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Complex formation of the neuron-specific ELAV-like Hu RNA-binding proteins

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ABSTRACT

Hu proteins are RNA-binding proteins that are the vertebrate homologs of *Drosophila* ELAV, and are implicated in stabilization or enhanced translation of specific mRNAs with AU-rich elements (AREs) in the 3′-untranslated region. Here, using the yeast two-hybrid system, we show that neuron-specific Hu proteins can interact with themselves. Immunoprecipitation assays demonstrated that the interaction between Hu proteins occurs in mammalian cells and is strongly enhanced in the presence of cellular RNA. Furthermore, using *in situ* chemical crosslinking assays, we found that HuD, one of the neuron-specific Hu proteins, multimerizes in cells. The crosslinked HuD multimers retain specific RNA-binding ability and can interact with additional Hu proteins. Consistent with this biochemical property, HuD showed granular distribution in two neurogenic cell lines. These results suggest that the RNA-bound form of HuD multimerizes cooperatively to form a specific granular structure that may serve as a site of post-transcriptional regulation of ARE-containing mRNAs.

INTRODUCTION

Hu proteins are RNA-binding proteins that were initially identified as autoimmune antigens in human paraneoplastic neurologic disorders (1,2). Four Hu proteins, HuD, HuC, HuB/Hel-N1 and HuR, have been identified in vertebrates (3,4). Three of them, HuD, HuB (Hel-N1) and HuC, are specifically expressed in neurons (1,3,5–11), whereas HuR is expressed in all tissues examined thus far (3,10–12). Hu proteins contain three RNA-binding domains (RBDs) of the ribonucleoprotein (RNP)-consensus sequence family (13), and are also called ELAV-like proteins, as they share extensive similarity with *Drosophila* ELAV protein (1,14). The amino acid sequence of RBDs is well conserved among all of the Hu proteins, and two of these RBDs are located in tandem and separated from the third one near the C-terminus by a linker region that is somewhat diverse in Hu proteins.

Previous studies demonstrated that mammalian Hu proteins bind specifically to AU-rich elements (AREs) in the 3′-untranslated region (3′-UTR) of various mRNAs (6,8,9,15–20). It was shown that RBD1 and RBD2 are responsible for the binding to AREs *in vitro* (9,21). In addition to ARE-binding, Hu proteins also bind to the poly(A) sequence through RBD3 *in vitro* (9,22). These observations strongly suggest that Hu proteins specifically recognize ARE-containing mRNAs by simultaneous binding to AREs and the poly(A) tail, and regulate the expression of the bound mRNAs post-transcriptionally. Indeed, several recent studies have implicated Hu proteins in such post-transcriptional regulation via the stabilization or enhanced translation of various mRNAs (18–20,23–26). In addition, it was demonstrated that the neuron-specific Hu proteins positively regulate neuronal differentiation of two neuronal cell lines, rat PC12 and mouse embryonal carcinoma P19, possibly by post-transcriptional regulation of mRNAs of neuronal genes (19,24,27–29). However, how neuron-specific Hu proteins achieve such post-transcriptional gene regulation remains unclear, although it was reported previously that HuB associates with polysomes and the cytoskeleton (30,31).

In this study, to gain insights into the molecular mechanism underlying the function of Hu proteins, we took advantage of the yeast two-hybrid system to identify the proteins that interact with neuron-specific Hu proteins, and found that Hu proteins can interact with themselves. Further *in vitro* and *in vivo* analyses demonstrated multimer formation of HuB and HuD in mammalian cells. Our findings suggest that the Hu complex may act as a site where stabilization and/or efficient translation of ARE-containing mRNAs occur.

MATERIALS AND METHODS

Yeast two-hybrid analysis

Yeast two-hybrid screening was carried out using the MATCHMAKER GAL4 Two-Hybrid System 2 (Clontech). Various cDNA fragments of mouse HuC, HuD or HuB were PCR-amplified using appropriate synthetic primers, introduced into the two-hybrid vectors, pAS2-1 and pACT2, and transformed into yeast strain Y190. A human fetal brain cDNA library (Clontech) was used for screening of proteins interacting with HuB. β-Galactosidase activity of the transformants was measured by the method of Guarente (32).

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Plasmid construction and preparation of fusion proteins

The plasmids encoding T7-tagged or FLAG-tagged fusion proteins were described previously (28). The deletion constructs of HuD lacking amino acids 48–127 (RBD1-del) and 303–385 (RBD3-del) were subcloned into pEF-BOS-T7, which were described in our previous paper as D14–47/128–385 and D14–302, respectively (28). For construction of glutathione *S*-transferase (GST)-fusion proteins, coding regions of the cDNAs for each protein (amino acids 14–385 for HuD, 1–367 for HuC, 1–360 for HuB) were PCR-amplified and introduced into the *Bam*HI site of pGEX-3X (Pharmacia) for GST–HuD, or between the *Bam*HI and *Eco*RI sites of pGEX-2T for GST–HuC and GST–HuB. For the *in vitro* transcription and translation of HuD, a PCR-amplified fragment encoding HuD was subcloned between the *Bgl*II and *Bam*HI sites of pSP73 (Promega) or the *Bam*HI site of pGBKT7 (Clontech). The mutant HuD (HuDmt), in which Tyr94, Phe96 and Tyr99 of RBD1, Val180, Phe182 and Phe185 of RBD2, and Phe345, Phe347 and Met350 of RBD3 were all replaced by Asp, was constructed by site-directed mutagenesis (Stratagene). Plasmids of GST-fusion constructs were transformed into *Escherichia coli* XL1-blue. GST-fusion proteins were induced with 1 mM IPTG for 3 h and affinity-purified with glutathione–Sepharose 4B (Amersham Pharmacia).

Cell culture and transfection

PC12 and HeLa cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) and 5% horse serum (for PC12 cells) or 10% FBS (for HeLa cells), respectively. SH-SY5Y cells were cultured in DMEM/F-12 (Gibco) supplemented with 10% FBS. Transient transfection into PC12 or SH-SY5Y cells was performed by electroporation using a GenePulser (BioRad). Transient transfection into HeLa cells was carried out using PolyFect transfection reagent (Qiagen).

