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Mammalian ELAV-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both the central and the peripheral nervous systems

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ABSTRACT **Hu proteins are mammalian embryonic lethal abnormal visual system (ELAV)-like neuronal RNA-binding proteins that contain three RNA recognition motifs. Although *Drosophila* ELAV is required for the correct differentiation and survival of neurons, the roles played by the Hu genes in the mammalian nervous system remain largely unknown. To explore the *in vivo* functions of mouse Hu proteins, we overexpressed them in rat pheochromocytoma PC12 cells, where they induced neuronal phenotype in the absence of nerve growth factor. We have characterized the functions of various forms of mHuB and mHuC bearing point mutations or deletions. Mutants of mHuC that had amino acid exchanges in the RNP1 domain of the first or second RNA recognition motifs (RRMs) lost biologic activity as well as RNA-binding activity. In addition, the mutants containing only the third RRM failed to induce the neuronal phenotype in PC12 cells and inhibited the biologic activity of cotransfected wild-type mHuB and mHuC, thus acting as a dominant-negative form. However, these mutants could not suppress the nerve growth factor-induced differentiation of PC12 cells. Further, we misexpressed wild-type and dominant-negative Hu in E9.5 mouse embryos, by using electroporation into the neural tube at the level of the rhombencephalon. mHuB and mHuC induced the ectopic expression of neuronal markers, whereas the dominant-negative forms of mHuB and mHuC suppressed the differentiation of central nervous system motor neurons. From these results, we suggest that Hu proteins are required for neuronal differentiation in the mammalian nervous system.**

Neurons use a variety of means to regulate posttranscriptional gene expression, including alternative splicing, RNA transport, local translation, and RNA editing. Neural RNA-binding proteins are likely to play essential roles in mediating this regulation (1–4). *Drosophila* embryonic lethal abnormal visual system (ELAV) is a member of an evolutionarily conserved family of neural RNA-binding proteins, the structures and expression patterns of which are highly conserved from *Drosophila* (5–7) to mammals (8, 9). The mammalian ELAV-like neuronal RNA-binding proteins, Hu proteins, were identified as autoimmune antigens of human paraneoplastic encephaloneuropathies associated with small lung-cell carcinomas (Hu-syndrome) (8). Hu antisera recognize a predominantly nuclear antigen present in all neurons but not expressed in other tissues (10, 11), consistent with the expression pattern of *Drosophila* ELAV. Hu antigens are a series of immunologically related proteins generated from several distinct genes. In mammals, cDNAs encoding four Hu family members, HuA (HuR), HuB (Hel-N1), HuC (Ple-21), and HuD, have been cloned (8, 12–14). The expression patterns for each Hu gene (except HuA) are overlapping and similar, with each family member showing a unique expression pattern in the developing mouse nervous system (9). Hu proteins as well as *Drosophila* ELAV contain three well-characterized RNA recognition motifs (RRMs) 1–3 of approximately 80 aa each (8).

RNA-binding proteins with RRMs have been shown to be required for various aspects of RNA metabolism as well as developmental regulation. *Drosophila* ELAV, for example, is required for the terminal differentiation and survival (5–7, 15, 16) of postmitotic neurons. Misexpression of *Drosophila* ELAV increases expression of the neuronal isoform of Neuroglian, a neural cell adhesion molecule, by regulating its alternative splicing in a neuron-specific manner (17). The major function identified for the mammalian Hu genes is in regulation of mRNA metabolism through binding to RNA stability elements [AU-rich element (ARE)] (13, 18–25). The AREs in the mRNAs of various genes, including GAP-43 (a neuronal growth cone-associated protein) (26, 27), p21 (inhibitor of cyclin-dependent kinases) (28), and neurofilament-M (29), have been shown to be potential targets of Hu proteins. Structure-function analyses of HuA (HuR) have shown that it binds to AU-rich mRNA sequences in the nucleus and may be involved in their nuclear export (18, 30). Misexpression of HuB (Hel-N1) in 3T3 cells was shown to increase the expression of glucose transporter protein by stabilizing its mRNA (31). Hu proteins have also been suggested to play important roles in neuronal development. In cultured chicken neural crest cells, it has been shown that misexpression of HuD induces neuronal differentiation (32). HuB (Hel-N1) proteins are up-regulated during neuronal differentiation of embryonic carcinoma P19 cells (33), and its misexpression can induce formation of neurites in human tetracarcinoma cells (hNT2) (29). However, it remains to be studied whether the Hu genes are authentically required for the differentiation of neurons *in vivo*, especially those in the central nervous system (CNS).

