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cDNA cloning and characterization of Drb1, a new member of RRM-type neural RNA-binding protein☆

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Abstract

Neural RNA recognition motif (RRM)-type RNA-binding proteins play essential roles in neural development. To search for a new member of neural RRM-type RNA-binding protein, we screened rat cerebral expression library with polyclonal antibody against consensus RRM sequences. We have cloned and characterized a rat cDNA that belongs to RRM-type RNA-binding protein family, which we designate as *drb1*. Orthologs of *drb1* exist in human and mouse. The predicted amino acid sequence reveals an open reading frame of 476 residues with a corresponding molecular mass of 53 kDa and consists of four RNA-binding domains. *drb1* gene is specifically expressed in fetal (E12, E16) rat brain and gradually reduced during development. In situ hybridization demonstrated neuron-specific signals in fetal rat brain. RNA-binding assay indicated that human Drb1 protein possesses binding preference on poly(C)RNA. These results indicate that Drb1 is a new member of neural RNA-binding proteins, which expresses under spatio-temporal control. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Neural RRM-type RNA-binding protein; Developmental regulation; RNA binding protein; RNA recognition motif

Neurons and glial cells are generated from multipotent neural stem cells during central nervous system (CNS) development [1]. Several basic helix-loop-helix (bHLH) transcription factors have been implicated to regulate neuronal or glial differentiation in the developing CNS [2]. On the other hand, recent discoveries show us that post-transcriptional regulation by neural RNA-binding proteins also plays an important role in neural development [3–8]. In several RNA-binding motifs, RNA recognition motif (RRM)-type RNA-binding domain (RBD) is most widely found and best characterized [9]. RRM-type RBD consists of ~90 amino acids, and demonstrates structural conservation of two α-helices and four β-strands [10–12].

elav and *musashi* are major gene families of neural RRM-type RNA-binding proteins in *Drosophila* [13,14]. Mutations in the *elav* locus of *Drosophila* result in a dysfunctional nervous system in which neuroblasts continue to proliferate, fail to differentiate, and migrate inappropriately [15]. Hu family, the mammalian homolog of Elav, consists of four different members (HuR [16], HuB/Hel-N1 [3], HuC [17], and HuD [18]). All four Hu proteins contain three RRM-type RNA-binding domains (RBD) and a linker region separating the two N-terminal RBDs (RBD I and RBD II) from the C-terminal RBD (RBD III). In vitro studies demonstrated that RBD I and RBD II tightly bind to RNA stability elements (AU-rich elements [AREs]) in 3′-untranslated region (UTR) of various mRNAs [19–22]. HuB/Hel-N1, HuC, and HuD have been demonstrated to induce neurite outgrowth when over-expressed in rat PC12 cells

☆ *Abbreviations:* Drb1, developmentally regulated RNA-binding protein 1; RRM, RNA recognition motif; *i*PABP, inducible poly(A) binding protein; RBD, RNA-binding domain; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; EST, expressed sequence tag; CNS, central nervous system; 3′-UTR, 3′-untranslated region; ISH, in situ hybridization; IHC, immunohistochemistry; PB, phosphate buffer; PBS, phosphate-buffered saline. DDBJ Accession Nos.: rat Drb1, AB036990; human Drb1, AB036991; mouse Drb1, and AB036992.

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without nerve growth factor (NGF) treatment [6]. These results indicate a crucial role of Hu proteins for terminal differentiation from neural progenitor cells to neurons through regulation of target mRNA stability by binding its AREs.

Another neural RRM-type RNA-binding protein family, *Drosophila musashi* (*d-msi*) [14], *Xenopus laevis nrp-1* [23], and their mammalian homolog *msi1* [24] and *msi2* [25], express in neural stem cells. Loss-of-function mutations in *d-msi* result in the frequent appearance of mechanosensory organs containing extra shaft and socket cells at the expense of neuron and glial cells [9]. In contrast to the Elav family, the members of Msi family are expressed in neural immature cells during embryonic CNS development and the expression levels of *msi1* and*msi2* genes are gradually decreased during development [24,25]. Recently, Okano and his colleagues demonstrated that Msi1 regulates Notch signaling by repressing translation of *tramtrack69 ttk69*) and *m-numb* mRNA through binding to 3′-UTR of these mRNAs [7,8].

Although these results demonstrate an essential role of neural RNA-binding proteins, the posttranscriptional regulatory mechanism in neural development is not fully understood. Lasko analyzed *Drosophila* genome database and reported 117 different RRM-containing proteins. Of those genes, 74 have not been described previously [26]. This result also suggests the possible existence of unknown RRM-type RNA-binding protein. To search for a new member of the neural RRM-type RNA-binding protein, we have screened rat cerebral expression library by polyclonal antibody against RRM of inducible poly(A) binding protein (*i*PABP). Here, we report the molecular cloning of a novel gene in rat embryonic day 17 (E17) fetal brain, which designated Developmentally regulated RNA-binding protein 1 (*drb1*). The gene is characterized by four RRM-type RNA binding domains and by its spatiotemporal expression pattern. A physiological role of Drb1 protein will be discussed.

