A recent genome-wide bioinformatic analysis indicated that 54% of human genes undergo alternative polyadenylation. Although it is clear that differential selection of poly(A) sites can alter gene expression, resulting in significant biological consequences, the mechanisms that regulate polyadenylation are poorly understood. Here we report that the neuron-specific members of a family of RNA-binding proteins, Hu proteins, known to regulate mRNA stability and translation in the cytoplasm, play an important role in polyadenylation regulation. Hu proteins are homologs of the Drosophila embryonic lethal abnormal visual protein and contain three RNA recognition motifs. Using an in vitro polyadenylation assay with HeLa cell nuclear extract and recombinant Hu proteins, we have shown that Hu proteins selectively block both cleavage and poly(A) addition at sites containing U-rich sequences. Hu proteins have no effect on poly(A) sites that do not contain U-rich sequences or sites in which the U-rich sequences are mutated. All three RNA recognition motifs of Hu proteins are required for this activity. Overexpression of HuR in HeLa cells also blocks polyadenylation at a poly(A) signal that contains U-rich sequences. Hu proteins block the interaction between the polyadenylation cleavage stimulation factor 64-kDa subunit and RNA most likely through direct interaction with poly(A) cleavage stimulation factor 64-kDa subunit and cleavage and polyadenylation specificity factor 160-kDa subunit. These studies identify a novel group of mammalian polyadenylation regulators. Furthermore, they define a previously unknown nuclear function of Hu proteins.

Polyadenylation, a process through which a poly(A) tail of 150–250 adenosines is added to the 3′-end of a newly synthesized mammalian pre-mRNA, plays an essential regulatory role in almost every aspect of gene expression including transcription termination, splicing, mRNA transport, mRNA stability, and protein translation (1). A recent genome-wide bioinformatic analysis indicated that a significant number of human and mouse genes undergo alternative polyadenylation (54 and 32%, respectively) (2). Of the human genes with multiple polyadenylation sites, 41% have polyadenylation sites located on different exons, indicating an important role for polyadenylation in gene regulation. A well characterized example of this type of regulation is the human calcitonin/calcitonin gene-related peptide (CGRP) gene, which has two alternative 3′-terminal exons that are selectively included in two different tissues to produce two functionally distinct polypeptides (3).

Polyadenylation of eukaryotic pre-mRNA is a two-step reaction including cleavage and addition of the poly(A) tail to the newly generated 3′-end (1, 4). In mammals, two major cis-acting elements define a poly(A) site, a highly conserved AAUAAA hexanucleotide located 10–30 nucleotides upstream of the cleavage site and a G/U- or U-rich downstream element located 20–40 downstream of the cleavage site. Some poly(A) sites also contain one or more U-rich upstream sequence elements (USEs) surrounding the AAUAAA. Trans-acting factors are required to assemble on a poly(A) site to carry out the polyadenylation reaction. The multiprotein cleavage and polyadenylation specificity factor (CPSF) complex binds at the AAUAAA sequence through the CPSF160 subunit. The heterotrimeric complex cleavage stimulation factor (CstF) binds at the downstream element sequence through the CstF64 subunit. In addition to these two complexes, poly(A)/polymerase, and cleavage factors I and II (CFI, CFII), the carboxyl-terminal domain of RNA polymerase II and nuclear poly(A)-binding protein I are also involved in polyadenylation (1, 4).

Although nearly all of the components of the basal polyadenylation machinery have been identified and reconstituted in vitro, regulation of polyadenylation is not well understood. The factors that have been shown to regulate polyadenylation include two basal polyadenylation factors, CFIm68 and CstF64 (5–8), heterogeneous nuclear ribonucleoproteins (H/H′, F, and polypyrimidine tract-binding protein) and the U1 small nuclear ribonucleoprotein-associated proteins (8–12). Tissue-specific regulation of polyadenylation is an understudied area. Although a testis-specific basal polyadenylation factor, CstF64 protein, was proposed to change the preference of the machinery as a whole to favor poly(A) sites containing non-canonical
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hexanucleotides (13, 14), factors that regulate transcript-specific events in a tissue-specific manner have not been identified in mammalian cells. Interestingly, this type of factor has been demonstrated in flies. The embryonic lethal abnormal visual (ELAV) protein was recently shown to inhibit the non-neuronal 3′-end processing, thereby promoting the neural splicing of the erect wing (ewg) pre-mRNA (15, 16).