Immunoprecipitation analysis

PC12 or HeLa cells co-transfected with FLAG-tagged HuD (FLAG–HuD) and various T7-tagged constructs (T7–HuB, T7–HuC, T7–HuD or T7–GFP) were lysed in TNE buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and immunoprecipitated with anti-T7 (Novagen) or anti-FLAG (Eastman Kodak) monoclonal antibody and protein A Sepharose 6MB (Amersham Pharmacia). The precipitated proteins were separated by electrophoresis on 12% SDS–polyacrylamide gels, transferred to PVDF membranes (Millipore) and immunoblotted with anti-FLAG or anti-T7 antibody.

In vitro binding between Hu proteins

For the GST pull-down assay, ^{35}S-methionine-labeled HuD and myc-tagged HuD were synthesized *in vitro* using the TNT SP6 Coupled Reticulocyte Lysate System (Promega). The *in vitro* translated products were mixed with GST or GST–Hu proteins in TNE buffer, and pulled down with glutathione–Sepharose 4B beads. After the beads were washed, the bound proteins were separated by SDS–PAGE and autoradiographed or immunoblotted with anti-myc monoclonal antibody (Roche). For *in vitro* binding analysis using cell extracts, cytoplasmic extracts of HeLa or PC12 cells expressing T7–HuD or FLAG–HuD were prepared by lysing the cells with buffer B4 (20 mM Tris–HCl pH 7.5, 150 mM KCl, 3 mM $MgCl_2$, 0.1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) (31). These extracts were separately incubated with or without ribonuclease (RNase) A (0.4 mg/ml; Sigma) for 1 h at 37°C. After RNase treatment, the extracts were mixed and immunoprecipitated with anti-T7 antibody on ice in the presence of heparin (2 mg/ml). The immunoprecipitates were subjected to western blotting analysis with anti-FLAG antibody. To analyze the interaction of HuD complexes with additional HuD and HuB molecules, a cytoplasmic extract of HeLa cells containing chemically crosslinked T7–HuD complexes was mixed with a cytoplasmic extract containing either FLAG–HuD or FLAG–HuB in the presence of heparin (2 mg/ml), and the samples were subjected to immunoprecipitation with anti-FLAG antibody, followed by western blotting with anti-T7 antibody.

RNA-binding assay

Cytoplasmic extracts of HeLa cells containing T7–HuD, T7–HuDmt or chemically crosslinked T7–HuD complexes were mixed with poly(U) Sepharose 4B (Amersham Pharmacia) or Sepharose 4B beads in the presence of heparin (2 mg/ml) at 4°C. After the beads were washed, the bound proteins were analyzed by electrophoresis on 8% SDS–polyacrylamide gels and subjected to western blotting with anti-T7 antibody.

In situ chemical crosslinking analysis

PC12 or HeLa cells expressing each T7–Hu protein, T7–ELAV or FLAG–HuR, were suspended in PBS at 10^7 cells/ml and incubated with or without 1 mM bis-maleimido hexane (BMH; Sigma) or disuccinimidyl suberate (DSS; Pierce) for 30 min at room temperature (33). The intactness of cells treated with BMH or DSS was confirmed by trypan blue staining. After the treatment, the cells were lysed with TNE buffer, and the extracts were separated by electrophoresis on 8% SDS–polyacrylamide gels, followed by western blotting with anti-T7 antibody.

Immunofluorescence analysis

For indirect immunofluorescence analysis, cells were fixed with 4% formaldehyde and then incubated with anti-T7 antibody. Subsequently, the cells were incubated with FITC-labeled anti-mouse IgG secondary antibody (DAKO) and analyzed by fluorescence microscopy.

RESULTS AND DISCUSSION

Protein–protein interaction between neuron-specific Hu proteins

Neuron-specific Hu proteins have been implicated in positive regulation of neuronal differentiation or synaptic plasticity by post-transcriptional regulation of neuronal genes (18,19,23,24,27,28,34). Although neuron-specific Hu proteins have been reported to regulate the stability or translation of several mRNAs (18,19,23,26,34), the molecular mechanism underlying such post-transcriptional regulation remains unclear. To