Materials and methods

cDNA cloning using polyclonal antibody against RRM oligo peptide. The peptide fragment corresponding to RRM1 of *i*PABP as "KGYAFVHFETQEAADKA" was synthesized. About 200 μg KLH-conjugated synthetic peptide was used to immunize rabbits. Using this anti-serum, a rat fetus (embryonic day 17) cerebral expression library packaged in λgt11 (Clontech, Palo Alto, Calfornia) was screened as previously described [27]. Out of 5×10^5 phage plaques, 32 positive clones were obtained and inserts were subcloned into pBluescript II SK+ (Stratagene) and sequenced on both strands by dideoxy chain termination method using BigDye Terminator (Applied Biosystems). Nucleotide sequences were subjected to homology search against GenBank database. One partial cDNA encoding a novel RRM-type RNA-binding protein was obtained. The full-length cDNA and human and mouse orthologs were obtained by PCR based on homology informations from expressed sequence tag (EST) and human genome database.

RNA blot analysis. Total RNAs were prepared from various rat tissues of indicated developmental stages or cultured neuronal precursor cells as previously described [28]. Total RNA (10 μg/lane) was separated by electrophoresis in 1% agarose–formaldehyde gels and transferred to nylon membrane (Amersham Biosciences). A rat *drb1* DNA fragment extending from the position at 480 to the position at 1700 was labeled with [α-^{32}P]dCTP by Ready-To-Go-DNA Labeling beads (Amersham Biosciences) and used as a probe.

In vitro translation and RNA-binding assays. In vitro translation of 1 μg of full-length human *drb1* cDNA was performed with the TNT-coupled reticulocyte lysate systems (Promega) in the presence of 40 μCi [^{35}S]methionine (Amersham Biosciences). Binding of ^{35}S-labeled human Drb1 to Sepharose bead-bound poly(A) or agarose bead-bound poly(C), (G), and (U) (Sigma) was performed as described previously [29]. After binding reaction, the RHP beads were washed and resuspended in sample buffer, boiled for 2 min, and loaded on a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel. Following electrophoresis, the gels were dried and visualized by BAS 2000 Bioimaging Analyzer (FUJIFILM).

Epitope tagging. A 5′-end of cDNA encoding residues 2–476 of human Drb1 was tagged with FLAG, by PCR using a set of primers, *Eco*RI-linked 5′ primer, 5′-CGGAATTCCGACGAAGCTG GCAGC TCTGCGAG-3′ (underline shows *Eco*RI restriction site) and *Bam*HI-linked 3′ primer, 5′-CGGGATCC*TCA*GTAAGTTCTTTGCCGTTT GTT-3′ (underline shows *Bam*HI restriction site and italic shows stop codon). The amplified fragment was digested with *Eco*RI and *Bam*HI, and then ligated into pFLAG-CMV-2 expression vector (Eastman Kodak).

Cell culture and DNA transfection. Rat pheochromocytoma cell line, PC12 cells, and mouse teratocarcinoma cell line, P19 cells, were obtained from American Type Culture Collection. PC12 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% horse serum and 5% fetal bovine serum (FBS). For the neuronal differentiation of PC12 cells, cells were treated with 50 ng/ml nerve growth factor (NGF, obtained from TaKaRa Shuzo). P19 cells were cultured in Eagle's minimum essential medium-α (αMEM) supplemented with 10% FBS. For neuronal differentiation of P19 cells, cells were treated with 0.3 μM all-*trans*-retinoic acid (Sigma). Cell cultures were maintained at 37 °C with 5% CO_2. For DNA transfection, LipofectAMINE 2000 reagent (Invitrogen) was used for transfection of plasmid DNA according to manufacturer's instruction.

Immunocytochemistry. The cells were harvested on glass-bottomed dish for 24 h, fixed in PBS containing 4% paraformaldehyde (PFA) and 0.4% Triton X-100 for 20 min at room temperature. The cells were then incubated with the mouse anti-FLAG antibody (M2, Sigma) for 1 h at a final concentration of 0.3 μg/ml in PBS-T containing 2% FBS, washed, and stained with Alexa-488 conjugated goat anti-mouse IgG (Molecular Probes) at a final concentration of 2 μg/ml. Immunofluorescent images were visualized by using Radiance 2000 Laser Scanning Confocal Microscope System (Bio-Rad).