Mammalian Hu proteins are a group of RNA-binding proteins closely related to the Drosophila ELAV protein. The three neuron-specific members of the Hu family, HuB (HelN1 in human), HuC, and HuD, were cloned as autoimmune antigens of a paraneoplastic neurodegenerative disorder called Hu syndrome (17). All members of the Hu protein family have been shown to interact with AU-rich elements and regulate mRNA stability; the human HuB protein HelN1 has also been shown to modulate translation (18). Recently, we demonstrated a nuclear function of Hu proteins as regulators of alternative RNA processing (19), although the underlying mechanism remains to be determined. Given that ELAV and Hu proteins belong to a well conserved protein family, we have investigated the possibility that Hu proteins regulate polyadenylation in mammalian cells.

In this report, we have provided evidence for a novel function of the neuron-specific Hu proteins as polyadenylation regulators. Using an in vitro polyadenylation assay with HeLa cell nuclear extract and recombinant Hu proteins, we have shown that both cleavage and poly(A) addition at two of the four tested poly(A) sites (SV40 late (SVL) and the non-neuronal alternative 3′-terminal exon 4 of the calcitonin/CGRP pre-mRNA) are blocked by Hu proteins. We have demonstrated that the polyadenylation inhibition is specific to poly(A) sites that contain a U-rich sequence near cleavage sites and depends on binding of Hu proteins to the U-rich sequences. Furthermore, we have shown that the third RRM on Hu proteins, which is dispensable for RNA binding, is required to block polyadenylation. We have also discovered interactions between Hu proteins and two poly(A) factors, CstF64 and CPSF160, and shown that Hu proteins block binding of CstF64 to the U-rich sequence-containing poly(A) site. These studies identify a novel nuclear function of Hu proteins as polyadenylation regulators. They also suggest a novel mechanism of regulated alternative RNA processing by Hu proteins in neurons.

EXPERIMENTAL PROCEDURES

Plasmids—To generate cDNA sequences of the mouse HuB and HuC, reverse transcription (RT)-PCR was carried out using RNA isolated from the mouse F9 cells and mHuB- or mHuC-specific oligonucleotides. The PCR products were digested with BamHI and EcoRI and cloned into the BamHI and EcoRI sites in pGEX-2TK vector (Amersham Biosciences). The Hu/A/HuR cDNA in pTet.MyC.HuR (a gift from Dr. Ann-Bin Shyu, University of Texas Health Science Center, Houston, TX) was PCR-cloned digested with EcoRV and subcloned into pcDNA3.1HisB. The HuA/HuR cDNA in pTet.Myc.HuR (a gift from Dr. Ann-Bin Shyu, University of Texas Health Science Center, Houston, TX) was PCR-cloned digested with EcoRV and subcloned into pcDNA3.1HisB.

Sequences—A subset of mammalian cellular and viral poly(A) sites were retrieved from GenBank (15, 16). The reporter, 1

A. Poly(A) site sequence:

SVL: GCCUUUAACUGGUAAGAUAGCUGAUUGUCUAAUUGUAAACCAUUAUAAAGCAAGG
Calcitonin Ex4: AUCUAACUGGCAGGCUGCUCCUUGGCAUGCUUGCUUGGAGG
APRT: GAGUCGUGGACAGCACACACAGCAGGCGAUC
GH: AGUGCCUCUCUCGCCDCCGAAGCAGCAUCACCGCAGCAGCCACCGUGGCGC

B. RNA substrates:

C. Recombinant proteins:

FIGURE 1. Sequence and structure of the polyadenylation substrates used in this study. A, sequences upstream and downstream of the cleavage site of the four Poly(A) sites are shown. The AAUAAA hexanucleotide and the CA dinucleotide preceding the cleavage sites are underlined. B, diagram showing the cleavage and poly(A) addition (precleaved) substrates. C, recombinant proteins used in this study. 8 μg of each GST protein was run on an SDS-polyacrylamide gel and stained with Gelcode Blue Stain (Pierce).

hGH poly(A) site. The reporters were generated by PCR-directed cloning.