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Abbreviations: NGF, nerve growth factor; RRM, RNA recognition motif; ARE, AU-rich element; CNS, central nervous system; PNS, peripheral nervous system; RHP, ribohomopolymer; GFP, green fluorescent protein; ERK, extracellular signal-regulated kinase; ELAV, embryonic lethal abnormal visual system.

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To address these issues, in the present study we designed point and deletion mutants of Hu proteins to perform detailed structure-function analyses to identify dominant-negative forms of the proteins. The *in vivo* functions of the Hu proteins in the peripheral nervous systems (PNS) and CNS were examined by overexpressing the full-length Hu (mHuB and mHuC) proteins and their dominant-negative forms in the neural tube of developing mouse embryo. On the basis of our data, we conclude that mHuB and mHuC are required for and capable of inducing neuronal phenotype in both CNS and PNS.

MATERIALS AND METHODS

Construction of Hu Expression Vectors. The cDNA clones of mouse HuB and HuC were isolated by reverse transcription–PCR by using primers whose design was based on previous reports (34, 35), and their DNA sequences were confirmed. Subsequently, FLAG tags were added to the N termini of the coding regions of mHuB and mHuC by PCR. A series of deletion mutants (R1–2, lacking aa 205–360 of mHuB and 205–367 of mHuC and R3, lacking aa 1–277 of mHuB and 1–284 of mHuC) were constructed by PCR. Amino acid substitution mutations (RRM1 mt, in which Tyr-82, Phe-84, and Tyr-87 were replaced by Asp, and RRM2 mt, in which Val-168, Phe-170, and Phe-173 were replaced by Asp) were constructed by site-directed mutagenesis. Three repeated sequences of the human cMyc-tag were added to the C terminus of the R3 mutant or negative control green fluorescent protein (GFP) by subcloning it into a Myc-pBS plasmid (unpublished data; a gift from K. Matsuno, Osaka University, Japan). The full-length and mutant HuB and HuC were subcloned into the mammalian expression vector pCXN2, carrying β actin-promoter and G418 resistance gene (a gift from J. Miyazaki, Osaka University, Japan) (36). For the expression of H-Ras mutants, we used the HA-tagged expression vectors pEF-BOS H-Ras^{V12}-HA and pEF-BOS H-Ras^{N17}-HA (gifts from M. Nakafuku, Tokyo University, Japan) (37). pCXN2-FLAG-GFP and pCXN2-GFP-Myc, which were used as negative controls, were constructed by inserting the DNA fragments encoding the FLAG-GFP (a gift from Y. Wakamatsu, University of Oregon) (32) or the GFP-Myc fusion protein into the pCXN2 vector.

Cell Culture, DNA Transfection, and Immunoblotting. PC12 cells were cultured according to Shimizu *et al.* (38). For the DNA transfection, 1×10^7 cells were suspended in 0.8 ml PBS (Nissui Seiyaku, Tokyo) and added to 40 μg of DNA (for double transfection, each 40 μg) prepared by using a Qiagen (Chatsworth, CA) Plasmid Kit. After incubation on ice for 10 min, electroporation was performed by using a Gene Pulser (Bio-Rad) at 260 V, 960 μF. Cells were then incubated 10 min on ice and plated on collagen-coated coverslips in 12-well plates. Twenty-four hours after transfection, G418 (GIBCO/BRL) (300 μg/ml) was added to the medium to select for DNA-transfected cells. Immunoblot was performed essentially as described (39). Transfected cells (1×10^6) were lysed in 150 μl of SDS sample buffer. Ten-microliter aliquots of the lysates were run in 15% SDS/PAGE. Quantitative loading of the proteins was confirmed by Coomasie blue staining. Antibody dilutions of 1:100 of 16A11 (Hu-monoclonal antibody) (40), 1:1,000 of anti-FLAG (Kodak), and 1:100 anti-Myc MYC1–9E mAb (obtained from American Type Culture Collection) were used.

Immunocytochemistry of PC12 Cells. Immunohistochemical staining was performed according to the method described previously (41). A 1:1,000 dilution of anti-FLAG M2 mAb (Kodak) or a 1:100 dilution of anti-Myc mAb was used as the primary antibody. For double staining of the FLAG and Myc tags, a 1:1,000 dilution of mouse M2 anti-FLAG mAb (Kodak) and a 1:100 dilution of rabbit anti-Myc polyclonal antibody (Upstate Biotechnology) were used as above. Neurofilament H was detected by polyclonal antibody (Sigma). Activated extracellular signal-regulated kinase (ERK) was detected by a polyclonal antibody to the doubly phosphorylated form (Promega). Rhodamine-conjugated anti-mouse IgG (Chemicon) and horseradish peroxidase-conjugated anti-rabbit IgG (Chemicon) were used as secondary antibodies. For the enhanced immunostainings, TSA-direct kit was used (NEN Life Science). Immunofluorescent images were visualized by using an Axioplan2 microscope equipped for fluorescence imaging (Zeiss).

