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Two different RNA binding activities for the AU-rich element and the poly(A) sequence of the mouse neuronal protein mHuC

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ABSTRACT

HuC is one of the RNA binding proteins which are suggested to play important roles in neuronal differentiation and maintenance. We cloned and sequenced cDNAs encoding a mouse protein which is homologous to human HuC (hHuC). The longest cDNA encodes a 367 amino acid protein with three RNA recognition motifs (RRMs) and displays 96% identity to hHuC. Northern blot analysis showed that two different mRNAs, of 5.3 and 4.3 kb, for mouse HuC (mHuC) are expressed specifically in brain tissue. Comparison of cDNA sequences with the corresponding genomic sequence revealed that alternative 3′ splice site selection generates two closely related mHuC isoforms. Iterative *in vitro* RNA selection and binding analyses showed that both HuC isoforms can bind with almost identical specificity to sequences similar to the AU-rich element (ARE), which is involved in the regulation of mRNA stability. Functional domain mapping using mHuC deletion mutants showed that the first RRM binds to ARE, that the second RRM has no RNA binding activity by itself, but facilitates ARE binding by the first RRM and that the third RRM has specific binding activity for the poly(A) sequence.

INTRODUCTION

Recently, a group of neuronal RNA binding proteins have been reported in several organisms. Elav is a neuron-specific RNA binding protein which is required for proper differentiation and maintenance of neurons in *Drosophila melanogaster* (1–5). Rbp9 is another fly Elav-like protein and is expressed only in the nervous system (6). Three human proteins, HuD, HuC and Hel-N1, belong to the group of Hu antigens that are recognized by autoimmune Hu antibodies of patients with paraneoplastic neurological disorders (7–10). Hu antigen homologs have been identified in other mammals, *Xenopus* and zebrafish (11–14). These Hu proteins share extensive similarity to Elav in that all contain three RNA recognition motifs (RRMs) and a linker region separating the first two RRMs from the last and are believed to be members of the vertebrate Elav family. RRMs are found in many RNA binding proteins, consist of ~90 amino acids, are characterized by structural conservation of two α-helices and four β-strands (for review see 15–17) and have been demonstrated to function as core domains for RNA binding in many cases (18–22).

The physiological function of vertebrate Elav family proteins remains unclear. However, the neuron-specific expression of the Hu proteins suggests that, like Elav, they may play vital roles in neuronal cells via three RRMs at the level of post-transcriptional gene regulation (11,12,23,24). A hint about Hu protein function came from biochemical studies showing that several mammalian Hu proteins bind to the AU-rich element (ARE) within the 3′-untranslated regions (3′-UTRs) of mRNAs which encode cell proliferation regulators (8,12,24–26). As AREs have been demonstrated to influence mRNA stability (27–34), Hu proteins are believed to regulate the expression of particular mRNAs by altering their stability, thereby contributing to neuronal differentiation and maintenance. Moreover, a recent finding that human Hel-N1 and Hel-N2 are associated with polysomes in cultured cells has suggested involvement of Hu proteins in translational regulation in neurons (35).

In this study, we cloned and sequenced cDNAs for the mouse HuC homolog (mHuC) and found that alternative splicing generates two mHuC isoforms. By iterative *in vitro* ligand RNA selection and binding analyses, we have demonstrated that both mHuC isoforms can bind to ARE-like RNAs with almost identical specificity. Moreover, we show that three mHuC RRMs play distinct roles in its RNA binding activity: the first RRM is required for ARE binding; this binding is greatly enhanced when the first RRM is connected with the second RRM; the third RRM has specific affinity for the poly(A) sequence.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides used in this work were as follows:

mHuC-5-1, 5′-CTG GAT CCA TGG TCA CTC AGA TAC TG-3′;
mHuC-5-2, 5′-CTG GAT CCT GAT GGT CAC TCA GAT A-3′;
mHuC-3-1, 5′-CTG AAT TCA ATG CTC AGG CCT TGT G-3′;
mHuC-S1, 5′-TCA ACA CCC TCA ATG GC-3′;
RRM1-END, 5′-ATG GAT CCT CAC CGG ATA GAG GCA GAA CT-3′;
RRM2-START, 5′-ATG GAT CCT GGA TGC CAA CCT GTA TGT C-3′;
RRM2-END, 5′-ATG GAT CCT CAC AAA TTG TCC AGC CGG AA-3′;
RRM3-START, 5′-ATG GAT CCT GCT CAA TAT GGC CTA CGG A-3′;
ID, 5′-ATG AAT TCT GCC AGG CCA CTC ATG CCA TC-3′.