Recombinant Proteins—Glutathione S-transferase (GST) fusion proteins were prepared using the B-PER GST spin purification kit (Pierce).

Nuclear Extract—Nuclear extracts were prepared from HeLa cells and the mouse brain as previously described (20, 21).

In Vitro Assays—Polyadenylation cleavage, poly(A) addition, UV cross-linking reactions, and gel mobility shift assay were carried out as described previously (22–24). Cross-linked polypeptides were immunoprecipitated using Hu patient sera (a gift from Dr. Jerome Posner, NY) or anti-CstF64 antibody 3A7.

In Vitro Translation, GST/His Pulldown, and Co-immunoprecipitation—In vitro translation was performed with the TnT-coupled transcription-translation rabbit reticulocyte system (Promega). GST/His pulldown and co-immunoprecipitation experiments were carried out as described previously (23). RNase A was included in the pulldown assays.

HeLa Cell Transfection—Transfection, RNA isolation, RT-PCR analysis, protein isolation, and Western blot analysis were carried out as previously described (23). One microgram of reporter, 1 μg of LacZ plasmid, and 0.4 μg of HuR or pcDNA3.1HisB plasmid were used in the transfections. Radioactivity in RT-PCR products was measured by PhosphorImager (Amersham Biosciences) analysis. RT-PCR products of the pre-mRNA and mRNA were excised from a gel and sequenced to confirm their identity.

RESULTS

Hu Proteins Block Polyadenylation at Sites Containing U-rich Sequences—A subset of mammalian cellular and viral poly(A) sites contain U-rich USE sequences that function to promote
efficiency of polyadenylation at these sites (23). The fact that Hu proteins specifically bind to the AU-rich elements in the 3′-untranslated regions of a number of mammalian mRNAs to regulate mRNA stability prompted us to examine whether Hu proteins might also play a role in regulating polyadenylation.

To address this question, we carried out in vitro polyadenylation assays using HeLa cell nuclear extract and recombinant Hu proteins. Both cleavage and poly(A) addition were assayed. We chose poly(A) sites derived from four pre-mRNAs based on U content in the vicinity of the poly(A) sites. Of the four sites, the SVL poly(A) site and the human calcitonin/CGRP exon 4 poly(A) site contained more than one U-run that contained three or more Us, whereas the hamster adenine phosphoribosyl transferase and hGH poly(A) sites did not (Fig. 1A). The SVL poly(A) site had several U-runs both upstream and downstream of the cleavage site, whereas the calcitonin exon 4 poly(A) site had one long U-run between the hexanucleotide and cleavage site and another one upstream of the hexanucleotide. The sizes of the cleavage and precleaved poly(A) addition substrates for all four poly(A) sites are shown in Fig. 1B. All but the growth hormone poly(A) site have been previously documented in similar polyadenylation cleavage assays (22). In establishing the poly(A) addition assay, we initially confirmed that, under our assay conditions, poly(A) addition at the calcitonin exon 4 poly(A) site is dependent on the AAUAAA hexanucleotide (data not shown).

As shown in Fig. 2, A and B, the addition of GST-mHuB blocked polyadenylation of the SVL and calcitonin exon 4 poly(A) sites at both the cleavage and poly(A) addition steps but had no effect on adenine phosphoribosyl transferase and growth hormone poly(A) sites. Addition of the GST protein had no effect on any of the poly(A) sites. Note that the low cleavage activity of the calcitonin exon 4 poly(A) site made it somewhat difficult to assess the effect of Hu proteins on polyadenylation cleavage of this site. However, the reproducible reduction of the cleavage activity coupled with a significant reduction of the poly(A) addition activity (Fig. 2B) and the CstF64 binding activity (see Fig. 5A) in the presence of the Hu proteins clearly indicates that Hu proteins affect polyadenylation at this site. We tested all of the Hu proteins (HuR, mHuB, mHuC, and hHuD) in polyadenylation cleavage assays on the SVL poly(A) site and found no difference in their ability to block polyadenylation (supplemental Fig. S1A). Results with only GST-mHuB are shown in Figs. 2 and 3. The ability of the Hu proteins to block polyadenylation does not correlate with the strength of the poly(A) sites, because SVL and calcitonin exon 4 represent the two extremes, the former being a very strong site and the latter