Ribohomopolymer (RHP)-Binding Assays. T7 epitope-tagged and histidine-tagged full-length and mutant (R1–2, R3, RRM1mt, and RRM2mt) mHuC bacterial fusion proteins and a control (human cdr2) fusion protein were produced after cloning into pET21b (Novagen). Ten picomol of each fusion protein was incubated with RHP beads (Sigma) in RHPA buffer (10 mM Tris·HCl, pH 7.4/2.5 mM $MgCl_2$/0.5% Triton X-100) with the indicated NaCl concentration and 1 mg/ml heparin in a volume of 500 μl. Binding was carried out at 4°C for 15 min. The beads were pelleted and washed five times in RHPA buffer with the indicated NaCl concentration, boiled in SDS buffer, and run on Western blots. Filters were probed with T7-tag monoclonal antibody (Novagen), and the relative amount of protein bound to RNA was visualized with ECL (Amersham Pharmacia) and quantitated by using National Institutes of Health IMAGE 1.61. These assays were able to detect binding of as little as 2–5% of the input protein.

Electroporation of Embryos, Whole-Mount Embryo Culture, and Immunostaining. Embryos were obtained from ICR mice at 9.5 d postcoitum (dpc). Whole embryos were cultured *in vitro* according to the method described previously (42). Each embryo was cultured in rat serum with 2 mg/ml glucose. After 2 hr of preculture, a DNA solution containing 5 μg/μl of the expression plasmid (pCXN2-FLAG-HuB, FLAG-HuC, HuB-R3, HuC-R3, or GFP-Myc) and 5 μg/μl of the marker plasmids pCAX-AFP (a mutant form of the GFP-expressing plasmid with the β-actin promoter, a gift from K. Umesono, Kyoto University, Japan) (43) mixed 1:1 with 0.01% fast green dye (Sigma) was injected into the neural tubes of embryos at the level of the rhombencephalon. DNA was injected by using micropipets made from capillary tubes pulled with a vertical microelectrode puller (Narushige, Tokyo) to a 10-μm internal diameter. Immediately after the injection of DNA, embryos were placed in Tyrode's solution and electroporated by using a T820 electroporator and a BTX500 (BTX, San Diego) optimizer (pulse 50 msec, 90 V $\times$ 5). After electroporation, embryos were cultured to 10.5 dpc and fixed in 4% paraformaldehyde and 1 $\times$ PBS. Frozen sections (12 microns thick) were cut on a cryostat and immunostaining was performed essentially as described (44) by using 2H3 monoclonal anti-165-kDa neurofilament antibody, anti-Islet1 antibody (both hybridoma supernatant, obtained from the Developmental Studies Hybridoma Bank, University of Iowa), or anti TuJ-1 antibody (Sigma). Detection of immunoreactivity was performed by using an ABC kit (Vector Laboratories) and a metal-enhanced DAB kit (Pierce).

RESULTS

Neuronal Phenotypes Are Induced by Overexpression of Hu Proteins in PC12 Cells. To elucidate the function of Hu proteins, they were first overexpressed in rat pheochromocytoma PC12 cells, which have been widely used for molecular analyses of the signaling pathways that lead to differentiation of PNS neurons. We transiently transfected pCXN2-FLAG-HuB or -FLAG-HuC into PC12 cells. The efficiency of transient transfection was approximately 10% based on immunostainings to the tag. However, we could drastically enrich the cells expressing exogenous Hu through G418 selection (more than 90% of the survived cells after the selections over 9 d)