In situ hybridization to tissue sections. Rat *drb1*-specific cRNA probe was synthesized using a 1221-base pair *Hin*dIII fragment spanning nucleotides 480–1700 of the full-length rat cDNA subcloned into pCMV-SPORT vector (Invitrogen). The cRNA transcriptions were performed with RNA colour kit (Amersham Biosciences) and fluorescein-labeled nucleotide. Brains obtained from adult and fetal rat were fixed for 12 h at 4 °C with 4% PFA in PBS, and then frozen by liquid nitrogen. Slices (20 μm) were obtained using a cryostat, collected on APS-coated Superfrost slides (Matsunami Glass Ind.). After 20 min of permeabilization at 37 °C in permeabilization buffer (100 mM Tris–HCl [pH 8.0], 50 mM EDTA, and 1 μg/ml RNase-free Proteinase K), tissue sections on slides were hybridized in hybridization buffer (50% formamide, 10 mM Tris–HCl [pH 7.6], 1 mM EDTA, 600 mM NaCl, 10 mM DTT, 1× Denhardt's solution, 0.25% SDS, 10% dextran sulfate, and *E. coli* tRNA [0.2 mg/ml, Sigma]) with fluorescein-labeled probes (200 ng/ml). After hybridizatiom, the sections were washed in 1 SSC for 10 min at room temperature and digested with 10 μg/ml RNase

A at 37 °C for 20 min. Sequentially, the sections were incubated in PBS containing 5% bovine serum albumin for 30 min, incubated with 1:200 dilution of alkaline phosphatase-conjugated anti-fluorescein antibody for 1 h. Color development with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate was carried out for 3 h.

Immunohistochemistry. Immunohistochemistry (IHC) was performed by as previously described [30]. Briefly, rats' brains were fixed with 4% PFA dissolved in 0.1 M phosphate buffer (PB). Sections of the brains were made by a freezing microtome at 50 μm. Following 1 h of incubation in block solution (3% normal goat serum in PBS), sections were incubated with anti-rat Nestin (BD Bioscience, 1:50 dilution in block solution) and anti-rat NeuN (Chemico, 1:100 dilution in block solution) overnight at 4 °C. Sections were washed twice for 15 min each in PBS and incubated for 1 h with biotin-conjugated anti-rat IgG (Vector laboratories). Sections were washed in PBS and further incubated for 1 h with an avidin–biotin complex (Vector laboratories) applied according to manufacturer's instruction. Following a final wash in PBS, sections were stained with the VIP staining kit (Vector laboratories) for 5–10 min until staining became apparent. Staining was terminated by washing twice for 5 min each in PB.

Results

Isolation of rat, mouse, and human drb1 cDNA

We have isolated a rat cDNA encoding a novel RRM-type RNA-binding protein from embryonic day 17 (E17) rat fetal brain as described in Materials and methods. In addition, we also cloned human and mouse orthologs by PCR based on homology informations from EST and human genome databases. The predicted amino acid sequences of rat, mouse, and human Drb1 proteins show 476 residues with a corresponding molecular mass of 53 kDa and are characterized by four RRM-type RNA-binding domains (Fig. 1A). The nucleotide sequences surrounding the AUG initiation codon are consistent with Kozak's rule [31]. From comparison among rat, mouse, and human orthologs, Drb1 proteins in *Mus musculus* and *Homo sapiens* are very similar to rat Drb1 (91.8% and 91.6% identical, respectively) (Fig. 1B).

Homology search of the rat *drb1* gene by the program BLAST showed us that several EST clones are identical to this sequence. Comparison of the rat Drb1 protein sequence with GenBank database using the program BLASTX showed extensive amino acid sequence similarities against *Drosophila* putative RNA-binding protein CG1316 (47% identity) (Fig. 1B). Although RRM-type RNA-binding domain is consisted of ~90 amino acid residues in general [10–12], RBD III of Drb1 protein contains ~120 amino acid residues and displays less similarities to evolutionarily conserved consensus sequences. In addition, RBD III of *Drosophila* putative homolog CG1316 lacks the amino terminus region including RRM2. These results suggest that RBD III of Drb1 protein is pseudo-RNA binding domain. Homology search by the program (BLASTP also displayed that RBD I and II of the Drb1 protein share extensive amino acid sequence similarities to mouse neural RNA-binding protein HuB and HuC (both 50% similarity). So we concluded that we had successfully cloned a novel gene encoding a protein similar to neural RRM-type RNA binding proteins.

Rat drb1 gene expression is brain-specific and developmentally regulated

To examine the tissue distribution pattern of rat *drb1* gene expression, each 10 μg total RNA from E21 rat fetus tissues was subjected to RNA blot analysis using 3′ half portion of rat *drb1* cDNA as a probe. Three *drb1* transcripts were detected in rat tissues (Fig. 2A). One is a major transcript of ~2 kb in length, which agrees with the estimated size of the rat full-length cDNA clone. Other transcripts of ~3 and ~4.4 kb in length appear to be less abundantly expressed and these species would utilize another poly(A) additional sites. The RNA blot analysis also indicates that the high *drb1* gene expression is limited to brain; however, less abundant expression is observed in kidney and spleen. Only trace amount of expression is observed in lung and heart (Fig. 2A). These results indicate that rat *drb1* gene predominantly expresses in E21 fetal brain.

Next, we analyzed spatiotemporal expression profile of *drb1* in rat brain by RNA blot analysis. The expression of *drb1* is highest in E16 brain and then gradually decreased along brain development (Fig. 2B, left half). When *drb1* gene expressed less abundantly in adult brain, relatively abundant *drb1* gene expression is observed in non-neural tissues (for example, kidney, spleen, heart, and lung) in adult rat (data not shown). Although total expression level in adult brain seems to be decreased, *drb1* gene is still expressed in the restricted region of adult brain, i.e., cerebellum, olfactory bulb, and hippocampus (Fig. 2B, right half). These results indicate that *drb1* gene expression is developmentally and spatially regulated in rat brain and that these precise controls of expression level suggest an importance of its physiological role for brain development.