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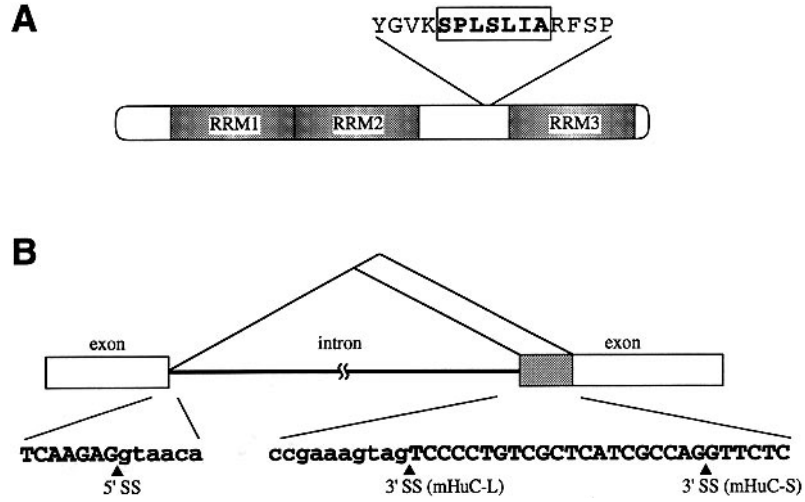

Figure 1. Two mHuC isoforms generated by alternative splicing. (**A**) Schematic representation of the mHuC-L structure. Three RRMs are shown and the alternative seven amino acids in the linker region are boxed. (**B**) Schematic representation of alternative 3′ splice site selection generating the two mHuC isoforms. The nucleotide sequences of both splice site regions are shown below and intron sequences are in lower case. Open and shaded boxes indicate the common and alternative exons respectively.

Screening and sequencing

A newborn mouse brain cDNA library (Stratagene) was screened according to previously described methods (36). Positive recombinant λ ZAPII phages were subjected to plasmid rescue using helper phage and plasmid DNA was obtained by conventional methods (36). The genomic DNA fragment corresponding to the linker region between RRM2 and RRM3 was PCR amplified using the RRM3-START and ID primers, cloned into pUC119 and then sequenced. Sequencing was performed using Sequenase version 2.0 (US Biochemicals) and an autosequencer (ALF express; Pharmacia).

Northern blot analysis

A mouse MTN Blot (Clontech) was used for Northern blot analysis. The RNA from each tissue was checked both qualitatively and quantitatively with a human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA (Clontech) control probe. Hybridization was performed as described under high stringency conditions (36). A Fuji BAS 2000 Image Analyzer and standard autoradiography were used to analyze the hybridization patterns.