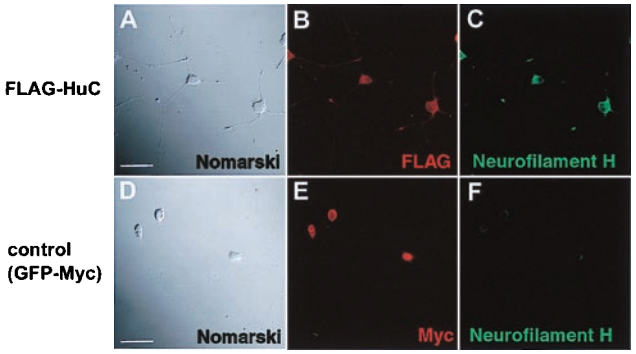

FIG. 1. HuC-induced neuronal phenotype of PC12 cells. (*A*) Morphology of pCXN2-FLAG-HuC-transfected PC12 cells (Nomarski differential interference contrast optics). Neuron-like morphology, similar to NGF-induced differentiation, appeared 9 d after transfection. (*D–F*) Negative control (pCXN2-GFP-Myc transfected PC12 cells) showed no morphological changes 9 d after transfection. Double staining for overexpressed FLAG-HuC (or GFP-Myc) (*B* and *E*) and neurofilament H (*C* and *F*). Antibodies for tagged sequence were used for the detection of transfected fusion protein. Overexpressed HuC protein localized mainly to the cytoplasm, and these cells showed increased expression of Neurofilament H (*C*), which is also known to increase in the NGF-induced differentiation of PC12 cells (53). (Bar = 15 μm.)

without increase of nontransfected cells. About 6 d after transfection, pCXN2-FLAG-HuC-transfected cells began to extend neurites. About 9 d after transfection, most of the FLAG-HuC (Fig. 1 *A–C*) or FLAG-HuB (data not shown) transfected cells were neuron-like in appearance and stopped proliferating (data not shown), a phenotype associated with neuronal differentiation. In contrast, control cells (transfected with pCXN2-GFP-Myc) (Fig. 1 *D–F*) showed no morphological changes.

Transfection of HuB or HuC without the FLAG tag showed similar morphological changes in PC12 cells as with the FLAG tag (data not shown). Expression of the neuronal marker neurofilament H, which is not known to be a direct target of Hu protein, was increased in PC12 cells expressing FLAG-HuC (Fig. 1 *B* and *C*), in contrast to the control cells (Fig. 1 *E* and *F*).

Mutation Analysis of Hu Proteins and Design of Dominant-Negative Forms. To further examine these findings, the functional domains of Hu proteins essential for the induction of neuronal phenotype were identified by mutation analysis. Hu proteins have three RRMs (RRM 1–3) (8, 9). We constructed deletion mutants lacking one or two of the RRMs from full-length mHuB and mHuC (Fig. 2*A*): mutants lacking the third RRM (termed "R1–2") or the first and second RRMs (termed "R3"). Furthermore, to examine the role of the RNA-binding activity of HuC protein, amino acid residues that are essential for RNA binding were mutated in the RRM1 (termed "RRM1mt") or RRM2 (termed "RRM2mt") of HuC.

To assess the ability of each of these mutants to bind RNA, binding to RHPs was assessed under varying conditions of stringency (Fig. 2*B*). Full-length HuC and HuC-R1–2 bound RHP avidly with a preference for ribouridine (rU) under high stringency (e.g., 54% and 39% of input protein, respectively, bound in 0.5M NaCl). RRM3 bound RHP only weakly, with a sequence preference for rU (e.g., 5% of input protein in 0.5 M NaCl). Similar results were found for full-length and R3