We also examined whether the expression control of the gene is shown in cultured cells, i.e., PC12 cells (rat pheochromocytoma cells) and P19 cells (mouse teratocarcinoma cells), before and after induction of neuronal differentiation. PC12 cells are differentiated to neuron by treatment with nerve growth factor (NGF) and P19 cells are differentiated to neuron with retinoic acid (RA). The *drb1* gene abundantly expresses in PC12 and P19 cells before induction (Fig. 2C). When these cells are treated with NGF and RA, respectively, *drb1* gene expression is gradually reduced. These results suggest that *drb1* transcripts are related to maintenance of neuronal progenitor cells.

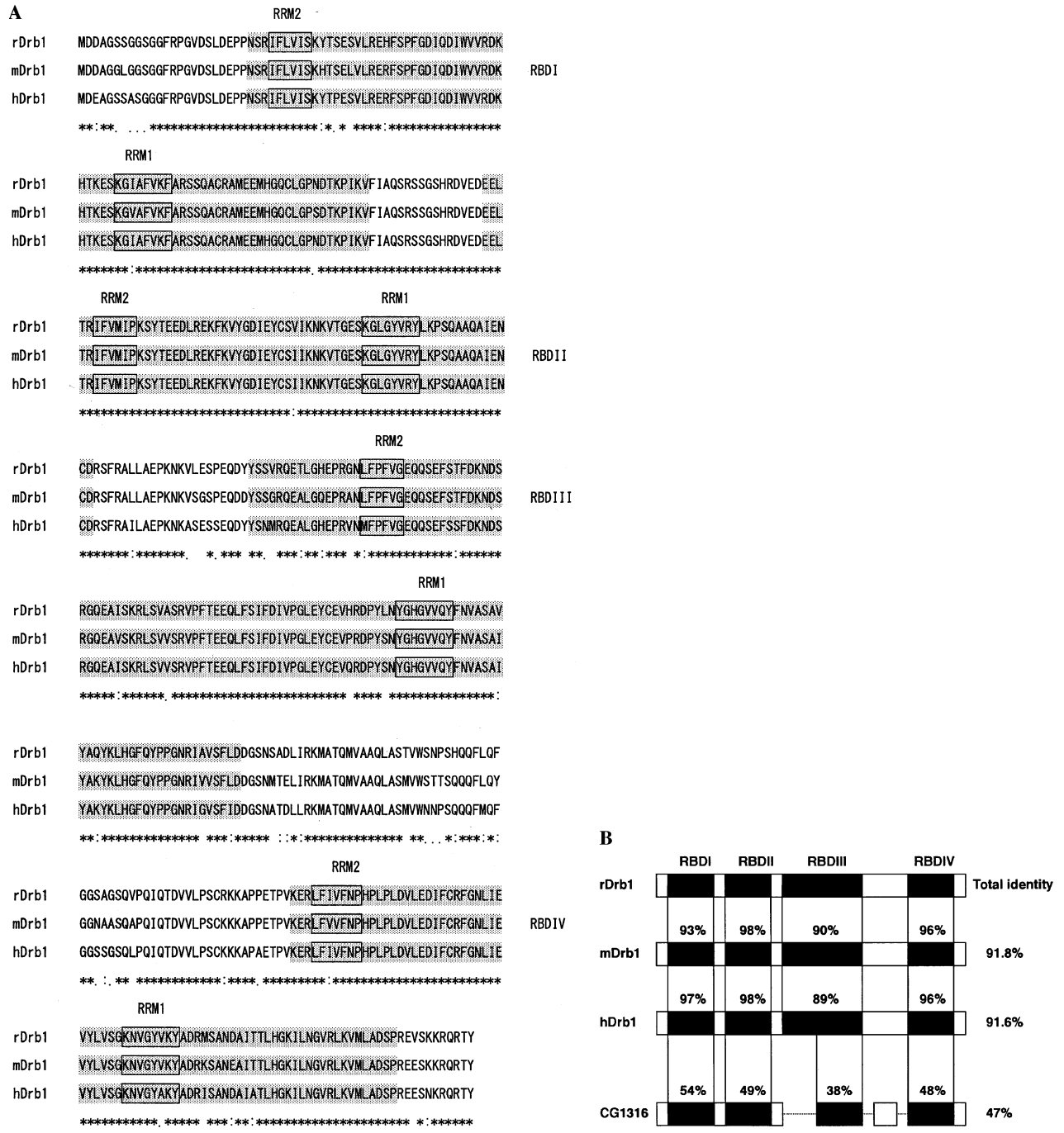

Fig. 1. Structure of Drb1 protein. (A) Multiple sequence alignment of rat, mouse, and human Drb1. The rat, mouse, or human protein is preceded by rat Drb1 (rDrb1), mouse Drb1 (mDrb1), and human Drb1 (hDrb1), respectively. The shaded area indicates RBD I through RBD IV. The box indicates the RRM consensus sequence. Asterisks indicate identical amino acids; dots and colons indicate conserved amino acids change. (B) Comparison of domain structures of rat, mouse, and human Drb1 and *Drosophila* CG1316. rDrb1 was compared with mouse and human orthologs and CG1316 (*Drosophila* putative RNA-binding protein). RBDs were indicated as closed box and percentage of identities to corresponding RBD of rDrb1 protein was indicated above each RBD. Total identities against rDrb1 were indicated at the right of schematic structure of protein.

