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Nucleotide sequences of two embryonic drebrins, developmentally regulated brain proteins, and developmental change in their mRNAs

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Drebrins are developmentally regulated proteins found in chicken brain and are classified into two forms of the embryonic type (E1 and E2) and one form of the adult type (A). Although the time courses of their appearance are different from each other, the structures of the 3 forms are closely related. Two kinds of drebrin cDNA, designated gDcw6 and gDcw17, were isolated from the cDNA library of the chicken embryo and their nucleotide sequences were determined. Their sequences were entirely identical except for a deletion of an internal 129-nucleotide sequence, and the gDcw17 insert contained an open reading frame capable of encoding 607 amino acids. These cDNAs seemed to correspond to two embryonic forms of drebrin mRNAs. The predicted drebrin molecules are highly hydrophilic and have proline-rich sequences and long stretches of glutamate in the carboxyl-terminal region. RNA dot-blot analysis using the drebrin cDNA as a probe demonstrated that the amounts of drebrin mRNAs were also developmentally regulated as those of drebrins. Southern blot analysis showed that the chicken genome has a single copy of the drebrin gene per haploid complement. These findings suggest that the multiple forms of drebrins result from alternative splicing of the single drebrin gene during neural development.

INTRODUCTION

The differentiation and further development of neurons must involve particular sets of the gene expression arranged temporally and spatially. Such genes are likely to encode molecules that are concerned with the processes of neural development, such as differentiation and migration of neurons, elongation of axons and dendrites and the formation of specific connections.

We previously demonstrated that the amounts of 3 proteins, named drebrins E1, E2 (embryonic types) and A (adult type), were developmentally regulated in chicken brain[20,21]. The molecular weights of drebrins E1, E2 and A measured by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) are 95, 100 and 110 kDa, respectively, and their isoelectric points are about 4.5. Drebrin E1 first appears as soon as neuronal cells have finished the final mitosis. While neuronal cells are extending their axons and dendrites, drebrin E2 takes the place of drebrin E1 and then declines as drebrin A emerges in parallel with further maturation of neurons. Finally, drebrin A is shown to be present throughout the remaining life span. Immunocytochemical studies have shown that all 3 drebrins are localized in the cytoplasm of neurons, drebrin A being especially localized in certain types of the dendrites[22]. It is proposed that drebrins E1, E2 and A might play some role in cell migration, extension of neuronal processes and plasticity of dendrites, respectively[21,22]. Since the time courses of appearance of these 3 drebrin molecules are completely different from each other, expression of each drebrin gene might be independently regulated during neural development. However, it has been shown that these structures are closely related to each other by analysis of peptide mapping[20] and cross-reactivity of monoclonal antibodies against

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drebrins[21]. These findings led us to conclude that the genes encoding these 3 molecules seemed to be closely related to each other. In order to elucidate their molecular structures and genetic relationship to each other, we attempted to isolate and characterize the cDNAs encoding drebrins. In a previous paper[23], we reported the isolation of cDNA clones encoding part of the drebrin molecule. In the present study, we obtained two types of cDNA clones which covered the whole stretch of drebrins, and determined their nucleotide sequences. Using these different cDNAs as probes, we investigated the genes encoding drebrins and their expression during neural development.

MATERIALS AND METHODS

cDNA cloning

A cDNA library of 10-day-old chicken embryo tissues constructed with λgt11 (Clonetech Labs), was used for screening. Approximately 1×10^6 recombinant phages were plated with *E. coli* LE392 and transferred to a nylon membrane (Hybond-N, Amersham). Plaque hybridization was performed using a previously isolated cDNA fragment, gDcw1 (see ref. 23), as a probe. The probe was labeled with ^{32}P using nick-translation kits (Nippon Gene and Amersham).