**FIGURE 2.** Hu proteins block polyadenylation of and interact with two poly(A) sites. A, effect of mHuB on in vitro polyadenylation cleavage. The 32P-labeled in vitro transcribed RNA cleavage substrates were assayed in HeLa cell nuclear extract under polyadenylation cleavage condition in the presence of buffer alone (lane 1) or increasing concentrations (0.3 and 1.2 μM) of GST (lanes 2 and 3) or GST-mHuB (lanes 4 and 5) protein. P, precursor; C, cleavage product. B, effect of mHuB on in vitro poly(A) addition. The 32P-labeled in vitro transcribed precleaved RNA substrates were assayed in HeLa cell nuclear extract under poly(A) addition in the presence of buffer alone (lanes 1), 1.2 μM GST (lanes 2), or GST-mHuB (lanes 3) protein. P, precursor; pA, poly(A) addition product. C, Hu proteins interact with poly(A) sites containing U-rich sequences. The 32P-labeled in vitro transcribed precleaved RNA substrates were UV cross-linked in HeLa cell nuclear extract supplemented with GST-mHuB protein (0.3 μM) and immunoprecipitated with antibodies specific to Hu proteins.
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A. Mutated sequence

|     | Wild type | Mut 1 | Mut 2 | Mut 3 |
|-----|-----------|-------|-------|-------|
| 1   | AAUAAAAAUAAUUUUUCCCCA |       |       |       |
| 2   | AAUAAAAACUAACUAUUCCCCCA |       |       |       |
| 3   | AAUAAAAAUAAUUUUUGACUA |       |       |       |
| 4   | AAUAAAAUAAUUGCAACCA |       |       |       |

B. UV cross-linking/IP

C. Poly(A) addition

![Diagram showing poly(A) addition with wild type and mutated sequences](image)

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FIGURE 3. Binding of Hu protein is required for blocking poly(A) addition of the calcitonin exon 4 poly(A) site. A, sequences of the U-rich region of the wild type and mutant calcitonin exon 4 poly(A) sites. B, the 32P-labeled in vitro transcribed cleavage precursor RNA substrates were UV cross-linked in HeLa cell nuclear extract in the presence of 0.3 μM GST-mHuB and immunoprecipitated with antibodies specific to Hu proteins. C, poly(A) addition analysis of wild type (lanes 1–3) or mutated (lanes 4–12) exon 4 precleaved precursor RNA substrates. The RNA substrates were assayed in HeLa cell nuclear extract in the presence of buffer alone (lanes 1, 4, 7, and 10), 1.5 μM GST-mHuB (lanes 2, 5, 8, and 11), or GST protein (lanes 3, 6, 9, and 12).

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a weak site (Fig. 2 A and B, compare the product to the precursor ratio in lane 1 of the SVL and calcitonin exon 4 panels).

The Polyadenylation-blocking Activity of Hu Proteins Depends on Their Binding to U-rich Sequences Surrounding the Hexanucleotide—Hu proteins have been shown to have a strong affinity for U-rich sequences (25–27). We suspected that Hu proteins bind to the U-rich sequences located close to the AAUAAA hexanucleotide in the SVL and calcitonin exon poly(A) sites, which is the binding site of the 160-kDa subunit of cleavage/polyadenylation specificity factors (CPSF160) required for both steps of polyadenylation. To determine whether Hu proteins bind to any of the four poly(A) sites, UV cross-linking/immunoprecipitation assays were carried out using HeLa cell nuclear extract supplemented with the recombinant GST-mHuB protein. Anti-Hu sera derived from patients who suffer from the Hu syndrome that can detect all three neuron-specific Hu proteins (HuB, HuC, and HuD) (but not the ubiquitously expressed HuA (HuR)) were used to immunoprecipitate the cross-linked proteins. Precleaved substrates that contained only the sequences upstream of the cleavage site were used in this assay. Interestingly, strong binding of mHuB to both the SVL and calcitonin exon 4 poly(A) sites (but not to the adenine phosphoribosyl transferase and growth hormone sites) was detected (Fig. 2B). A gel mobility shift assay using the same RNA substrate was also carried out, the result of which is consistent with the cross-linking/immunoprecipitation assay (supplemental Fig. S2). These results imply that Hu proteins preferentially block poly(A) sites containing U-rich sequences. To test specificity of the Hu effect in polyadenylation, we generated three mutant RNA substrates that contained different point mutations in the U-rich region (Fig. 3A). When we examined these mutant substrates for binding to Hu proteins and poly(A) addition activity, we observed a strong inverse correlation between the two activities. A U-rich sequence mutant (Fig. 3A, mut 1) that completely abolished Hu protein binding also lost its ability to be regulated by Hu proteins in the poly(A) addition assay (Fig. 3B, compare lane 2 to 1, and 3C, compare lane 5 to 2). A milder mutant (Fig. 3A, mut 3) that showed reduced but still detectable Hu binding activity was partially blocked by an Hu protein in the poly(A) addition assay (Fig. 3B, compare lane 4 to 1, and 3C, compare lane 11 to 2). Finally, in mutant 2, where the C-rich (but not the U-rich) sequence was disrupted, Hu protein binding was not affected nor was poly(A) addition (Fig. 3B, compare lane 3 to 1, and 3C, compare lane 8 to 2). These experiments establish a strong correlation between binding of Hu proteins and their ability to regulate polyadenylation.