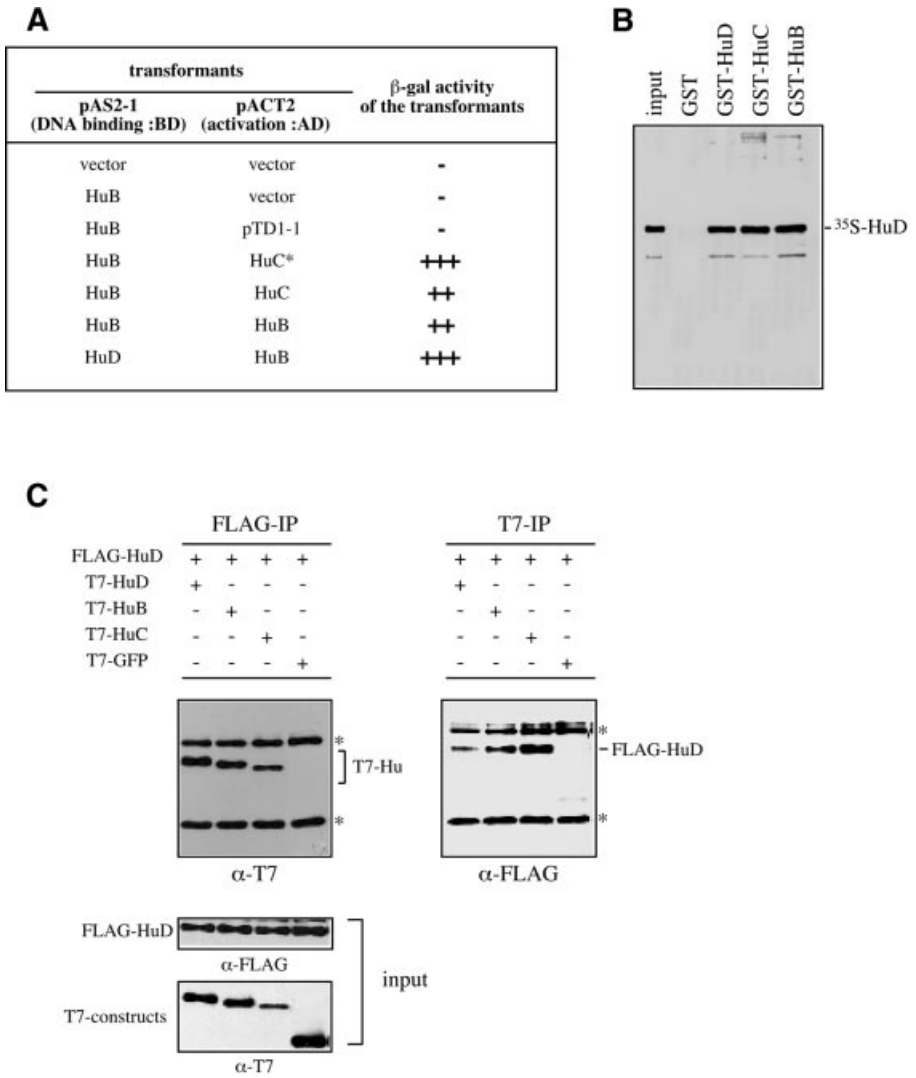

Figure 1. Protein–protein interaction between Hu proteins. (**A**) The results of yeast two-hybrid analysis using neuron-specific Hu proteins. The β-galactosidase activity of transformants expressing each combination of proteins is normalized by the activity of the combination of vector plasmids (pAS2-1 and pACT2): –, <3-fold; +, 3–10-fold; ++, 10–20-fold; +++, >20-fold over the control value. HuC* and pTD1-1 represent human HuC lacking the N-terminal 20 amino acid residues and the negative control plasmid encoding SV40 large T antigen, respectively. (**B**) *In vitro* binding of ^{35}S-methionine-labeled HuD to the indicated recombinant GST–Hu fusion proteins in the presence of RNase A. (**C**) Hu proteins can self-associate in mammalian cells. HeLa cells were co-transfected with FLAG–HuD and either T7–HuD, T7–HuB or T7–HuC. The cell extracts were immunoprecipitated with anti-FLAG antibody (FLAG–IP) or anti-T7 antibody (T7–IP), and the precipitants were analyzed by western blotting with anti-T7 antibody (α-T7) or anti-FLAG antibody (α-FLAG). The asterisks indicate the heavy and light chains of the IgG used for immunoprecipitation. As controls, the levels of each proteins in the cell extracts used are shown below (input: α-FLAG, α-T7).

gain insights into the mechanism, it should be important to identify the proteins that interact with neuron-specific Hu proteins. In order to identify such proteins, we used the yeast two-hybrid system in this study. As all neuron-specific Hu proteins are highly expressed in brain tissues containing maturely differentiated neurons (10), we used a human fetal brain cDNA library for the two-hybrid screening. When we used mouse HuB as bait (mouse HuB is 99% identical to human HuB in amino acid sequence) and screened 6×10^6 transformants of the library, we were successful in obtaining one positive clone encoding a fragment of human HuC (HuC*) that contained all RBDs but lacked the N-terminal 20 amino acid residues. Based on the result that mouse HuB can interact with human HuC in yeast, we thought that neuron-specific Hu proteins may interact with each other and form a homo- or hetero-complex, since the amino acid sequences of all Hu proteins are well conserved (3). We then examined, by two-hybrid analysis, whether HuB could interact with itself or other members of Hu proteins. As expected, the results showed that HuB can interact with itself, HuD and HuC in yeast (Fig. 1A).

There is a difference in expression profiles during neuronal development among three neuron-specific Hu proteins (10). However, several similarities are also found between HuB and HuD: for example, similar subcellular localization and almost the same neurite-inducing activity when overexpressed into PC12 cells (28). Therefore, we further examined the interaction properties of HuD to clarify whether HuD could show

the same homo- and hetero-complex formation as HuB. To confirm the homo- or hetero- interaction between Hu proteins *in vitro*, we prepared three GST-fusion proteins, GST–HuD, GST–HuC and GST–HuB, and tested their interaction with *in vitro* translated HuD in the presence of RNase. In this experiment, we added RNase A to exclude the possibility of indirect interaction that might occur by bridging via RNA. All GST-fusion proteins, but not GST alone, pulled down the *in vitro* translated HuD (Fig. 1B). We cannot exclude strictly the possibility of indirect interaction via poly(A) RNA which might be present in the pull-down reactions, since RNase A does not cleave poly(A) sequence. However, we found that a mutant HuD, HuDmt, with almost no poly(A) binding activity still interacted with GST–HuD at almost the same degree as the *in vitro* translated HuD did with GST–HuD in the presence of RNase A (see Fig. 3D). Therefore, we concluded that the interaction between Hu proteins is basically mediated by protein–protein interaction.

Next, we examined whether the Hu interaction occurs in mammalian cells. To do this, we utilized epitope-tagged Hu proteins. FLAG-tagged HuD (FLAG–HuD) was transfected into HeLa cells together with T7-tagged HuD (T7–HuD), HuB (T7–HuB) or HuC (T7–HuC), and then the cell extracts were subjected to immunoprecipitation with anti-FLAG antibody in the absence of RNase A (Fig. 1C). The anti-FLAG antibody pulled down all T7-tagged Hu proteins. Conversely, immunoprecipitation of the same extracts with anti-T7 antibody also pulled down FLAG–HuD. The same results were obtained when PC12 cells were used for the immunoprecipitation analysis (data not shown). These results indicate that the interaction between Hu proteins also occurs in mammalian cells.

RBDs are responsible for the interaction between Hu proteins

To identify the region responsible for the self-interaction of Hu proteins, we constructed several deletion mutants of HuC and tested their interaction with HuB using the two-hybrid system (Fig. 2). HuC fragments containing only the third RBD (CRBD3) or both the second and third RBDs (CRBD2+3) strongly interacted with HuB, as did the full-length HuC (Fig. 2B). The fragment containing the first and second RBDs (CRBD1+2) also interacted moderately with HuB, although fragments containing the first RBD (CRBD1) or the second RBD (CRBD2) alone showed only very weak interaction with HuB. Further analysis showed that CRBD3 strongly interacted with itself or CRBD1+2 (Fig. 2C). It should be noted that the linker region between RBD2 and RBD3 of HuC did not show such an interaction. Thus, these results suggest that the RBDs of Hu proteins function as self-interaction domains.