Drb1 protein specifically binds to poly(C) RNA

To examine its RNA binding activity of Drb1 protein, RNA-binding assay using RNA homopolymer (RHP) beads is performed. Drb1 protein was synthesized by in vitro translation with [^{35}S]methionine from human full-length *drb1* cDNA. Two protein products of ~53 and ~37 kDa in molecular weight are detected, of which the larger product is corresponding to intact Drb1 protein and the small one is speculated as a degraded form or premature stop products (Fig. 3A). These labeled proteins were incubated with each RHP beads:

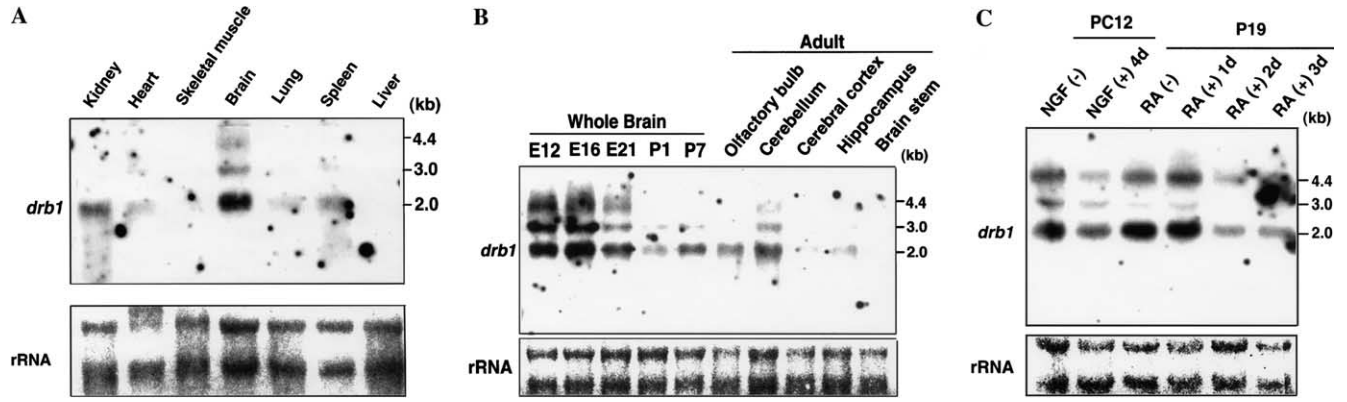

Fig. 2. Spatiotemporal distribution of rat drb1gene expression. (A) Tissue distribution of *drb1* mRNA in rat. RNA blot analysis of *drb1* mRNA for various rat E21 tissues was performed. The top panel shows the blot probed for *drb1* cDNA. The bottom panel shows the ethidium bromide-stained rRNAs. (B) Expression of *drb1* gene is developmentally regulated. Developmental changes of rat *drb1* gene expression in the whole brain (left half) are analyzed. Spatial expression of *drb1* gene in rat adult brain is demonstrated (right half). The top panel shows the blot probed for *drb1* cDNA. The bottom panel shows the ethidium bromide-stained rRNAs. (C) Expression of *drb1* gene in cultured neural precursor cells. *drb1* expression in PC12 and P19 cells was demonstrated before and after differentiation induction. The top panel shows the blot probed for *drb1* cDNA. The bottom panel shows the ethidium bromide-stained rRNAs.

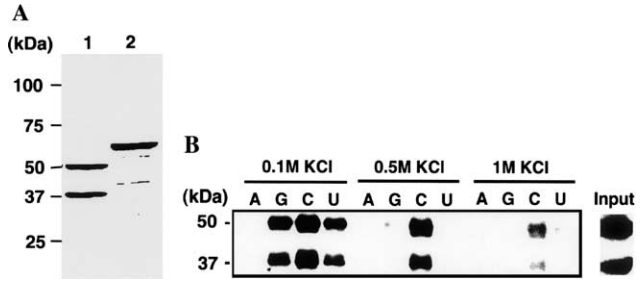

Fig. 3. RNA-binding assay of Drb1 protein. (A) In vitro translation of human Drb1 protein. Prior to RNA-binding assay, in vitro translation product of human full-length *drb1* cDNA was confirmed by SDS–PAGE (lane 1). Luciferase is in vitro translated as a positive control (lane 2). (B) RNA-binding assay. Binding analysis of [^{35}S]methionine-labeled hDrb1 to Sepharose bead-bound poly(A) or agarose bead-bound poly(C), (G), and (U) was performed. The KCl concentration of the binding buffer varied from 0.1 to 1 M. The lane marked "Input" shows 25% of the volume of the in vitro-translated product used for the binding reactions.