All of the Three RRMs on Hu Proteins Are Required to Block Polyadenylation—It is possible that Hu proteins block polyadenylation by binding to the U-rich sequences close to a poly(A) site and simply block the access of poly(A) factors to the polyadenylation signals. We carried out two sets of experiments to address this issue. First, because U-rich sequences can also be bound by other proteins such as polypyrimidine tract-binding protein and TIAR, we tested whether these proteins would block polyadenylation of the SVL poly(A) site. Both proteins were similar to Hu proteins in size. The addition of GST-TIAR did not affect poly(A) cleavage at this site, even though it can
Hu Proteins Block Binding of CstF64 to Poly(A) Sites and Interact with Two Polyadenylation Factors—To probe the mechanism whereby Hu proteins inhibit polyadenylation of the SVL RNA, we tested whether the addition of GST-polypyrimidine tract-binding protein did not affect polyadenylation at this site (data not shown). These results indicate that the polyadenylation-inhibitory effect is specific to the Hu proteins. Second, specific RNA binding was demonstrated previously for the RRM1 and RRM2 domains of HuD and the RRM3 domain for HuB (26–28). To test which RRMs are required to block polyadenylation, we generated two truncation mutants of mHuB, one containing RRMs 1 and 2 and the other RRM3 (Fig. 4B). Neither of these two mutants blocked SVL polyadenylation cleavage (Fig. 4B). However, RRM12 (but not the RRM3 mutant) showed strong binding to RNA (Fig. 4C). The exact same result was observed with the hHuD full-length and RRM12 mutant (data not shown). Taken together, these results indicate that binding to RNA by itself is not sufficient for Hu proteins to block polyadenylation. It is likely that Hu proteins interact with polyadenylation factors to block their polyadenylation activity.

Hu Proteins Block Binding of CstF64 to Poly(A) Sites and Interact with Two Polyadenylation Factors—To probe the mechanism whereby Hu proteins inhibit polyadenylation of SVL and calcitonin exon 4, we first tested whether the addition of Hu proteins to HeLa cell nuclear extract blocks CstF64 binding to the exon 4 poly(A) site. The 32P-labeled in vitro transcribed cleavage precursor RNA (Fig. 1B) was UV cross-linked in HeLa cell nuclear extract in the presence of buffer alone (lane 1), increasing amounts (0.3 and 1.5 μg) of GST-mHuB (lanes 2 and 3) or GST (lanes 4 and 5) and immunoprecipitated with the antibody specific to CstF64. GST/His-pulldown experiment with GST/His-Hu proteins and 35S-labeled CPSF160 (left panel) or CstF64 (right panel). 10% of input 35S-labeled protein was included in lane 1 in each panel. GST/His-tagged proteins (2 μg of each protein) used in this assay include GST (lane 2), GST-mHuB (lane 3 on left panel), GST-mHuC (lane 4 on left panel and lane 3 on right panel), and His-hHuD (lane 5 on left panel and lane 4 on right panel). Co-immunoprecipitation of CstF64 with Hu proteins. The mouse brain nuclear extract was subjected to immunoprecipitation with the Hu patient sera. Proteins in total nuclear extract (NE) (50% input) (lane 1), supernatant (Sup) (5%) (lane 2) or pellet (lane 3) after immunoprecipitation were separated on SDS-PAGE and probed with antibody specific to CstF64 (top panel) or TIA-1/TIAR (bottom panel) in Western blot analysis.
molecular weight of the His-hHuD protein was detected (data not shown). This result is interesting in light of the observation that the Drosophila ELAV protein is co-immunoprecipitated with dCstF64 when added to the non-neuronal nuclear extract to examine binding of dCstF64 to the non-neuronal poly(A) site of the ewg pre-mRNA. However, in that case, binding of dCstF64 to the poly(A) site was not reduced by the ELAV protein (16).