DNA sequencing

The cDNAs encoding drebrins were subcloned into pUC119, and then the subclones having various lengths of cDNA were prepared by a kilo-sequencing method[24]. The intact clones and their deleted mutants, which overlapped each other and permitted to duplicate the sequence determinations, were sequenced by the dideoxy chain-termination method of Sanger et al.[19] using denatured double-stranded DNAs as templates[7]. A deoxy-7-deaza sequencing kit (Takara Shuzo) was used according to the conditions recommended by the supplier. The orientation and the reading frame of these cDNAs were determined by immunoblot analysis of the fusion proteins[25] of these clones and a series of their deleted mutants.

RNA dot-blot analysis

The optic tectum was removed from chick embryos (4–20 days of incubation) and newly hatched chicks (White Leghorn) and then quickly frozen on dry ice. Total RNA was extracted from the optic tectum (10–100 mg wet wt./sample) by the guanidium thiocyanate method[4] and precipitation with lithium chloride using an RNA extraction kit (Amersham). Extracted RNA was denatured with formaldehyde and then 10 μg of RNA was spotted onto nylon membrane (GeneScreen*Plus*, New England Nuclear) using a Bio-Dot apparatus (Bio-Rad). Hybridization was performed as described below. The relative intensities of hybridized probes on autoradiograms were measured with a laser densitometer (Chromoscan 3, Joyce-Loebl).

Southern blot analysis

For Southern blot analysis, genomic DNA was prepared from adult chicken blood corpuscles, digested with restriction endonucleases, and electrophoresed (5 μg per lane) in a 0.8% agarose gel. As previously described[10], the nucleic acids were transferred to nylon membrane and hybridized with nick-translated or multi-prime labeling (Amersham) probes in a solution containing 0.02% each of Ficoll, bovine serum albumin and polyvinylpyrrolidone, 6 × SSC, 1% SDS and 100 μg/ml of denatured salmon sperm DNA at 68 °C for 16–24 h. The nylon membranes were washed twice in 0.1 × SSC containing 1% SDS at 42 °C. Hybridized probes were detected by autoradiography at −70 °C using an intensifying screen.

Generation of antiserum against the synthetic peptide

A peptide consisting of 25 amino acid residues deduced from the cDNA was produced using a solid-phase peptide synthesizer (430A, Applied Biosystems). Ten mg of the synthetic peptide was conjugated with 5 mg of porcine thyroglobulin using glutaraldehyde, and then emulsified with Freund's complete adjuvant. A rabbit (New Zealand White) was injected with the emulsion (ca. 50 μg of conjugates) into each side of the inguinal lymph node as previously described[6,17]. A booster hypodermic injection (ca. 100 μg of conjugates) was then administered 3 weeks after the initial injection. Collection of antiserum was started at the same time. Antiserum was purified with an affinity column (Affigel-10, Bio-Rad) coupled with the synthetic peptide before use for immunoblot analysis.

Gel electrophoresis and immunoblot analysis

The prodecures used for immunoblot analysis have been described in detail previously[5,21]. In brief, SDS–PAGE and two-dimensional gel electrophoresis were performed by the methods of Laemmli[14] and O'Farrell[18], respectively. After the electrophoresis, the proteins were transferred to nitrocellulose membrane sheets (Toyo Roshi) by electroblotting. The sheets were blocked with Tris-buffered saline, pH 7.4, containing 3% bovine serum albumin, followed by incubation with the antibodies. After washing, the sheets were further incubated with horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG. The blots were developed using 0.05% 4-chloro-1-naphthol and 0.01% hydrogen peroxide.