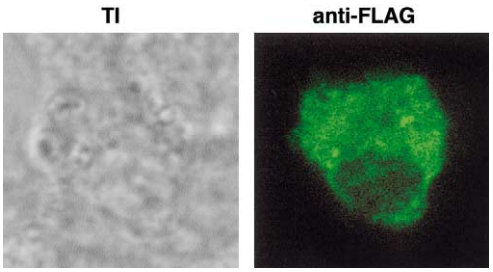

Fig. 4. Subcellular localization of Drb1 protein in PC12 cell. PC12 cells transfected with the FLAG-tagged *drb1* cDNA show cytoplasmic staining with the anti-FLAG monoclonal antibody. TI (Transmission image) and anti-FLAG (fluorescent) views are shown.

Sepharose bead-bound poly(A) or agarose bead-bound poly(C), (G), and (U). RNA binding properties were examined as described in Materials and methods. The RNA binding specificity is demonstrated as poly(C) > poly(G) = poly(U) ≫ poly(A) (Fig. 3B). These results indicate that Drb1 protein possesses binding preference on poly(C) RNA.

Drb1 predominantly localized in cytoplasm

To determine the subcellular localization of Drb1 protein, immunocytochemsitry was performed using PC12 cells. FLAG-tagged human *drb1* cDNA in a mammalian expression vector was transfected into PC12 cells and then 24 h after transfection the FLAG-tagged human Drb1 protein was detected with anti-FLAG antibody. FLAG-hDrb1 protein predominantly localized to cytoplasm (Fig. 4). In addition, a small population of cells demonstrated dual subcellular distribution, nucleus and cytoplasm (data not shown). These results indicate that Drb1 protein mainly exists in cytoplasm and occasionally may shuttle between nucleus and cytoplasm.

Drb1 expresses in neurons, not in glial cells

To determine the spatial localization of Drb1 protein in rat CNS, we performed in situ hybridization (ISH) with an antisense RNA probe synthesized from cDNA encoding rat Drb1 protein. In E16 rat fetal CNS, strong signals were detected in subventricular zone and neural tube (Fig. 5, panels A and C, compared with B and D as control). And in postnatal day 3 (P3) rat CNS, we found significant signals in hippocampal pyramidal neurons and cortical neurons (Fig. 5, panel E) in contrast to control (Fig. 5, panel F).

On the other hand, the developmental stage of neural cells in similar regions of E16 and P3 rat brain