The CstF64 binding result indicates that Hu proteins block polyadenylation of SVL and the calcitonin exon 4 by interfering with the interaction of poly(A) factors to the poly(A) sites and also suggests that CstF64 interacts with Hu proteins. To test this hypothesis, we examined the potential interaction between Hu proteins and poly(A) factors by a GST/His pulldown assay using recombinant Hu proteins (GST-mHuB, GST-mHuC, and His-hHuD) and 35S-labeled CstF64, CPSF160, or CFIm68. RNase was included to ensure that any interaction detected was not mediated by RNA. The result of this experiment demonstrates that Hu proteins interact with CstF64 and CSPF160 but not CFIm68 (Fig. 5B and data not shown). Thus, Hu proteins block polyadenylation by interacting, mostly likely directly, with CstF and CPSF, which are required for both cleavage and poly(A) addition steps. To test whether the interaction between Hu proteins and poly(A) factors occurs in vivo in cells where Hu proteins are naturally expressed, we carried out a co-immunoprecipitation assay using nuclear extract isolated from the mouse brains. CstF64 was detected in the complex immunoprecipitated with the anti-Hu sera, whereas the control proteins TIA-1/TIAR were not detected (Fig. 5C).

**HuR Blocks Polyadenylation of the SVL Poly(A) Site in Cultured Cells**—To demonstrate the biological relevance of our findings in vitro, we tested whether the results could be duplicated in a cell transfection experiment. We made two reporter constructs that were identical, except for the poly(A) sites; one contained the SVL poly(A) site and the other the hGH poly(A) site (Fig. 6A). The two reporters were individually transfected with either a vector control or the HuR expression plasmid in HeLa cells. A third plasmid, LacZ expression vector, was included as an internal control. Semicontinuous RT-PCR was carried out using total RNA isolated from the transfected cells and two sets of primers. One set of primers was used to detect the precursor transcript and the other set, including an oligo(dT) primer, to detect the polyadenylated mRNA. As shown in Fig. 6B, the level of the SVL poly(A) site-containing mRNA was decreased when HuR was overexpressed. Importantly, this change in the mRNA level was accompanied by an increase in the pre-mRNA level, suggesting that the HuR effect was not the result of a change in mRNA turnover. No significant change of either the mRNA or the pre-mRNA level was observed with the reporter containing the hGH poly(A) site in the absence or presence of overexpressed HuR.

Interestingly, we observed reduced splicing when the transcript containing the SVL poly(A) site was blocked at polyadenylation. This result is consistent with the previously demonstrated coupling of polyadenylation and splicing during RNA processing (Refs. 29–31 and reviewed in Ref. 32). It has been documented that mutations of polyadenylation signal AAUAAA or downstream sequences decreases not only polyadenylation but also splicing efficiency.

**DISCUSSION**

In this report, we have demonstrated that the mammalian Hu proteins regulate polyadenylation by blocking poly(A) sites containing U-rich sequences. Our studies expand the very short list of polyadenylation regulators in mammals. Given that >50% of human pre-mRNAs undergo alternative polyadenylation and alternative poly(A) sites are frequently associated with AU-rich sequences within 100 nucleotides from the cleavage site, our results provide a basic understanding of how Hu proteins regulate alternative polyadenylation is of great importance.