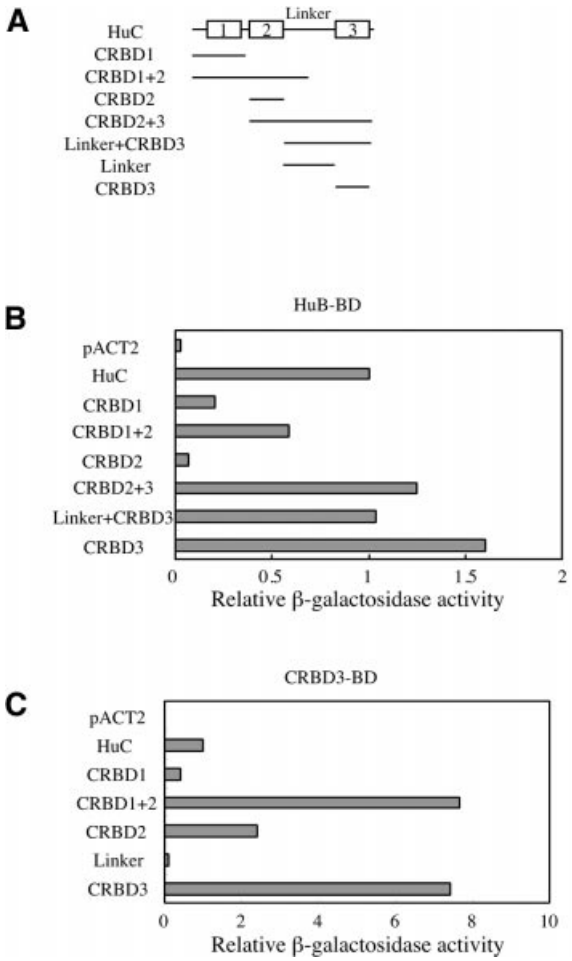

Figure 2. RBDs are responsible for self-association of Hu proteins. (**A**) Schematic representation of the fragments of HuC used for the yeast two-hybrid analysis. RBDs are represented by boxes. (**B**) The relative β-galactosidase activity of the transformants expressing HuB fused to the DNA-binding domain (BD) and various fragments of HuC fused to the activation domain (AD). The β-galactosidase activity was normalized by the activity of the combination of HuB and HuC. (**C**) The relative β-galactosidase activity of the transformants expressing CRBD3 fused to BD and various fragments of HuC fused to AD. The β-galactosidase activity was normalized by the activity of the combination of CRBD3 and HuC.

Efficient interaction between Hu proteins requires RNA-binding activity

Several RNA-binding proteins have been reported to self-associate via their RNA-binding regions, including RBDs, KH-motifs and other RNA-binding motifs (33,35–39). Some of these proteins require RNA for their efficient interaction, whereas others do not. Therefore, we further tested the possible involvement of RNA in the Hu interaction using mammalian cell extracts, although the interaction between Hu proteins occurs in the absence of RNA (Fig. 1B). To do this, T7–HuD and FLAG–HuD were expressed independently in HeLa cells, and the two cell extracts were mixed with or without RNase treatment, followed by immunoprecipitation with anti-T7 antibody (Fig. 3A and B). The HuD–HuD interaction occurred efficiently in the absence of RNase treatment. In contrast, the interaction became very weak after RNase treatment. The same result was obtained with PC12 cells (data not shown). These results suggest that some cellular RNA(s) strongly activates the Hu interaction, although Hu proteins have a weak intrinsic ability to interact together even in the absence of such RNA.

If the RNA-bound forms of Hu proteins have increased self-interaction, mutant Hu proteins with reduced RNA-binding activity would be expected to show only an intrinsic ability to interact with other Hu proteins. To test this possibility, we constructed an HuD mutant (HuDmt) that has reduced RNA-binding activity because it contains amino acid

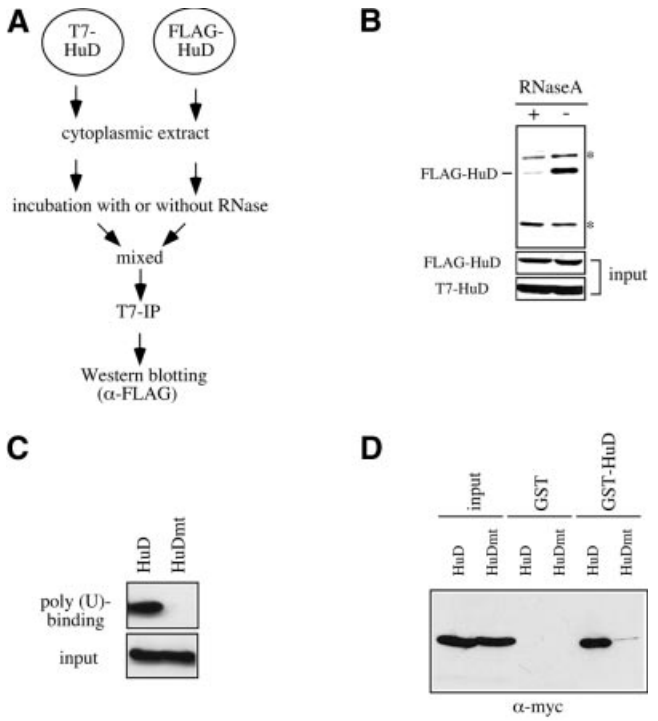

Figure 3. Cellular RNA is required for efficient interaction between HuD molecules. (**A**) Scheme of the *in vitro* binding experiment using HeLa cell extracts containing T7–HuD and FLAG–HuD. (**B**) Immunoprecipitation of the mixed extracts with anti-T7 antibody in the presence or absence of RNase A. RNase treatment had no effect on the levels of tagged HuD in the extract (two bottom panels). The asterisks indicate the heavy and light chains of IgG used for immunoprecipitation. (**C**) Binding of wild-type HuD and a mutant HuDmt to the poly(U) sequence. HeLa cell extracts containing T7–HuD or T7–HuDmt were incubated with poly(U)–Sepharose beads and the bound proteins were analyzed by western blotting with anti-T7 antibody. As controls, the levels of T7–HuD and T7–HuDmt in the extracts used are shown (input). (**D**) Pull-down of the *in vitro* translated myc-HuD or myc-HuDmt by GST or GST–HuD. Proteins pulled-down by GST or GST–HuD were analyzed by western blotting with anti-myc antibody. As controls, the levels of myc-HuD and myc-HuDmt in the *in vitro* translation reactions used are shown (input).