Preparation of GST fusion proteins

To construct plasmid pGST-mHuC-L, the fragment containing the mHuC-L coding region was PCR amplified using the mHuC-5-1 and mHuC-3-1 primers, cut with *Eco*RI and *Bam*HI and then cloned into the pGEX-2T plasmid (Pharmacia). For pGST-mHuC-S, a PCR fragment was amplified from cDNA which lacked the 21 nt exon using the mHuC-S1 and mHuC-3-1 primers. The fragment was mixed with a restriction fragment containing the 5′-region (~720 bp) of the longest cDNA and then subjected to crossover PCR using the mHuC-5-1 and mHuC-3-1 primers. The resultant crossover PCR fragment was cut with *Eco*RI and *Bam*HI and then cloned into the pGEX-2T plasmid. For pGST-RRM1, a PCR fragment made from pGST-mHuC-L using the RRM1-END and RRM3-START primers was cut with *Eco*RI and the resultant large fragment was blunt ended and self-ligated. For pGST-RRM1+2, a PCR fragments made from pGST-mHuC-L using the RRM2-END and RRM3-START primers was cut with *Eco*RI and the resultant large fragment was blunt ended and self-ligated. For pGST-RRM2, a PCR fragment made from pGST-mHuC-L using the RRM2-START and RRM2-END primers was cut with *Bam*HI and cloned into the pGEX-3X plasmid (Pharmacia). For pGST-RRM2+3, a PCR fragment made from pGST-mHuC-L using the RRM2-START and mHuC-3-1 primers was cut with *Eco*RI and *Bam*HI and cloned into pGEX-3X. Each expression plasmid was transformed into *Escherichia coli* XL1-blue, GST fusion proteins were induced with 1 mM IPTG for 4 h and affinity purified on glutathione–Sepharose.

In vitro RNA selection and UV crosslinking

In vitro selection using GST–mHuC-L or GST–mHuC-S was performed as described previously (12,37) with the following modifications. A total of seven rounds of selection and amplification were performed and during the last two rounds of selection the KCl concentration was raised to 350 mM in the binding and washing buffers. Washing buffer for the final round contained 0.5 M urea. *In vitro* selected RNAs were synthesized by T7 RNA polymerase in the presence of [α-^{32}P]GTP and purified by denaturing polyaclyamide gel electrophoresis (PAGE) as described previously (37,38). The binding reaction mixture contained, in 10 µl, D′K200T buffer (20 mM HEPES–NaOH, pH 7.9, 200 mM KCl, 5% glycerol, 0.1% Triton X-100, 1 mM DTT, 0.1 mM PMSF), 100 µg/ml yeast RNA, labeled RNA (~2 × 10^4 c.p.m.) and ~5 pmol each GST fusion protein. The mixture was incubated for 20 min at 20°C followed by UV irradiation (600 mJ/cm^2) on ice. The irradiated sample was mixed with 1 µl RNase A (10 mg/ml), incubated for 30 min at 37°C and then subjected to SDS–PAGE as described previously (38). The efficiency of label transfer to each fusion protein was analyzed by densitometry of dried gels using a Fuji BAS 2000 Image Analyzer.

Poly(A) binding assay

The reaction mixture contained, in 30 µl, the binding buffer (10 mM Tris–HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 1 mM DTT, 0.1 mM PMSF), 15 µl poly(A)–Sepharose beads (50% slurry in binding buffer), 50 µg/ml yeast RNA and ~5 pmol each GST fusion protein. The mixture was incubated for 20 min at room temperature with gentle mixing and then the poly(A)–Sepharose beads were washed five times with 300 µl binding buffer. The GST fusion proteins bound to the beads were fractionated on a 12% SDS–polyacrylamide gel and analyzed with the ECL Western blot detection system (Amersham) using an anti-GST rabbit polyclonal antibody (700-fold dilution) and a secondary anti-rabbit Ig antibody (6000-fold dilution).

RESULTS

Nucleotide sequences of mHuC cDNAs

When we isolated cDNA encoding the HuD homolog from a mouse brain cDNA library previously, we obtained a cDNA fragment which possibly corresponded to the third RRM of human HuC (hHuC) (11,25). Using the cDNA fragment as a probe, we screened another mouse brain cDNA library (~5×10^5 recombinants) for clones containing the entire coding region of the protein and isolated seven positive cDNA clones. The longest cDNA contains an open reading frame encoding a 367 amino acid protein with three RRMs (RRM1, RRM2 and RRM3), showing 96% identity to human HuC (Fig. 1). Thus, we concluded that the protein encoded by the cDNA is the mouse HuC homolog (mHuC).