RESULTS

Isolation of drebrin cDNAs

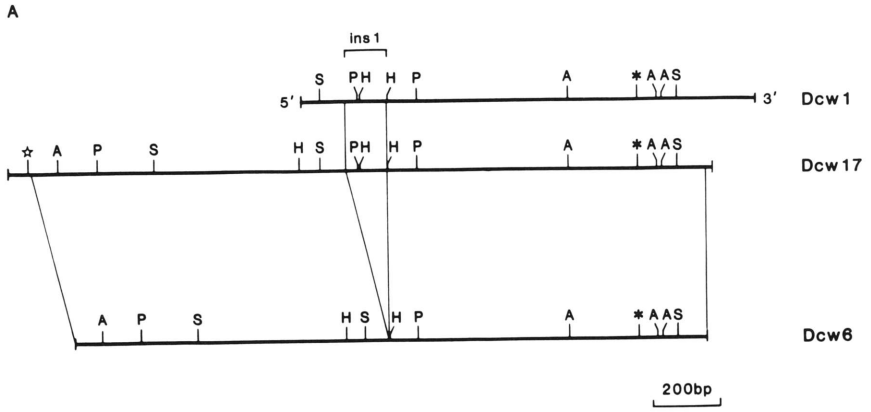
As described in a previous paper[23], one cDNA clone, designated gDcw1 (Fig. 1A), was isolated from the chicken embryo cDNA library by immunological screening with anti-drebrin antiserum. The fusion protein of gDcw1 reacted with 3 of 5 individual monoclonal antibodies for drebrins. The length of the gDcw1 insert was about half of that of drebrin mRNA (ca. 2.7 kb), detected by Northern blot analysis[23]. These results demonstrate that gDcw1 does not cover the entire cDNAs for drebrins. To isolate the cDNAs encoding the full-length drebrin sequence, gDcw1 was employed as a probe to rescreen the same library. Consequently, 74 positive clones were isolated. These cDNA inserts were strongly hybridized to the probe, but their digestion patterns produced by restriction endonucleases were divided into two groups. One had ca. 0.2 kb of the internal *Pst* I fragment, but the other did not (Fig. 1A). For further characterization of these cDNAs, gDcw17 and gDcw6, each of which was longer than the other clones of each groups, were subcloned into a plasmid, pUC119 (designated pDcw17 and pDcw6, respectively).

Sequences of the cDNAs and amino acid sequences of drebrins

The nucleotide sequences of pDcw6 (1889 bp) and pDcw17 (2102 bp) were separately determined. The complete nucleotide sequence and the deduced amino acid sequence of pDcw17 are shown in Fig. 1B. The pDcw6 insert covered the sequence from position 68 to position 2081 in the pDcw17 insert. Within this region, both sequences of pDcw6 and 17 were entirely identical except that an internal 129-nucleotide sequence (positions 1005–1133), designated ins1, was deleted in pDcw6. Both of the nucleotide sequences were rich in G+C (ca. 68%). The characteristic sequence, GAGGAGGAG, including sequences in which one or two substitutions occur, was repeated 26 times throughout the entire regions of the cDNAs. In pDcw17, nucleotide sequences around the presumed initiation codons satisfied Kozak's criteria[12], supporting the possibility that the first ATG (position 60) was the translation initiation site. The pDcw6 insert was 8 nucleotides shorter than the site in the pDcw17 insert. In-frame termination codons (TAG) were found at position 1822. Therefore, the pDcw17 insert contains an open reading frame capable of encoding 607 amino acid residues. The sequence of ins1 corresponds to 43 amino acid residues. The predicted drebrin molecules from pDcw6 and pDcw17 were estimated to have molecular weights of 62,165 and 66,553, respectively. Prominent features of the amino acid sequences of drebrins were an abundance of proline and stretches of 2–9 glutamate residues in the carboxyl-terminal region. The calculated net charge of both translates was −66.5, being consistent with the fact that drebrins are acidic proteins, as reported previously[20].

The hydropathy profiles of the deduced amino acid sequences of drebrins were analyzed by the method of Kyte and Doolittle[13] (Fig. 2). The predicted drebrin molecules were highly hydrophilic. Although a small hydrophobic region was observed at residues 42–120, the degree of hydrophobicity was not great for constitution of the membrane-spanning regions[11]. The grand averages of hydropathy values (−0.53 and −0.56, respectively) are significantly smaller than the mean for soluble proteins (−0.41)[13]. These results are consistent with the facts that drebrin molecules were enriched in the soluble fraction[20] and that immunoreactivity with anti-drebrin antibodies was observed in the cytoplasm of neurons[21,22].