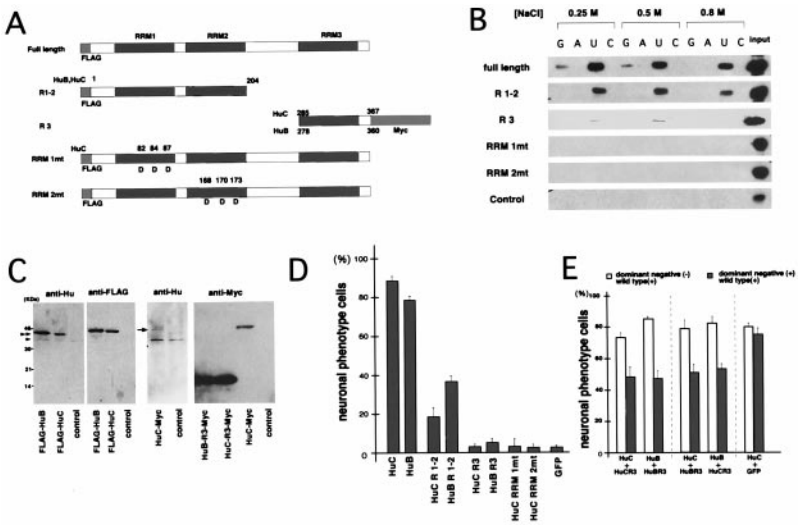

FIG. 2. Mutation analyses of Hu (HuB and HuC) proteins. (*A*) Mutants of Hu proteins used for functional analysis. HuB and C deletion mutants R1–2 and R3, and HuC RRM amino acid replaced mutants (RRM1mt, RRM2mt) were constructed as described in *Material and Methods*. (*B*) RNA-binding assay of HuC mutants. Full-length and mutant HuC fusion proteins and a control fusion protein were incubated with poly(rG)-, poly(rA)-, poly(rU)-, and poly(rC)-Sepharose RHP beads in either 0.25 M, 0.5 M, or 0.8 M NaCl. After being washed, protein bound to the RHP was analyzed by Western blot analysis with an anti-T7 antibody (Novagen). (*C*) Expression of exogenous and endogenous Hu proteins in PC12 cells 2 d after transfection. To detect these proteins, immunoblot analyses were performed. The expression level of exogenous FLAG-tagged full-length transgene products (indicated by double arrowheads) were quantitated in comparison to the endogenous Hu proteins of control cells (pCXN2 transfected PC12 cells) (indicated by arrowhead). The expression level of Myc-tagged HuB/C-R3 transgene products was indirectly quantitated by comparing to those of Myc-tagged full length transgene product (indicated by small arrow) and endogenous Hu proteins in the control cells. (*D*) Neuronal differentiation-inducing activities of PC12 cells by Hu mutants. The differentiation-inducing activities of mutants were evaluated by observing the morphology of transfected PC12 cells. G418 was added to the medium to enrich Hu-transfected cells. The cells that had dendrites longer than the diameter of their cell bodies were termed "neuronal phenotype" cells. This graph shows the proportion of differentiated cells among the transgene-containing cells (detected by using antibodies against the FLAG or Myc tags) 9 d after transfection. FLAG-GFP fusion protein was used as a negative control. One hundred transgene-expressing cells were examined three times (total 300 cells) per construct. (*E*) Hu-R3 mutants act as dominant-negative forms of Hu. Full-length Hu and Hu-R3 mutants were cotransfected into PC 12 cells and analyzed by using the same criteria as in *D* (single positive and double positive, each 100 cells, three times). Differentiation of cells transfected with both full-length Hu and the Hu-R3 mutant (double-positive cells) was reduced compared with cells transfected with full-length Hu alone (single-positive cells). Transfection of HuC with HuB-R3 or HuB with HuC-R3 showed the same results. Negative controls (pCXN2-FLAG-HuC with pCXN2-GFP-Myc) did not show any inhibition of neuronal differentiation.

constructs of HuD tested, whereas constructs harboring the hinge region between RRM2 and RRM3 together with RRM3 bound no better than R3 alone (data not shown). In addition, no significant RNA binding was detected with the RRM1mt and RRM2mt constructs, consistent with prior structure-function studies of the RRMs (45). Moreover, RNA binding of Hu constructs was tested against specific RNA ligands (fos-ARE and additional RNA ligands identified by RNA selection, H.J.O. and R.B.D., unpublished data) by filter-binding assay. Full length HuC and HuD constructs showed high-affinity binding to these ligands (K_d value $\approx$ 7–12nM), R1–2 constructs bound with $\approx$5–10 fold lower affinity, consistent with prior gel shift studies on similar R1–2 constructs (27, 34, 46), and R1 mt, R2 mt, and R3 Hu constructs showed no measurable RNA binding.

Expression levels of transfected Hu proteins were examined by using immunoblotting (Fig. 2*C*). Quantification by densitometric scanning (Scanning Imager, Molecular Dynamics) demonstrated high levels of overexpression of both full-length and R3 constructs (approximately 10-fold) relative to endogenous Hu proteins.

The biologic activities of these mutants were then assayed in PC12 cells (Fig. 2*D*). The R1–2 mutants were still capable of inducing the neuronal phenotype (neurite extension) in PC12 cells, but their effects were weaker than those of the wild-type proteins (20 $\approx$ 25% of the wild-type control). The R3 mutants, however, essentially had no neuronal phenotype-inducing activity and exerted no significant effect on proliferating activity of transfected cells. RRM1mt and RRM2mt also showed no significant neuronal phenotype-inducing activity (Fig. 2*D*).

The R3 mutants, which essentially lack (or have greatly diminished) neuronal phenotype-inducing and RNA-binding activities, may inhibit the normal functions of HuC as dominant-negative forms. To examine this possibility, R3 mutants were coexpressed with wild-type HuB or HuC. PC12 cells expressing both the wild-type FLAG-HuC, and mutant HuC-R3 showed a significantly lower percentage of cells with neuronal phenotype than the cells expressing only FLAG-HuC (Fig. 2*E*). Interestingly, combinations of different Hu proteins (HuC/HuB-R3 and HuB/HuC-R3) showed essentially the same results (Fig. 2*E*), indicating that the R3 mutant of one type of Hu protein is capable of inhibiting other member(s) of the Hu family as a dominant-negative form.