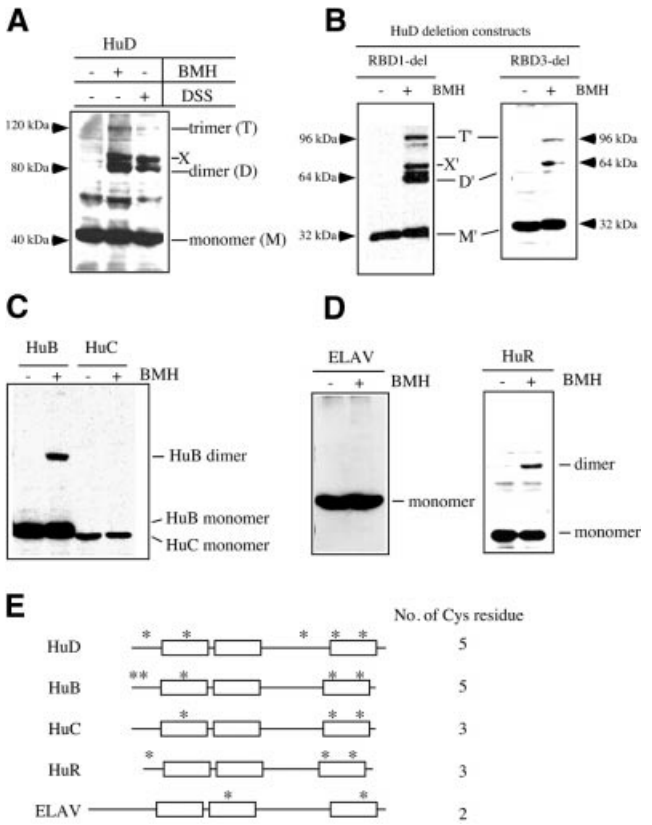

Figure 4. Complex formation of neuron-specific Hu proteins in mammalian cells revealed by *in situ* chemical crosslinking. PC12 cells expressing each T7–Hu protein were treated with BMH or DSS, and the cell extracts were then analyzed by western blotting using anti-T7 antibody. (**A**) Formation of dimer and trimer complexes of HuD. T, D and M indicate trimer, dimer and monomer of HuD, respectively. X indicates a specific HuD complex that has yet to be identified. (**B**) Crosslinked complexes of deletion constructs of HuD, RBD1-del and RBD3-del. T′, D′ and M′ indicate the trimer, dimer and monomer of them, respectively. X′ corresponds to the X complex of HuD. (**C**) The results of *in situ* crosslinking with T7–HuB and T7–HuC. (**D**) The results of *in situ* crosslinking with T7–ELAV or FLAG–HuR. (**E**) Cysteine residues within Hu proteins and ELAV. The asterisks indicate the cysteine residues that are the possible sites for chemical crosslinking by BMH.

substitutions in the RNP1 motif, which is known to be essential for RNA binding, of all RBDs. The substituted amino acid residues are expected to be solvent-exposed and it is thought that the mutation to these sites does not destroy the overall domain structure (27,40). In our case, these substitutions were found to abolish the RNA-binding activity of HuD, since the HuDmt showed almost no binding to poly(U) RNA (Fig. 3C) and to poly(A) RNA (data not shown). As expected, HuDmt showed significantly reduced ability to interact with GST–HuD (Fig. 3D). This result is consistent with our prediction that efficient interaction between Hu proteins occurs only when they bind to some cellular RNA. Considering the RNA-binding specificity of Hu proteins, it is possible that the occupation of RBDs by ARE-containing mRNAs may promote some conformational change of the domains that results in more efficient interaction of Hu proteins.

Complex formation of Hu proteins in mammalian cells

The results described above indicate that Hu proteins can form homo- and hetero-complexes in cells. To detect such complexes more directly in mammalian cells, we performed *in situ* chemical crosslinking analysis (33) (Fig. 4). PC12 cells expressing T7–HuD were treated with thio-specific (BMH) or amine-specific (DSS) chemical crosslinking reagents, and the resultant crosslinked complexes in the cell extracts were analyzed by immunoblotting with anti-T7 antibody. In addition to the monomeric HuD of 41 kDa, three specific crosslinked complexes could be detected using either of the crosslinkers (Fig. 4A). Two of the complexes had molecular masses of ~80 and 120 kDa, and were likely to be the dimer and trimer of HuD, respectively.

To confirm this conclusion, we examined the crosslinked complexes of two kinds of HuD deletion constructs, RBD1-del and RBD3-del, which are smaller than the wild-type HuD by ~9 kDa because of deletion of the first RBD and the third RBD, respectively. As in the case of wild-type HuD, the crosslinked complexes of the dimer and trimer of both deletion constructs could be detected as judged by their molecular masses (which reflected the difference in size from those of the wild-type HuD) (Fig. 4B), indicating that the two crosslinked

complexes of HuD are the dimer and trimer. Although RBD3-del lacked RBD3 that was suggested as a strong self-interaction region (Fig. 2), it could form multimeric complexes. The formation of multimeric complexes of RBD3-del may be mediated by the region between RBD1 and RBD2 (RBD1+2), since the region has a moderate interaction activity with Hu protein (Fig. 2). Another crosslinked complex of ~90 kDa (Fig. 4A, indicated by X) is clearly different from the homo-oligomers of HuD. This crosslinked complex, like the dimer and trimer of HuD, was not sensitive to treatment with RNase or phosphatase (data not shown). In addition, a crosslinked complex corresponding to the X complex (X′) could be detected for RBD1-del but not for RBD3-del (Fig. 4B). Therefore, although we do not know the identity of the X complex of HuD at present, it may contain some protein(s) that associate with the third RBD of HuD.