The polyadenylation-regulating activity of Hu proteins correlates with their binding to the U-rich sequences upstream of the cleavage site. These U-rich sequences are similar to the previously characterized USEs present in some of the cellular and viral poly(A) sites and necessary for efficient polyadenylation at those sites harboring them (23). In the case of the calcitonin exon 4 poly(A) site, mutation of the U-rich sequence did not reduce the polyadenylation efficiency (Fig. 3C, compare lanes 1, 4, 7, and 10). Thus, whether the U-rich sequence functions as a classical USE in this poly(A) site remains to be further determined. Recently, Kaufmann et al. (23) have demonstrated that a newly discovered human CPSF subunit, Fip1, binds to the L3 U-rich USEs containing AAUAAA and stimulates poly(A) polymerase. Interestingly, in this study, Fip1 was also found to bind to the SVL poly(A) site, the same site that can be blocked by Hu proteins (23). In a more recent study by Zhao et al. (34),
a 57-nucleotide USE was identified in human papillomavirus 16 that interacts with several nuclear factors including Fip1. Taken together, these studies suggest an intriguing potential mechanism in which Hu proteins compete with and/or prevent Fip1 from binding to the U-rich sequences. In light of another recent discovery (7) that CFIm68 binds to a number of USE sequences containing UGUAN repeats and promotes polyadenylation and that the SVL poly(A) site contains such repeats, it remains a formal possibility that Hu proteins and CFIm may modulate the polyadenylation activity of sites that contain binding sites for both proteins through their competing activities. Given that USEs have been identified in an increasing number of cellular poly(A) sites (23, 35–40), it will be of particular interest to further investigate the role of Hu proteins in USE-mediated polyadenylation regulation.

How do Hu proteins regulate polyadenylation? We demonstrate that all three of the RRMs of Hu proteins are required for the polyadenylation-blocking activity of these proteins, suggesting that RNA-binding activity alone is not sufficient. Presumably, RM3 and the hinge region are involved in interacting with the poly(A) factors. We favor a model in which Hu proteins bind to RNA and poly(A) factors simultaneously. Such interactions may modulate the structure of the poly(A) complex formed on the U-rich sequence-containing sites in a way that renders the complex non-functional. Presumably, neither RNA binding nor interaction with poly(A) factors alone can induce such a rearrangement.

Given that three of the Hu proteins are neuron-specific and interact with CstF64 in brain nuclear extract (Fig. SC), our studies also suggest an appealing mechanism for neuron-specific alternative RNA-processing regulation. Calcinonin/CGRP pre-mRNA is differentially processed in neurons, where the non-neuronal calcinonin exon 4 is skipped and the exons 5 and 6 are included to produce CGRP (3). It was previously demonstrated that inclusion of exon 4 is promoted by a number of factors bound at an intronic element in non-neuronal cells (20, 24, 41, 42). Of these factors, U1 small nuclear ribonucleoprotein and SRp20 were shown to promote polyadenylation of this exon (20, 22). Recently, we demonstrated that Hu, as well as Fox-1/Fox-2 proteins promote the neuron-specific skipping of the calcinonin exon 4 (19, 43). It is likely that, in neurons, Hu proteins block polyadenylation of exon 4, thereby promoting the neuron-specific pathway. This mechanism may function in conjunction with other neuronal factors, such as Fox-1/Fox-2, to regulate the neuron-specific skipping of exon 4. A similar example is the Drosophila ewg pre-mRNA. ELAV protein, the Drosophila homolog of Hu proteins, inhibits 3′-end processing within the non-neuronal exon of the ewg pre-mRNA to promote neural splicing (16).

Although both Hu proteins and ELAV block polyadenylation, major differences exist between the functions of these proteins. ELAV binds to several runs of AU-rich sequence downstream of the cleavage site of the non-neuronal 3′-terminal exon close to where CstF64 binds, whereas Hu proteins bind at sequences adjacent to the AUAAA hexanucleotide, the CPSF160 binding site. However, it is possible that Hu proteins function through binding at the downstream U-rich sequences of those sites, such as SVL, that contain such sequences (Fig. 1). Furthermore, the addition of ELAV to nuclear extract isolated from non-neuronal cells did not block interaction of CstF64 and RNA in contrast to what we observed for the Hu proteins (Fig. 5) (16). These results suggest potential distinct or different variations of mechanisms for these two putative orthologous proteins in regulating polyadenylation.

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Hui Zhu, Hua-Lin Zhou, Robert A. Hasman and Hua Lou

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