Actions of Hu in PC12 Cells: Relationship to Nerve Growth Factor (NGF)/Ras Signaling. The molecular mechanisms underlying the neuronal phenotype-inducing activity of Hu proteins were examined in relation to NGF/Ras signals, which are also known to induce the neuronal differentiation of PC12 cells (47). First, we performed the *in situ* detection of activated ERK (the doubly phosphorylated form), which is known to be increased in the NGF/Ras-mediated differentiation of PC12 cells (48). PC12 cells that differentiated in response to NGF treatment showed strong expression of activated ERK, especially in their nuclei (Fig. 3*E*). In contrast, the PC12 cells, whose neuronal phenotype was induced by the misexpression of HuC, showed no detectable expression of activated ERK (Fig. 3*C*). Next, we examined whether the misexpression of the dominant-negative form of HuC (R3) could prevent the NGF-induced differentiation of PC12 cells. Although the forced expression of dominant-negative H-Ras (H-Ras^{N17}) (49) prevented NGF-induced PC12 cell differentiation, the misexpression of HuC-R3 did not, even at a lower dose of NGF (Fig. 3*F*). Furthermore, HuC-R3 could not suppress the differentiation of PC12 cells induced by the misexpression of active H-Ras (H-Ras^{V12}) (50) (Fig. 3*G*). However, wild-type HuC (FLAG-HuC) induced the neuronal phenotype of PC12 cells that otherwise should have been inhibited by H-Ras^{N17} (Fig. 3*G*). Taken together, these results indicate that Hu is likely to induce the neuronal phenotype of PC12 cells in a parallel and distinct pathway from NGF/Ras and its subsequent ERK activation. However, we cannot completely exclude the possibility that Hu proteins are involved in NGF/Ras pathway downstream of ERK activation. The dominant-negative effect of Hu-R3 was not complete in blocking the effects of the cotransfected wild-type Hu in PC12 cells (Fig. 2*E*). Thus, it is possible that the effect of Hu-R3 was not strong enough to inhibit the NGF or dominant-active Ras-induced neuronal differentiations of PC12 cells.

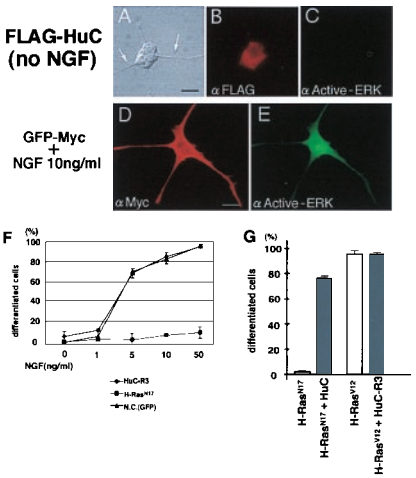

FIG. 3. Hu proteins act independently of the NGF/Ras cascade. (*A–E*) HuC-induced neuronal phenotype of PC12 cells is not accompanied by ERK activation. Expression of activated-ERK in PC12 cells overexpressing HuC in the absence of additional NGF (*A–C*) or cells expressing GFP-Myc in the presence of additional NGF (*D* and *E*) 9 d after transfection. There was no detectable expression of activated ERK in neuron-like PC12 cells that overexpressed HuC. Bar = 5 μm. (*F*) HuC-R3 cannot prevent NGF-induced PC12 differentiation. PC12 cells transfected with HuC-R3, H-Ras^{N17} (dominant negative) or FLAG-GFP (negative control) were cultured in medium containing additional NGF. Transfected cells were selected by using G418. Morphology of transfected cells was evaluated 9 d after transfection same as in Fig. 3. Although H-Ras^{N17} misexpression almost completely blocked NGF-induced differentiation, HuC-R3 misexpression showed no significant influence on differentiation. (*G*) Dominant-negative H-Ras cannot prevent HuC-induced neuronal phenotype. H-Ras^{N17} and HuC, or H-Ras^{V12}(dominant active) and HuC-R3 were cotransfected into PC12 cells. Morphology of the cells expressing both proteins was evaluated as in the legend for Fig. 2. Dominant-negative H-Ras (H-Ras^{N17}) could not suppress HuC-induced neuronal phenotype, and HuC-R3 could not suppress dominant active Ras (H-Ras^{V12})-induced differentiation.