The same crosslinked complexes were also detected when HeLa cells were used (data not shown), indicating that these complexes are not cell specific. Therefore, we concluded that HuD exists in mammalian cells as dimer and trimer complexes, as well as in the monomer form. We also examined the complex formation of other neuron-specific Hu proteins and found that under the same conditions as described above for HuD, HuB can form dimers but not trimers, whereas HuC cannot form any complexes (Fig. 4C). In Figure 4E, the positions of cysteine residues within Hu proteins and ELAV are shown, which are the possible sites for chemical crosslinking by BMH. As the RBD1 deletion mutant of HuD can form multimeric complexes efficiently (Fig. 4B), it is most likely that efficient chemical crosslinking between HuD molecules by BMH occurs within RBD3. Two cysteine residues within RBD3 are well conserved among Hu proteins, indicating that different complex formation among neuron-specific Hu proteins is not due to the chemical specificity of the crosslinker, but due to the intrinsic property of each Hu protein. These results also show that the crosslinked product X is specific for HuD. No biochemical feature specific for any one of the neuron-specific Hu proteins has been reported previously. Thus, the differential complex formation among Hu proteins revealed in this study is the first biochemical evidence that suggests a functional difference of the neuron-specific Hu proteins *in vivo*. Interestingly, under the same crosslinking conditions as for HuD, ubiquitously expressed Hu protein, HuR, showed only the dimer formation, like HuB, whereas *Drosophila* ELAV did not show any complex formation, like HuC (Fig. 4D). Although it has been shown recently that HuR associates with several specific proteins in cells (41), we could not detect such interactions in our *in situ* crosslinking assay. HuR and ELAV have been demonstrated to be involved in mRNA stabilization and alternative splicing regulation, respectively (20,42,43). The inability to form complexes may indicate that HuC has a function distinct from the control of mRNA stability and/or translation, perhaps acting as a splicing regulator, like ELAV.

Multimeric HuD complexes retain RNA-binding activity and interact with Hu proteins

Since Hu proteins can bind to RNAs containing AREs or U-rich sequences *in vitro*, we examined whether the crosslinked complexes of HuD retain such an RNA-binding activity. To do this, we prepared HeLa cell extracts containing

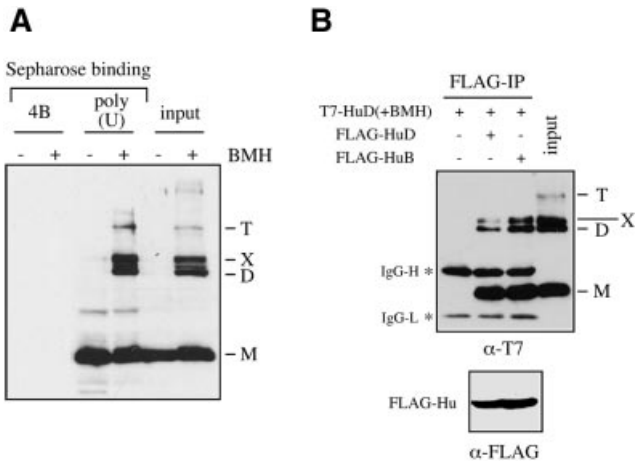

Figure 5. Multimeric HuD complexes retain RNA-binding activity and interact with Hu proteins. (**A**) Binding of multimeric HuD complexes to poly(U) RNA. HeLa cells expressing T7–HuD were treated with BMH, and the cell extracts were then pulled down with poly(U)–Sepharose [poly(U)] or control Sepharose 4B (4B) beads. The bound proteins were analyzed by western blotting using anti-T7 antibody. (**B**) Co-precipitation of the crosslinked HuD complexes with HuD and HuB. HeLa cell extracts containing the crosslinked T7–HuD complexes were mixed with the cell extract containing FLAG–HuD or FLAG–HuB, and the mixed samples were then immunoprecipitated with anti-FLAG antibody, followed by western blotting with anti-T7 antibody (α-T7). As controls, the levels of FLAG–HuD and FLAG–HuB in the cell extracts used are shown below (α-FLAG).

the crosslinked T7–HuD complexes, and then tested their binding to poly(U) RNA (Fig. 5A). The three crosslinked complexes of HuD strongly bound to poly(U) RNA, as did monomeric T7–HuD. This indicates that the dimer and trimer complexes of HuD retain the same RNA-binding activity as monomeric HuD. In addition, the RNA-binding ability of the dimer and trimer complexes of HuD implies that the RNA- and protein-binding surfaces of HuD, which may be located within RBDs, are essentially different. This is consistent with our interpretation that the inefficient interaction between HuD and HuDmt (Fig. 3D) is primarily caused by the RNA-binding deficiency of HuDmt, which leads to the inability to form an appropriate conformation for efficient protein–protein interaction. Moreover, the crosslinked complexes of HuD still retain the ability to interact with HuD and HuB proteins (Fig. 5B).

Granular distribution of HuD in cells

The results described above suggested that multiple HuD molecules form a large complex in cells which includes various ARE-containing mRNAs. Indeed, close inspection of immunostaining views revealed that wild-type HuD showed granular distribution when it was expressed in two neurogenic cell lines, PC12 and SH-SY5Y (Fig. 6, left). Consistent with its very low protein–protein interaction ability, HuDmt was distributed almost uniformly in the cytoplasm of both cell lines (Fig. 6, right). It should be noted that HuDmt has almost no neurite-inducing activity in PC12 cells (data not shown). It is likely that the granule formation of wild-type HuD reflects multimeric complex formation of the proteins together with some ARE-containing mRNAs. Previously, it was demonstrated that human ELAV-like protein Hel-N1/

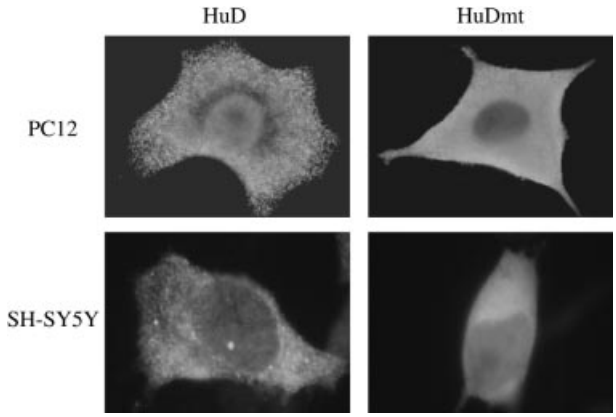

Figure 6. Granular distribution of HuD in two neurogenic cell lines. Immunostaining views of PC12 (top) or SH-SY5Y (bottom) cells expressing T7–HuD (left) or T7–HuDmt (right). After transfection, cells were fixed and immunostained with anti-T7 antibody.

Hel-N2 also exists as granular RNP complexes associated with polysomes and the cytoskeleton in cultured cells (30,31). Those authors' interpretation of this granular distribution was that multiple Hel-N1 molecules might assemble at their target site on some mRNA, and that the factor responsible for this nucleation is their target sequences. Based on the results of our present study, we propose that protein–protein interaction between Hu proteins is the principal nucleation factor for the formation of such granular RNP complexes and that the RNP complex is comprised of multiple Hu molecules and ARE-containing mRNAs.

