNAs (42–46). We cannot rule out the possibility that RRM1 and RRM2 play additional roles in Hu-mediated splicing regulation. For example, when HuR RRM1, but not RRM2, is artificially tethered to the Fas exon 6 RNA in a splicing reporter, it suppresses exon inclusion on its own, suggesting that it may have a splicing regulatory function beyond its ability to bind to RNA (24).

The splice variants arising from the hinge region of HuC showed minimal differences in ability to regulate splicing of the three targets studied, and all showed robust HuC–HuC interaction (Figures 5 and 6). This indicates that the hinge region alternative splicing of HuC does not play a major role in the regulation of alternative splicing by HuC. Thus, the functional consequences of the alternative splicing of the hinge region of Hu proteins, both in the context of alternative splicing regulation and other molecular functions, remain to be elucidated.

We did not observe significant differences in steady-state localization between HuC domain deletion mutants, indicating that decreases in splicing regulation by some mutants cannot be accounted for simply by failure to localize to the site of splicing regulation in the nucleus (Supplementary Figure S1). Of note is that the localization of the HuC mutant lacking the hinge region was similar to that of full-length HuC, even though for HuR and the Drosophila homologue ELAV, sequences required for nuclear localization have been identified in the hinge region (Supplementary Figure S1) (47,61). This indicates that the hinge region is not solely responsible for the ability of HuC to localize to the nucleus.

The HuC RRM3 domain was required for regulation of all alternative exons that we studied, whereas the hinge region was required for optimal regulation of splicing of only some targets in HeLa cells (Figure 1B, C and D). Supporting our finding, a mutant of the Hu family member HuR lacking both hinge and RRM3 was likewise unable to suppress Fas exon 6 inclusion from a splicing reporter (24). Splicing regulation by Hu proteins bears a resemblance to the regulation of mRNA stability, as studies have indicated that RRM3 is required for mRNA stabilization, whereas the hinge region is dispensable for the stabilization of at least some targets (48,62).

In addition to being required for splicing regulation, the RRM3 domain is critical for HuC–HuC interaction, and the hinge region is less critical but also plays a role in the interaction in cell lysates, as deletion of the hinge region abolishes the interaction when RNA is not present, and
deletion of the RRM3 domain abolishes the interaction with or without RNA present (Figure 6A). Supporting this result are studies indicating a role for the hinge and/or RRM3 in the HuR–HuR interaction, HuC–HuB interaction and the Drosophila homologue ELAV self-interaction (53,57,63). Our co-IP studies show that the 5′ portion of RRM3 is most important for HuC–HuC interaction (Figure 6). Three well-conserved sequences within the same portion of RRM3 were shown to be important in the Drosophila ELAV–ELAV interaction, suggesting that this conserved interaction may be functionally important (63). Intriguingly, these same portions of HuC RRM3 that were important for HuC–HuC interaction were also required for optimal regulation of splicing for all three targets that we studied (Figure 4).

These data suggest that the binding of HuC to itself, and possibly even multimerization of HuC along target RNAs, may play a role in splicing regulation. Although multimerization of HuC along RNA targets has not yet been shown directly, there is evidence that shows multimerization of HuR, HuB, HuD and Drosophila ELAV on RNA (52,53,55,57,63). Multimerization is involved in the regulation of eukaryotic splicing by Drosophila ELAV (64), and it is known to be important for non-splicing functions of Hu proteins. For example, HuR can alleviate microRNA (miRNA)-mediated repression of translation of target mRNAs by binding to sites away from the miRNA-binding site and then oligomerizing along the RNA to displace the miRNA–RNA-induced silencing complex from its binding site (56). An interesting possibility is that Hu proteins may regulate splicing in part by binding to their U-rich target sequences, which are often degenerate and spread out, and multimerizing along the RNA, allowing them to affect the interaction of splicesomal components or splicing factors even at sites away from the Hu-binding sites. Another splicing regulator, hnRNP A1, has been proposed to use a similar mechanism to suppress the inclusion of the HIV-1 tat exon 3 by binding to an exonic splicing silencer and cooperatively propagating along the exon (65).

An interesting question is how the binding of HuC to similar U-rich sequences surrounding different alternative exons can suppress the inclusion of some exons but enhance the inclusion of others. A recent genome-wide study in HuC/HuD knockout mouse brains identified at least 37 exons whose inclusion is regulated in an HuC/HuD-dependent manner, indicating that the regulation of splicing by HuC may be widespread. In this study, they observed that exons whose inclusion is positively regulated by HuC/HuD tend to have U-rich HuC-binding sites downstream of the alternative exon, whereas exons whose inclusion is suppressed by HuC tend to have binding sites both upstream and downstream of the exon (13). The RNA sequences required for HuC-mediated regulation of NF1 and HuD splicing also follow this pattern (23,26). Thus, one possibility is that the position of HuC-binding sites relative to alternative exons helps determine whether HuC promotes or inhibits inclusion, as has been observed for many other splicing regulatory proteins (34). Different splicing regulatory proteins may interact with HuC when it is bound to U-rich sequences in different contexts, influencing how it regulates exon inclusion. The next step is to identify such interacting proteins and to determine which domains of HuC are important for these interactions.

Of the four Hu protein family members, by far the most studied is HuR, followed by HuD, whereas relatively few studies have focused on the functions of HuB and HuC. Here, we add to the limited knowledge of how the domains of HuC affect its function. For instance, we determined the effects of deleting domains on protein localization and determined the domains required for HuC–HuC interaction. Although we focused on the splicing regulatory functions of HuC, our results could also provide insight into how HuC performs its other molecular functions. For example, it would be interesting to determine whether the same specific portions of the HuC hinge and RRM3 that we determined were essential for HuC–HuC interaction are also required for regulation of mRNA stabilization and protein translation by HuC, implying a potential role for HuC–HuC interaction in these functions.

The fact that each domain of HuC apart from the N-terminus plays a role in splicing regulation suggests that this regulation is complex. In addition, the finding that different portions of the HuC hinge and RRM3 domains are required for optimal regulation of different splicing events suggests that each exon may have evolved its own mechanisms for fine-tuning splicing regulation. As more of the many recently identified targets of Hu-mediated splicing regulation are characterized in detail, it will be interesting to contemplate whether and to what extent the roles identified here of individual HuC domains in splicing regulation are generalizable to the regulation of many alternative exons and to other Hu proteins.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2 and Supplementary Figure 1.

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REFERENCES