Roles of Hu Proteins in Neuronal Development Within Embryonic CNS. Hu proteins may be required for CNS neuronal development, because they begin to be expressed at very early developmental stages in essentially all neurons within the CNS (4, 9, 40, 41, 51). To investigate this possibility, we misexpressed wild-type (FLAG-HuC) or dominant-negative mutants (R3) of HuB and HuC in the developing mouse neural tube using a newly developed electroporation method followed by whole-embryo culture (42). Neuronal development of Hu-transfected embryos was evaluated by observing the expression of neuronal markers (neurofilament-M, TuJ-1 and islet-1) in sections. In the embryos transfected with pCXN2-FLAG-HuC at E9.5, ectopic neurofilament-M signals were observed in the transfected (right) side of the ventricular zone, corresponding to the site of transgene expression detected by anti-FLAG antibody (Fig. 4 *A* and *C*). Similar results were obtained in FLAG-HuB-introduced embryos (data not shown). These signals were never seen in nontransfected (left) side of the same slice (Fig. 4 *B* and *D*) or in negative controls (pCXN2-GFP-Myc-transfected embryos; data not shown). Another neuronal marker, TuJ-1, was induced in pCXN2-FLAG-HuC-transfected embryos (Fig. 4 *E*

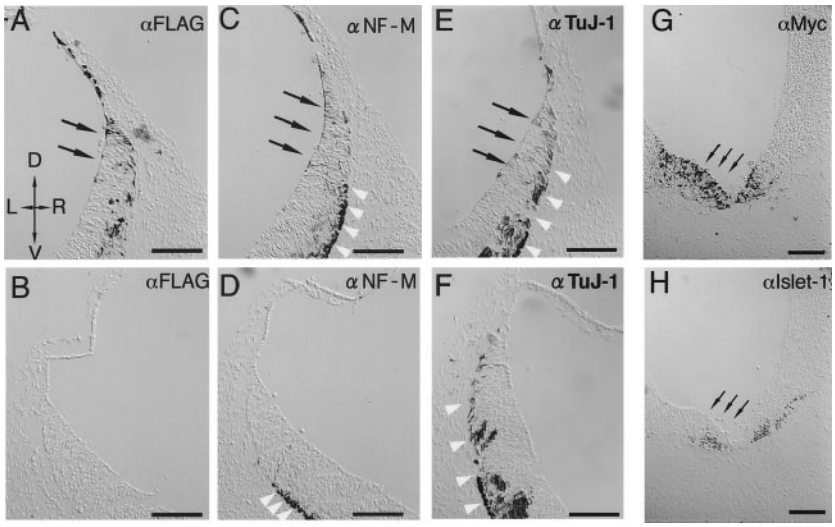

FIG. 4. Misexpression of Hu and Hu-R3 mutants in mouse embryonic CNS. Twelve-micrometer frozen serial sections of a HuC (FLAG-tagged)-transfected E9.5 mouse embryo (*A–F*) and a HuC-R3 (Myc-tagged)-transfected embryo (*G* and *H*) (D, dorsal; V, ventral; L, left; R, right). Sections of the HuC-introduced embryo were immunostained with antibodies against FLAG (*A* and *B*), neurofilament M (*C* and *D*) and TuJ-1 (*E* and *F*). Paired images of each individual section are shown from the transfected (*A, C*, and *E*) and nontransfected (*B, D*, and *F*) sides of the same slices. At the rhombencephalon of the normal mouse embryos in these stages (E9.5), the neurogenesis has already begun and neuronal markers are seen. Therefore, even in the nontransfected side (*D* and *F*), the expressions of authentic neuronal markers [TuJ1 (*D*); NF-M (*F*)] were observed in the outside of the ventricular zone (indicated by white arrowheads), corresponding to normal neuronal development. In the transfected side (*C* and *E*), however, ectopically induced expressions of neuronal markers were observed from within the ventricular zone [indicated by black arrows, TuJ-1 (*C*); NF-M (*E*)], coinciding with the expression of transfected FLAG-HuC [indicated by black arrows in (*A*)]. Such ectopic expressions of neuronal markers are never seen in normal neuronal development. Sections of a HuC-R3-transfected embryo were immunostained by using antibodies against Myc-tag (*G*) and Islet-1 (*H*). Islet-1-positive cells decreased in the HuC-R3 overexpressing region (indicated by small arrows). (Bar = 100 μm.)

and *F*). Thus, overexpression of HuB or HuC appears to be sufficient to induce the ectopic neuronal development within embryonic CNS.

To address whether Hu is required for normal neuronal development in the embryonic CNS, dominant-negative Hu proteins (R3) were misexpressed in the CNS of mouse embryos. When HuC-R3 (Fig. 4 *G* and *H*) or HuB-R3 (data not shown) was transfected into E9.5 mouse embryos, expression of the early motor neuronal marker, Islet-1, was shown to be suppressed coinciding with transgene expression. At this stage, endogenous expression of Hu proteins was already detected in this region by immunohistochemistry (data not shown). These findings suggest that the Hu-R3 mutants acted as dominant-negative forms to inhibit the ability of endogenous Hu proteins to promote the differentiation of CNS neurons.