To date, various RNA-binding proteins have been reported to form dimers or multimer complexes (33,35–39,44), and their important roles in regulating splicing or development have been discussed (38,44). However, to our knowledge, there are few reports about multimerization of RNA-binding proteins that are involved in neuronal differentiation. Our results suggest that multimerization of two neuron-specific Hu proteins also has an important effect on their function *in vivo*. Considering that Hu proteins have been implicated in the stabilization and/or enhanced translation of ARE-containing mRNA, one can speculate that Hu proteins that bind to ARE-containing mRNAs assemble efficiently via protein–protein interaction, which is facilitated by RNA binding, and eventually form large RNP complexes. Then, as described previously (31), these complexes efficiently interact with the translational apparatus so that the stability and/or translation of such mRNA(s) is up-regulated. In addition to such post-transcriptional regulation, it is possible that granular RNP complexes of Hu proteins may contribute to the temporal storage of ARE-containing mRNA(s) whose immediate and high expression in response to extracellular signals is needed for proper neuronal functions.

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REFERENCES

1. Szabo,A., Dalmau,J., Manley,G., Rosenfeld,M., Wong,E., Henson,J., Posner,J.B. and Furneaux,H.M. (1991) HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. *Cell*, **67**, 325–333.
2. Dalmau,J., Furneaux,H.M., Cordon-Cardo,C. and Posner,J.B. (1992) The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues. *Am. J. Pathol.*, **141**, 881–886.
3. Good,P.J. (1995) A conserved family of elav-like genes in vertebrates. *Proc. Natl Acad. Sci. USA*, **92**, 4557–4561.
4. Good,P.J. (1997) The role of elav-like genes, a conserved family encoding RNA-binding proteins, in growth and development. *Semin. Cell. Dev. Biol.*, **8**, 577–584.
5. Abe,R., Uyeno,Y., Yamamoto,K. and Sakamoto,H. (1994) Tissue-specific expression of the gene encoding a mouse RNA binding protein homologous to human HuD antigen. *DNA Res.*, **1**, 175–180.
6. King,P.H., Levine,T.D., Fremeau,R.T.,Jr and Keene,J.D. (1994) Mammalian homologs of *Drosophila* ELAV localized to a neuronal subset can bind *in vitro* to the 3′ UTR of mRNA encoding the Id transcriptional repressor. *J. Neurosci.*, **14**, 1943–1952.
7. Sakai,K., Gofuku,M., Kitagawa,Y., Ogasawara,T., Hirose,G., Yamazaki,M., Koh,C.S., Yanagisawa,N. and Steinman,L. (1994) A hippocampal protein associated with paraneoplastic neurologic syndrome and small cell lung carcinoma. *Biochem. Biophys. Res. Commun.*, **199**, 1200–1208.
8. Abe,R., Yamamoto,K. and Sakamoto,H. (1996) Target specificity of neuronal RNA-binding protein, Mel-N1: direct binding to the 3′ untranslated region of its own mRNA. *Nucleic Acids Res.*, **24**, 2011–2016.
9. Abe,R., Sakashita,E., Yamamoto,K. and Sakamoto,H. (1996) Two different RNA binding activities for the AU-rich element and the poly(A) sequence of the mouse neuronal protein mHuC. *Nucleic Acids Res.*, **24**, 4895–4901.
10. Okano,H.J. and Darnell,R.B. (1997) A hierarchy of Hu RNA binding proteins in developing and adult neurons. *J. Neurosci.*, **17**, 3024–3037.
11. Wakamatsu,Y. and Weston,J.A. (1997) Sequential expression and role of Hu RNA-binding proteins during neurogenesis. *Development*, **124**, 3449–3460.
12. Ma,W.J., Cheng,S., Campbell,C., Wright,A. and Furneaux,H. (1996) Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. *J. Biol. Chem.*, **271**, 8144–8151.
13. Burd,C.G. and Dreyfuss,G. (1994) Conserved structures and diversity of functions of RNA-binding proteins. *Science*, **265**, 615–621.
14. Robinow,S., Campos,A.R., Yao,K.M. and White,K. (1988) The elav gene product of *Drosophila*, required in neurons, has three RNP consensus motifs [published erratum appears in *Science* (1989), **243**, 12]. *Science*, **242**, 1570–1572.
15. Levine,T.D., Gao,F., King,P.H., Andrews,L.G. and Keene,J.D. (1993) Hel-N1: an autoimmune RNA-binding protein with specificity for 3′ uridylate-rich untranslated regions of growth factor mRNAs. *Mol. Cell. Biol.*, **13**, 3494–3504.
16. Liu,J., Dalmau,J., Szabo,A., Rosenfeld,M., Huber,J. and Furneaux,H. (1995) Paraneoplastic encephalomyelitis antigens bind to the AU-rich elements of mRNA. *Neurology*, **45**, 544–550.
17. Chung,S., Eckrich,M., Perrone-Bizzozero,N., Kohn,D.T. and Furneaux,H. (1997) The Elav-like proteins bind to a conserved regulatory element in the 3′-untranslated region of GAP-43 mRNA. *J. Biol. Chem.*, **272**, 6593–6598.
18. Aranda-Abreu,G.E., Behar,L., Chung,S., Furneaux,H. and Ginzburg,I. (1999) Embryonic lethal abnormal vision-like RNA-binding proteins regulate neurite outgrowth and tau expression in PC12 cells. *J. Neurosci.*, **19**, 6907–6917.
19. Antic,D., Lu,N. and Keene,J.D. (1999) ELAV tumor antigen, Hel-N1, increases translation of neurofilament M mRNA and induces formation of neurites in human teratocarcinoma cells. *Genes Dev.*, **13**, 449–461.
20. Brennan,C.M. and Steitz,J.A. (2001) HuR and mRNA stability. *Cell Mol. Life Sci.*, **58**, 266–277.
21. Chung,S., Jiang,L., Cheng,S. and Furneaux,H. (1996) Purification and properties of HuD, a neuronal RNA-binding protein. *J. Biol. Chem.*, **271**, 11518–11524.