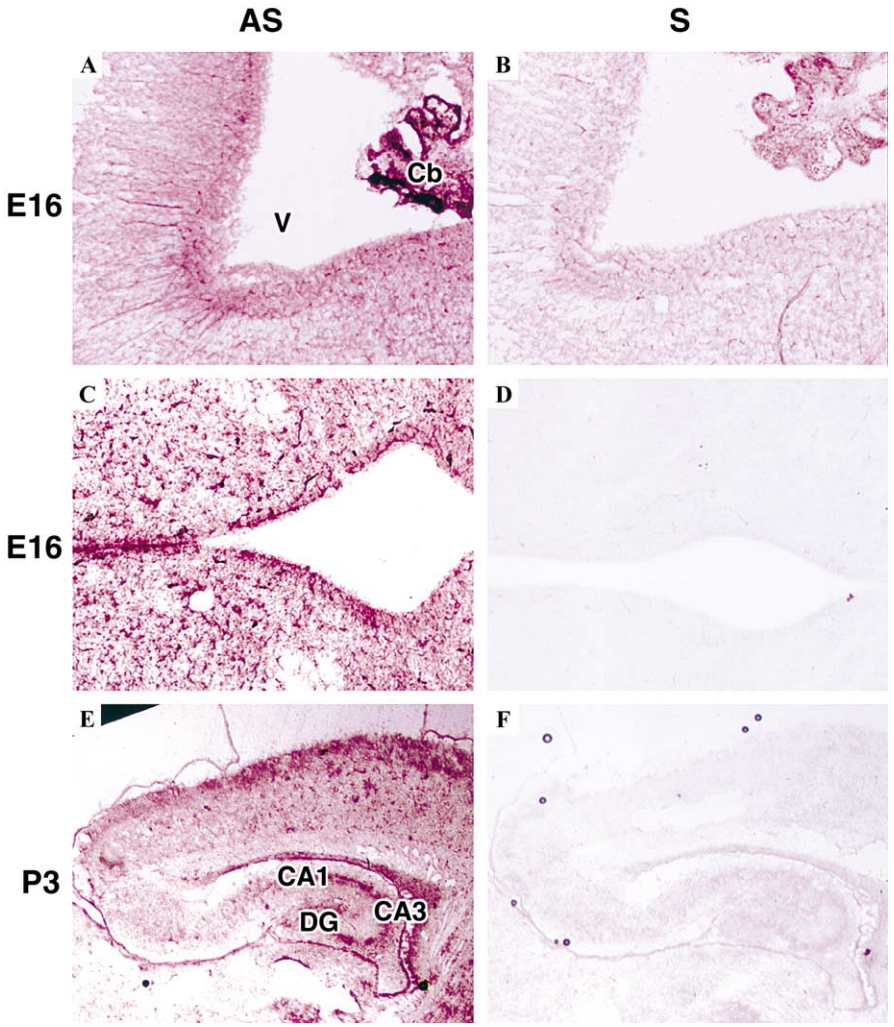

Fig. 5. In situ hybridization of *drb1* mRNA in rat brain. Using cDNA encoding rDrb1 as templates, sense and antisense RNA probes were prepared and hybridized with 20 μm frozen sections of normal embryonic or postnatal rat brain as described in Materials and methods. Panels A and B show sagittal section view of ventriculus, C and D show coronal section view of neural tube of embryonic day 16 (E16) rat brain. Panels E and F show sagittal section view of postnatal day 3 (P3) rat hippocampus. AS, antisense probe; S, sense probe; V, ventriculus; Cb, cerebellum, CA1, cornu ammonis 1; CA3, cornu ammonis 3; DG, dentate Gyrus.

was examined by immunohistochemical (IHC) analysis using monoclonal antibodies against Nestin, a marker protein for neural stem cells, and NeuN, for neural cells. In the E16 rat fetus, neural cells in the subventricular zone of brain and neural tube were significantly stained with anti-Nestin antibody (Fig. 6, panels A and D) and weakly stained with anti-NeuN antibody (Fig. 6, panels B and E). On the contrary, interneurons in P3 cerebral cortex and pyramidal cell layer in P3 hippocampus appeared to be significantly stained with anti-NeuN antibody and weakly with anti-Nestin antibody (Fig. 6, panels G and H, compared with panel I as control). IHC staining patterns by both antibodies and ISH signal patterns by *drb1* riboprobe were very similar. Especially, the signal pattern of ISH in CA region of P3 hippocampus seems to be identical with the distribution pattern of neurons that are stained with anti-NeuN antibody, not with that of glial cells. From these results, it is likely that rat *drb1* gene specifically expresses in neural cells of both fetal and postnatal brains.

In addition, RNA blot analysis previously indicated that peak of *drb1* gene expression in brain is E16, when immature neurons and neural stem cells are abundant [32]. Therefore, *drb1* gene would express in both immature neurons and neural stem cells.

Discussion

In the present study, we reported the isolation and characterization of a new member of neural RRM-type RNA-binding protein Drb1, abbreviated from developmentally regulated RNA-binding protein 1, among