Nucleotide sequence comparison of the mHuC cDNAs revealed that a short segment of 21 nt within the coding region of the longest cDNA was absent from one partial mHuC cDNA clone. The seven amino acid sequence encoded by the segment is located within the linker region between RRM2 and RRM3 (Fig. 1A). As the segment contained a short pyrimidine stretch and an AG dinucleotide, which are features of the vertebrate 3′ splice site consensus sequence (39), it seemed indicative of the occurrence of alternative 3′ splice site selection. To examine this hypothesis, we isolated a genomic DNA fragment of ~6 kb spanning RRM2–RRM3 by PCR using two specific primers outside the putative alternative exon sequence and sequenced exon–intron boundary regions. Sequence comparison of the genomic DNA with the cDNAs showed that the 21 nt segment is an alternative exon generated by 3′ splice site selection (Fig. 1B). Thus, we designated the larger protein, which contains the alternative amino acid sequence, mHuC-L, and the other smaller protein mHuC-S. As the previously described human counterpart hHuC lacks the alternative seven amino acid residues (7,10), it was concluded to correspond to mHuC-S.

Brain-specific expression of mHuC

Expression of mHuC in various mouse tissues was examined by Northern blot analysis (Fig. 2). Two mHuC mRNAs of 5.3 and 4.3 kb were detected only in brain tissue, where the amount of the longer one seemed much greater than that of the shorter one, suggesting the occurrence of alternative RNA processing or promoter utilization, although the detailed structural organization of the two mRNAs remains to be elucidated. The brain-specific mRNA expression indicated that mHuC is a member of the vertebrate Elav family (7–12).

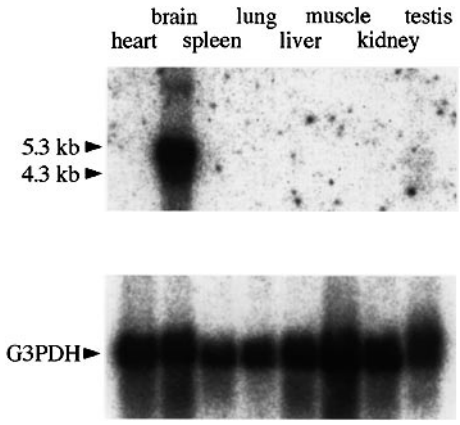

Figure 2. Brain-specific expression of mHuC mRNAs. Northern blot analysis was performed using poly(A)$^+$ RNAs from the indicated mouse tissues. The same blot was reprobed with a G3PDH probe as a control.

In vitro selection of RNAs with affinity for mHuC isoforms

The amino acid sequence deduced from the cDNA sequences suggested that both mHuC isoforms have RNA binding activity, because both contain three identical RRMs. To test this possibility and, if it is the case, to clarify whether there are differences in RNA binding specificity between the two isoforms, we took advantage of iterative *in vitro* ligand selection from a pool of random RNAs as described previously (12,37,40,41). We made a fusion protein in which glutathione S-transferase (GST) was fused to either mHuC-L or mHuC-S and mixed each fusion protein with a pool of RNAs containing 25 nt random sequences. After seven rounds of *in vitro* selection, RNAs bound by each GST–mHuC fusion protein were reverse transcribed and 21 cDNA clones (12 for mHuC-L, 9 for mHuC-S) were subjected to sequence analysis (Fig. 3). All mHuC-selected RNAs contained AU-rich sequences similar to the ARE. Enrichment of such a motif suggested that mHuC is actually an RNA binding protein with specificity like that of other neuronal RNA binding proteins (8,12,25).

Comparison of RNA binding specificity between two mHuC isoforms

To confirm the results from *in vitro* selection analysis, we performed *in vitro* binding analysis using the GST–mHuC isoforms as described previously (12,37). We arbitrarily chose three mHuC-S-selected (S1, S13 and S14) and two mHuC-L-selected (L1 and L3) RNAs for comparison of binding specificity of two mHuC isoforms and performed UV crosslinking assays (Fig. 4; see also Fig. 3). Both mHuC isoforms could bind to all RNAs tested, even if the relative affinity of each RNA varied from 1- to ~3-fold, but they showed no binding to a negative control BS RNA which contains the multi-cloning site sequence derived from the vector plasmid. Significantly, the binding profile of mHuC-S to the five kinds of *in vitro* selected RNAs was almost the same as that of mHuC-L (Fig. 4B). These results indicated that both mHuC isoforms have specific RNA binding activity to ARE-like sequences and that the seven amino acid portion