DISCUSSION

Here, we investigated the roles of Hu in the mammalian nervous system by misexpressing full-length and dominant-negative forms of HuB and HuC. The full-length HuB and HuC induced neuronal phenotypes of PC12 cells, resulting in neuron-like morphological changes, increased expression of a neuronal marker gene (Fig. 1 *C* and *D*), and mitotic arrest (data not shown). Overexpression of Hu in chicken neural crest-derived cells results in the premature appearance of neuronal markers (32) and the HuB protein family is up-regulated during neuronal differentiation of embryonic carcinoma P19 cells (33). Taken together, these results indicate that Hu proteins are likely to act as positive regulators of neuronal development in a way that is similar to *Drosophila* ELAV. Furthermore, we showed that Hu proteins function not only in the PNS but also in the developing CNS. In the developing mouse embryo, we observed misexpression of HuB/C or their R3-mutant affected the expression of neuronal markers. In addition to the possible direct translational activation of neurofilament-M by HuB (29), it is notable that expression of other two neuronal markers (TuJ-1 and Islet-1) are also affected. Collectively, we can conclude that Hu proteins promote neuronal development.

We have shown that neuronal differentiation in PC12 cells and *in vivo* can be mediated by full-length HuB and HuC expression constructs and, albeit with less efficiency, in PC12 cells by constructs harboring only RRM 1–2. In contrast, full-length Hu constructs harboring point mutations in either RRM1 or RRM2 failed to promote neuronal phenotype. These biologic results correlate with the RNA-binding activities of each construct—full-length or R1–2 constructs bound RNA avidly and promote neuronal phenotype in PC12 cells, whereas point mutants showed no RNA-binding to RHPs and no biologic activity. Thus the biologic activities we observed are likely to depend on the RNA-binding activity of the Hu proteins. The Hu proteins stabilize target RNAs through sequence-specific binding to AREs (18, 22, 24, 25, 31). Interestingly, Steitz and colleagues have examined the ARE binding with RRM1–2 constructs (aa 1–185 of HuA) nearly identical to the R1–2 construct used here (aa 1–204 of HuC) and found that the RRM1–2 failed to stabilize ARE containing target RNAs in L929 cells (18). Although directly comparable stability assays using HuB/C constructs have not been reported, these observations suggest that the biologic activity of HuB/C-R1–2 constructs in neuronal cells may not act through stabilization of ARE containing RNAs. Thus, one possible explanation is that Hu proteins may have other biologically important RNA targets in neurons in addition to ARE elements.

Our results also point to the importance of the RRM3 of Hu in neuronal differentiation, because HuB/C-R3 appears to act in a dominant-negative manner to inhibit the biologic activity of Hu in PC12 cells and *in vivo*. Previous studies have demonstrated not only that HuA RRM3 is necessary for stabilization of ARE-containing target RNAs (18), but that RRM3 may bind polyA RNA *in vitro* (34, 52). We did not find evidence for significant RNA binding of RRM3 to poly rA or

other RHPs, this discrepancy may be accounted for by the different stringency of binding assays—we examined RNA binding under stringent conditions (Fig. 2*B*), whereas previous studies examined polyA binding under nonstringent binding conditions (34, 52). Nonetheless, our observations do not rule out the possibility that HuC/B-R3 acts as a dominant-negative domain by competing for binding of full-length protein to specific RNA target sequences. Another possibility is that HuC/B-R3 blocked Hu function by competing at the level of protein–protein interaction. The only candidate protein interaction domain identified in Hu proteins is a tightly defined NLS/NES domain (amino acids 205–237 in HuA) (28), which is close to but distinct from RRM3. Notably, the analogous HuB/C sequence elements were absent from our dominant-negative constructs (HuC/B-R3), suggesting that the dominant-negative action may not result from direct interaction with nuclear-cytoplasmic shuttling activity. Finally, it is possible that the action of HuC-R3 involves inhibition of both RNA and protein binding.

Availability of a dominant-negative form of Hu is likely to provide a useful tool for exploring its *in vivo* functions. HuB/C and D proteins have more than 90% homology at the amino acid level. The expression pattern of each Hu gene is different, but they overlap one another considerably. Such a redundancy of Hu family members may hinder functional analysis by the targeted disruption of each gene. We have shown that one Hu-R3 may suppress other Hu proteins. Therefore, further studies that use combinations of overexpressed dominant-negative forms with targeted disruption will unequivocally reveal the roles of Hu family members in the nervous system.

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