22. Ma,W.J., Chung,S. and Furneaux,H. (1997) The Elav-like proteins bind to AU-rich elements and to the poly(A) tail of mRNA. *Nucleic Acids Res.*, **25**, 3564–3569.
23. Mobarak,C.D., Anderson,K.D., Morin,M., Beckel-Mitchener,A., Rogers,S.L., Furneaux,H., King,P. and Perrone-Bizzozero,N.I. (2000) The RNA-binding protein HuD is required for GAP-43 mRNA stability, GAP-43 gene expression and PKC-dependent neurite outgrowth in PC12 cells. *Mol. Biol. Cell*, **11**, 3191–3203.
24. Anderson,K.D., Morin,M.A., Beckel-Mitchener,A., Mobarak,C.D., Neve,R.L., Furneaux,H.M., Burry,R. and Perrone-Bizzozero,N.I. (2000) Overexpression of HuD, but not of its truncated form HuD I+II, promotes GAP-43 gene expression and neurite outgrowth in PC12 cells in the absence of nerve growth factor. *J. Neurochem.*, **75**, 1103–1114.
25. Dean,J.L., Wait,R., Mahtani,K.R., Sully,G., Clark,A.R. and Saklatvala,J. (2001) The 3′ untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR. *Mol. Cell. Biol.*, **21**, 721–730.
26. Manohar,C.F., Short,M.L., Nguyen,A., Nguyen,N.N., Chagnovich,D., Yang,Q. and Cohn,S.L. (2002) HuD, a neuronal-specific RNA-binding protein, increases the *in vivo* stability of MYCN RNA. *J. Biol. Chem.*, **277**, 1967–1973.
27. Akamatsu,W., Okano,H.J., Osumi,N., Inoue,T., Nakamura,S., Sakakibara,S., Miura,M., Matsuo,N., Darnell,R.B. and Okano,H. (1999) Mammalian ELAV-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both the central and the peripheral nervous systems. *Proc. Natl Acad. Sci. USA*, **96**, 9885–9890.
28. Kasashima,K., Terashima,K., Yamamoto,K., Sakashita,E. and Sakamoto,H. (1999) Cytoplasmic localization is required for the mammalian ELAV-like protein HuD to induce neuronal differentiation. *Genes Cells*, **4**, 667–683.
29. Anderson,K.D., Sengupta,J., Morin,M., Neve,R.L., Valenzuela,C.F. and Perrone-Bizzozero,N.I. (2001) Overexpression of HuD accelerates neurite outgrowth and increases GAP-43 mRNA expression in cortical neurons and retinoic acid-induced embryonic stem cells *in vitro*. *Exp. Neurol.*, **168**, 250–258.
30. Gao,F.B. and Keene,J.D. (1996) Hel-N1/Hel-N2 proteins are bound to poly(A)+ mRNA in granular RNP structures and are implicated in neuronal differentiation. *J. Cell Sci.*, **109**, 579–589.
31. Antic,D. and Keene,J.D. (1998) Messenger ribonucleoprotein complexes containing human ELAV proteins: interactions with cytoskeleton and translational apparatus. *J. Cell Sci.*, **111**, 183–197.
32. Guarente,L. (1983) Yeast promoters and lacZ fusions designed to study expression of cloned genes in yeast. *Methods Enzymol.*, **101**, 181–191.
33. Chen,T., Damaj,B.B., Herrera,C., Lasko,P. and Richard,S. (1997) Self-association of the single-KH-domain family members Sam68, GRP33, GLD-1 and Qk1: role of the KH domain. *Mol. Cell. Biol.*, **17**, 5707–5718.
34. Quattrone,A., Pascale,A., Nogues,X., Zhao,W., Gusev,P., Pacini,A. and Alkon,D.L. (2001) Posttranscriptional regulation of gene expression in learning by the neuronal ELAV-like mRNA-stabilizing proteins. *Proc. Natl Acad. Sci. USA*, **98**, 11668–11673.
35. Patel,R.C., Stanton,P., McMillan,N.M., Williams,B.R. and Sen,G.C. (1995) The interferon-inducible double-stranded RNA-activated protein kinase self-associates *in vitro* and *in vivo*. *Proc. Natl Acad. Sci. USA*, **92**, 8283–8287.
36. Sakashita,E. and Sakamoto,H. (1996) Protein–RNA and protein–protein interactions of the *Drosophila* sex-lethal mediated by its RNA-binding domains. *J. Biochem. (Tokyo)*, **120**, 1028–1033.
37. Perez,I., McAfee,J.G. and Patton,J.G. (1997) Multiple RRMs contribute to RNA binding specificity and affinity for polypyrimidine tract binding protein. *Biochemistry*, **36**, 11881–11890.
38. Chen,T. and Richard,S. (1998) Structure-function analysis of Qk1: a lethal point mutation in mouse quaking prevents homodimerization. *Mol. Cell. Biol.*, **18**, 4863–4871.
39. Piron,M., Delaunay,T., Grosclaude,J. and Poncet,D. (1999) Identification of the RNA-binding, dimerization and eIF4GI-binding domains of rotavirus nonstructural protein NSP3. *J. Virol.*, **73**, 5411–5421.
40. Lisbin,M.J., Gordon,M., Yannoni,Y.M. and White,K. (2000) Function of RRM domains of *Drosophila melanogaster* ELAV: Rnp1 mutations and rrm domain replacements with ELAV family proteins and SXL. *Genetics*, **155**, 1789–1798.
41. Brennan,C.M., Gallouzi,I.E. and Steitz,J.A. (2000) Protein ligands to HuR modulate its interaction with target mRNAs *in vivo*. *J. Cell Biol.*, **151**, 1–14.
42. Koushika,S.P., Soller,M. and White,K. (2000) The neuron-enriched splicing pattern of *Drosophila* erect wing is dependent on the presence of ELAV protein. *Mol. Cell. Biol.*, **20**, 1836–1845.
43. Lisbin,M.J., Qiu,J. and White,K. (2001) The neuron-specific RNA-binding protein ELAV regulates neuroglian alternative splicing in neurons and binds directly to its pre-mRNA. *Genes Dev.*, **15**, 2546–2561.
44. Wang,J. and Bell,L.R. (1994) The Sex-lethal amino terminus mediates cooperative interactions in RNA binding and is essential for splicing regulation. *Genes Dev.*, **8**, 2072–2085.