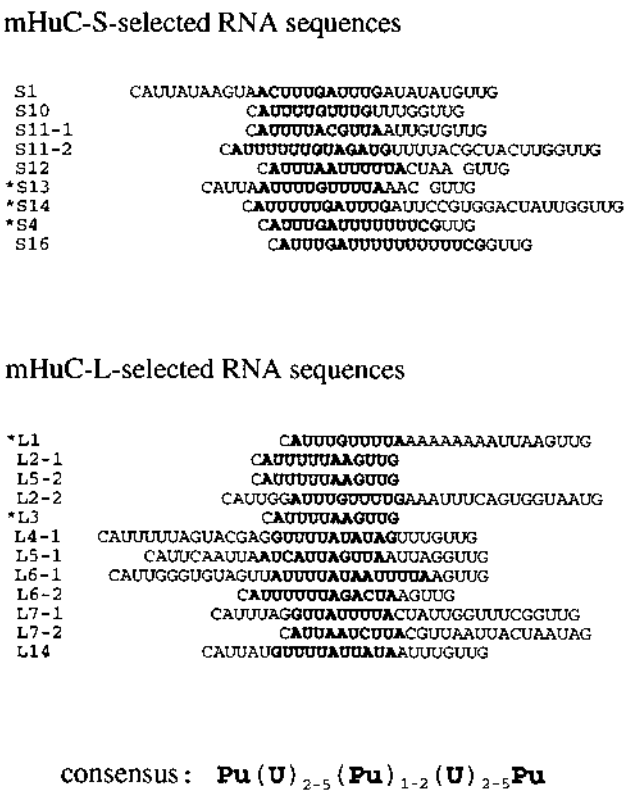

Figure 3. RNA sequences selected *in vitro* by mHuC-S and mHuC-L. Sequences similar to the ARE are in bold face. The consensus sequence is shown below. The RNA sequences which were used for the *in vitro* binding experiment are marked by asterisks.

encoded by the alternative exon does not change the binding specificity of mHuC.

Determination of the domain responsible for ARE binding

Both mHuC isoforms contain three RRMs which are well conserved in other neuronal RNA binding proteins. To determine which of these RRMs contribute(s) to the binding activity for ARE-like sequences, we made several deletion mutants of GST–mHuC-L and examined their binding efficiency to L1 RNA, which showed the highest affinity for both mHuC isoforms (Fig. 5). GST–RRM1 could bind to L1 RNA, but the efficiency was reduced to ~20% as compared with wild-type GST–mHuC-L. GST–RRM3 and GST–RRM2 showed very weak (~3%) and no binding respectively. Remarkably, GST–RRM1+2, containing both the first and second RRMs, showed higher binding activity (~80%), close to the wild-type level, whereas no binding could be detected with GST–RRM2+3. These results indicate that the first RRM1 is primarily responsible for ARE binding and that the second RRM2 has no binding activity *per se* but greatly enhances binding by RRM1 and represses the weak binding by RRM3.

Poly(A) binding by the third RRM

As RRM3 showed only very weak ARE binding and no binding when connected with RRM2, we suspected that it would preferentially bind to some sequence motif other than the ARE. In this respect, a possible *in vivo* function of the neuronal RNA binding protein family is thought to be regulation of stability of some growth-related mRNAs via the ARE in the 3′-UTR and the 3′-UTR of mRNAs also contains a poly(A) sequence. In addition, human Hel-N1 and Hel-N2 have been shown to be UV crosslinked to poly(A)$^+$ mRNAs in cell extracts (35) and human HuD and HuR show poly(A) binding activity *in vitro* (H.M.Furneaux, personal communication). Thus, one possibility is that the target of RRM3 is the poly(A) sequence at the 3′-end of mRNAs. If this is the case, the weak binding of RRM3 to L1 RNA could be interpreted as the result of a weak affinity for the nine consecutive adenine residues downstream of the ARE portion of L1 RNA. To test this possibility, we performed precipitation assays of several GST–mHuC fusions using poly(A)–Sepharose beads. After incubation of each mHuC fusion with poly(A)–Sepharose beads followed by extensive washing, the GST fusion proteins bound to the beads were examined by Western blot analysis using an anti-GST antibody (Fig. 6). Significant amounts of GST–mHuC-L and GST–RRM3 were bound to poly(A)–Sepharose, whereas no binding could be detected with GST alone, GST–RRM1, GST–RRM2 or GST–RRM1+2. These results clearly demonstrate that mHuC binds to the poly(A) sequence as well as to the ARE and that RRM3 is responsible for the poly(A) binding activity.