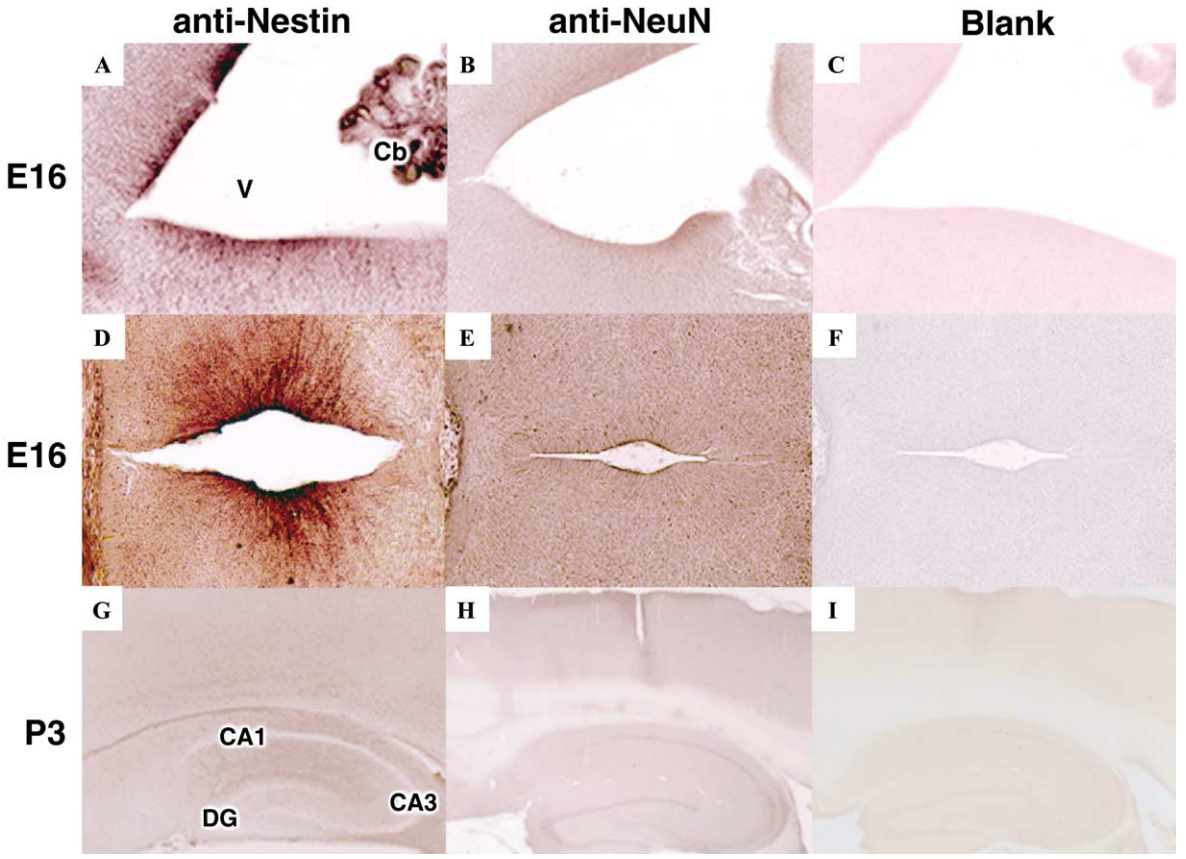

Fig. 6. Immunohistochemical staining of rat brain. Rat anti-Nestin or anti-NeuN is used for immunohistochemical staining. Blank indicates staining without primary antibody. Panels A and B show sagittal section view of lateral ventricle, D and E show coronal section view of neural tube of embryonic day 16 (E16) rat brain. Panels G and H show sagittal section view of postnatal day 3 (P3) rat hippocampus. V, Ventriculus; Cb, Cerebellum, CA1, Cornu ammonis 1; CA3, Cornu ammonis 3; DG, Dentate Gyrus.

rat, mouse, and human. The structures of these Drb1 proteins consist of four RRM-type RBDs and their RBDs are similar to those of another neural RNA-binding proteins, HuB and HuC. The *drb1* gene predominantly expresses in brain of rat fetus and its expression level is gradually decreased during brain development. The *drb1* gene also expressed in cultured neuronal progenitor cells and in fetal and postnatal neurons. The Drb1 protein localizes in cytoplasm of cultured cells and possesses binding preference on poly(C) RNA. From these results, Drb1 protein would play an important role during neurogenesis.

Structural domain organization of Drb1 protein is characterized by four RRM-type RBDs. As described previously, first and second domains display similarity to those of mouse HuB and HuC, and third domain is speculated as pseudo-RBD from its protein sequence. Previous reports have shown several RRM-type RNA-binding proteins containing four RBDs, i.e., poly(A) binding protein [33], polypyrimidinetract-binding protein [34], yeastnucleolar proteinnop 4 [35], and mouse nucleolin [36]. However, they have not been demonstrated as neural protein and other neural RNA binding proteins, i.e., Hu and Msi, do not possess four RBDs in their structural organization. Considering, above, Drb1 protein is a new family of neural RRM-type RNA-binding proteins.

The most significant character of Drb1 is the developmentally regulated gene expression during neurogenesis. As described previously, *m-msi1* gene demonstrates the highest expression in E12 brain [24] and *Hu* gene expression is observed in the postmitotic neuron [37]. In contrast, rat *drb1* gene expression is most abundant in E16 brain, and then gradually decreased along development (Fig. 2B). In addition, *drb1* gene expresses in neuronal progenitor cells, but reduced in differentiated neural cells (Fig. 2C), although Hu protein expresses in neurons and induces neural differentiation. Such a temporal regulation of *drb1* gene expression, actually before *Hu* and after *msi* expression, is very unique and strongly suggests a crucial role for neural development.

Physiological function of Drb1 is not precisely examined. However, it is revealed that Drb1 protein mainly localizes in cytoplasm of PC12 cell (Fig. 4) and specifically binds to poly(C) RNA in vitro (Fig. 3B).

Msi and Hu proteins predominantly localize in cytoplasm and it is proved that Hu has a shuttling property between nucleus and cytoplasm [38]. As occasional existence of Drb1 in nucleus is observed (data not shown), Drb1 might be like a shuttling protein as HuC or HuD [6,38]. Furthermore, a striking difference among Msi, Hu, and Drb1 proteins displays their RNA-binding property; Msi specifically binds to poly(G) (29), Hu to poly(U) [39], and Drb1 to poly(C) RNA. As Msi and Hu target to each specific mRNA species, for example, *ttk69* and *m-numb* mRNA to Msi (7,8), GAP-43, $p21^{(waf1)}$, and neurofilament M mRNA to Hu (20–22), it is likely that Drb1 also has its target mRNA and functions to control mRNA stability or translation efficiency like other RNA-binding proteins. Previously, another poly(C) RNA-binding protein, murine poly(C) binding protein (mCBP), has been reported [40]. Its RNA-binding property is similar to that of Drb1; however, mCBP belongs to K-homology (KH)-type RNA-binding protein [41]; therefore, its target RNA species would be different from that of Drb1 protein.

In conclusion, we identified mammalian Drb1 proteins as a new family of neural RRM-type RNA-binding proteins. Its distinct spatiotemporal expression pattern and poly(C)-specific RNA binding property are totally different from those of other neural RNA-binding proteins such as Msi and Hu, and strongly suggest its unique role for neural development. The exact physiological function of Drb1 protein should be further investigated for understanding the molecular mechanism of neural development.

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