DISCUSSION

Role of the alternative exon in mHuC function

Alternative 3′ splice site selection was found to generate two mHuC isoforms; one has an additional seven amino acid sequence in the linker region between RRM2 and RRM3, the other does not. Similar alternative splicing occurs in the same

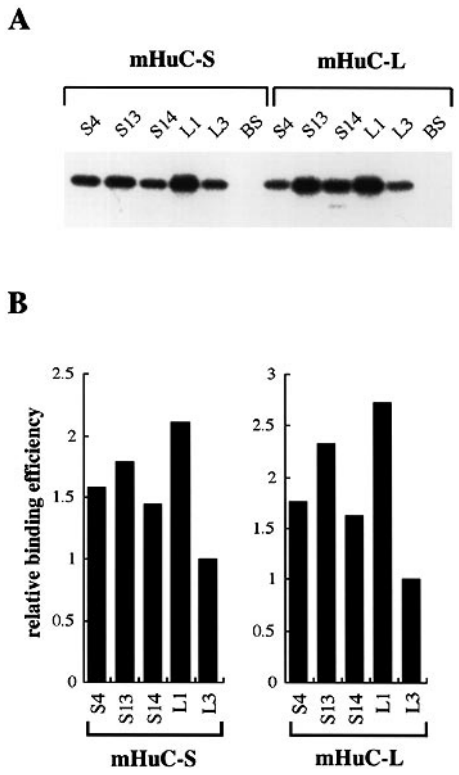

Figure 4. Comparison of the RNA binding profiles of two mHuC isoforms. (**A**) *In vitro* binding of mHuC-S and mHuC-L to their selected RNAs. The five *in vitro* selected RNAs indicated above were crosslinked to both mHuC-S and mHuC-L. BS is a negative control RNA containing the multi-cloning site sequence of the plasmid Bluescript II. (**B**) Relative binding efficiencies of each of the RNAs to the two mHuC isoforms were determined by densitometric analysis using a Fuji BAS-2000 imaging analyzer. The binding efficiency of L3 RNA was taken as 1.0.

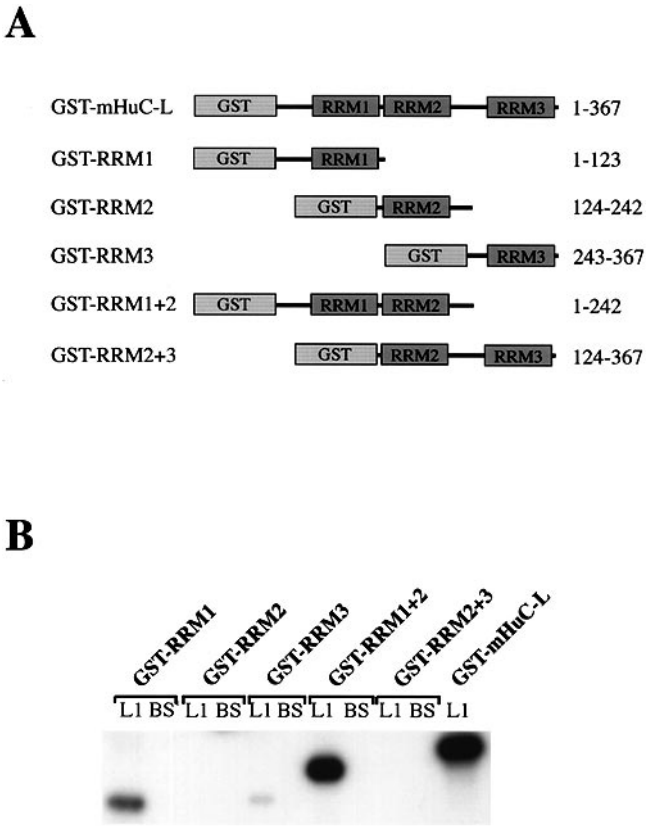

Figure 5. Determination of the ARE binding domain of mHuC-L. (**A**) Schematic representation of GST–mHuC-L and its deletion mutants. The locations of GST and RRMs 1–3 are labelled. The numbers on the right indicate the amino acid residues of mHuC-L, starting with the initiation methionine. (**B**) UV crosslinking experiment with mHuC fusion proteins. Each fusion protein was incubated with either L1 RNA or the negative control BS RNA described in Figure 4 and UV irradiated, followed by RNase treatment, electrophoresis on a 12% SDS–polyacrylamide gel and then autoradiography.

linker region of other Hu proteins (14,25,42). Therefore, the alternative splicing may have some regulatory role in Hu protein function. One possibility is that such an alternative amino acid sequence might modulate RNA binding specificity. However, at least *in vitro*, we could not observe any significant difference in ARE binding specificity between the two mHuC isoforms, although the effect on poly(A) binding remains to be examined. The second possibility is that it might be a domain which interacts with another factor(s) that may cooperate with mHuC and the alternative amino acid residues might affect the interaction and thereby regulate the mHuC function *in vivo*, because the region in the vicinity of the alternative sequence of mHuC is relatively rich in hydrophobic residues. In this respect, we have found that another mouse neuronal RNA binding protein, Mel-N1, seems to interact with mHuC isoforms, but we could not detect any difference in the interaction with the two isoforms (E.Sakashita and H.Sakamoto, unpublished data). At present, no known RNA processing factor interacting with Hu proteins has been reported. It will be necessary to look for such factors to understand the molecular function of Hu proteins in RNA metabolism.

Another attractive idea is that the alternative amino acid sequence might comprise the modification site of some protein kinase(s) in the signal transduction pathway which controls cell proliferation. Indeed, only when the seven amino acid residues are included does the sequence VKSPL arise. This matches the substrate consensus XKSPX of the cell cycle-dependent protein kinase cdc-28 or proline-dependent protein kinase (43). Thus, only mHuC-L may be phosphorylated at the serine residue encoded by the alternative exon and this may affect mHuC function. Considering the close relationship of Hu proteins with cell growth, i.e. their capability to bind ARE-containing mRNAs, including c-*fos* mRNA, it will be of great interest to examine whether such phosphorylation of mHuC occurs in coordination with the cell cycle.

Distinct roles of the three RRMs in RNA binding

In this study, we have demonstrated that mHuC has two different RNA binding activities: one for the ARE, another for the poly(A) sequence. The former activity is essentially carried by RRM1 and greatly enhanced by the concomitant presence of RRM2, which shows no ARE binding by itself. The molecular mechanism of the enhancement of RRM1 function by RRM2 is still unknown, but it is likely that RRM2 supports the RRM1 structure to optimally recognize the ARE. Such enhancer function of an RRM is novel and may explain why the tandem RRM1–RRM2 structure is conserved in all Hu proteins. In the case of human HuD (hHuD),

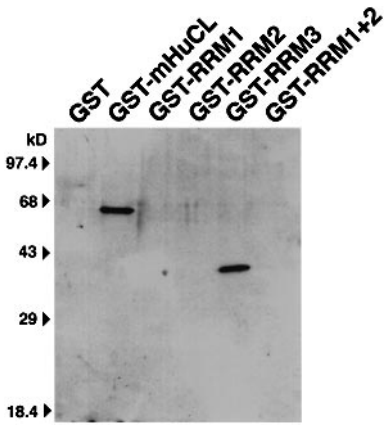

Figure 6. Poly(A) binding by the third RRM of mHuC. Each GST fusion protein indicated was incubated with poly(A)–Sepharose beads in the binding buffer (for details see Materials and Methods). After extensive washing, the protein bound to the beads was examined by Western blot analysis. The positions of size marker proteins (kDa) are shown on the right side.

RRM1 alone shows only very weak binding activity and the tandem array of RRM1 and RRM2 is required for efficient ARE binding (26). It remains to be elucidated whether this minor difference between mHuC and hHuD may result from the difference in the intrinsic nature of these proteins or from some difference in the assay methods used. In both cases, however, it is evident that the first two RRMs, but not the third, are directly involved in ARE binding. In contrast to hHuD and mHuC, the ARE binding domain of Hel-N1 has been reported to be the third RRM (8). At present we do not know why a protein so similar to both mHuC and hHuD uses a different RRM for ARE binding.

Another RNA binding activity of mHuC is for the poly(A) sequence and is carried by RRM3. In the cases of human HuD and HuR, similar activity by the third RRM, which prefers poly(A) chains >70 residues, has been observed (44; H.M.Furneaux, personal communication). In the *in vitro* selection results using full-length mHuC we could not detect poly(A) binding activity, since there is no evident feature of a consensus poly(A) stretch. This may be because the poly(A) binding activity of RRM3 is much lower than the ARE binding activity of RRM1–RRM2 or because restriction of the randomized region length (25 nt) in our *in vitro* selection system does not satisfy the optimal length requirement for the poly(A) binding activity of RRM3, as suggested by Furneaux and co-workers (H.M.Furneaux, personal communication). At present, we interpret the very weak binding of RRM3 to L1 RNA (Fig. 5) as due to a low affinity for the poly(A) residues and not for the ARE entity. The binding activity of RRM3 to the short poly(A) stretch of L1 RNA, even if not optimal, seems to have some additive effect on the total RNA binding activity of mHuC, because L1 RNA showed the highest affinity for GST–mHuC-L of the *in vitro* selected RNAs examined and the affinity was somehow reduced when GST–RRM1+2 was used in place of the full-length protein.

Possible physiological function of Hu proteins

It is well known that the major factors bound to the poly(A) stretch of mRNAs are poly(A) binding proteins, which are well conserved in many organisms. The yeast poly(A) binding protein Pab1p has been shown to be required for deadenylation, a critical step of the mRNA degradation pathway (45,46), and a poly(A) binding protein-dependent nuclease has been identified in yeast, where poly(A) stretch degrading activity is dependent on the 3′-UTR sequence (47,48). Similar poly(A)-specific nuclease activity has also been found in mammalian cells (49,50). Poly(A) shortening has been shown to be the first step in mRNA destabilization of ARE-containing mRNAs, including c-*fos* mRNA in mammalian cells, and to induce decapping and exonucleolytic cleavage of mRNAs in yeast (31,32,51,52).

In the present study, we have demonstrated that mHuC can bind to the poly(A) sequence as well as to the ARE via two distinct activities of its RRMs. Considering the similar results with the human proteins HuD and HuR, it is possible that all Hu proteins can bind concomitantly to both the ARE and the poly(A) stretch of growth-related mRNAs and bridge the two signal sequences. Thus, Hu proteins may mimic or enhance the conventional poly(A) binding protein function on ARE-containing mRNAs and facilitate rapid decay of the mRNAs via deadenylation by poly(A)-specific nuclease in neuronal cells. An opposite possibility also exists, that Hu proteins inhibit the deadenylation step by competing with poly(A) binding proteins for the poly(A) sequence. Also, like CPEB, they might activate cytoplasmic polyadenylation and stabilize ARE-containing mRNAs, leading to efficient translation (53,54). In this case, other ARE binding factors identified so far (55–60) may act directly in rapid mRNA decay by replacing Hu proteins on the ARE. In any case, regulation of Hu protein function in response to some cellular signals might occur and further analyses related to the signal transduction mechanism and on interactions with other proteins will be of importance to understand Hu protein function in neurons.

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