In order to compare the nature of the cDNA translate and that of the drebrins themselves, the fusion protein of pDcw17 was analyzed by gel electrophore-



B

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   1 CCGAGACACTGCCGCAGCGCCCGGTCCCTCCGGTCCCCCGGCAACATCCCGACCCCGCC ATG GCT GGC GTC GGC TTC GCG GCG CAC CGC
                                                                  Met-Ala-Gly-Val-Gly-Phe-Ala-Ala-His-Arg  10

  90 CTG GAG CTG CTC GCC TCC TAC CAG GAC GTG ATC GGC GAG GAC AGC CCC ACC GAC TGG GCC CTC TAC ACG TAT GAG
     Leu-Glu-Leu-Leu-Ala-Ser-Tyr-Gln-Asp-Val-Ile-Gly-Glu-Asp-Ser-Pro-Thr-Asp-Trp-Ala-Leu-Tyr-Thr-Tyr-Glu  35

 165 GAT GGC TCT GAT GAC CTG AAG CTG GCA GCA TCA GGA GGA GGG GGT CTG CTG GAG CTC TCT GGG CAC TTT GAG ATC
     Asp-Gly-Ser-Asp-Asp-Leu-Lys-Leu-Ala-Ala-Ser-Gly-Gly-Gly-Gly-Leu-Leu-Glu-Leu-Ser-Gly-His-Phe-Glu-Ile  60

 240 CAG AAG GTG ATG TAC GGC TTC TGC AGC GTC AAG GAG CCC CAG GCC GTG CTC CCC AAA TAT GTC CTT GTC AAT TGG
     Gln-Lys-Val-Met-Tyr-Gly-Phe-Cys-Ser-Val-Lys-Glu-Pro-Gln-Ala-Val-Leu-Pro-Lys-Tyr-Val-Leu-Val-Asn-Trp  85

 315 GTG GGT GAG GAT GTG CCT GAC GCC CGC AAA TGT GCC TGT GCC AGC CAC GTG GCC AAG ATC GCA GAG TTC TTC CAG
     Val-Gly-Glu-Asp-Val-Pro-Asp-Ala-Arg-Lys-Cys-Ala-Cys-Ala-Ser-His-Val-Ala-Lys-Ile-Ala-Glu-Phe-Phe-Gln 110

 390 GGT GTG GAT GTT ATC GTC AAT GCC AGC AGC GTG GAG GAC ATT GAC CCG GGG GCC ATC GGG CAG CGG CTC TCC AAC
     Gly-Val-Asp-Val-Ile-Val-Asn-Ala-Ser-Ser-Val-Glu-Asp-Ile-Asp-Pro-Gly-Ala-Ile-Gly-Gln-Arg-Leu-Ser-Asn 135

 465 GGG CTG GCG CGC GTC TCC AGC CCC GTG CTG CAC CGC CTG CGG CTG CGT GAG GAC GAG AAT GCC GAG CCC GTG GGC
     Gly-Leu-Ala-Arg-Val-Ser-Ser-Pro-Val-Leu-His-Arg-Leu-Arg-Leu-Arg-Glu-Asp-Glu-Asn-Ala-Glu-Pro-Val-Gly 160

 540 ACG ACC TAC CAG AAA ACC GAC GCC ACC GTG GAG ATG AAG CGG CTC AAC CGG GAG CAG TTC TGG GAA CAA GCC AAG
     Thr-Thr-Tyr-Gln-Lys-Thr-Asp-Ala-Thr-Val-Glu-Met-Lys-Arg-Leu-Asn-Arg-Glu-Gln-Phe-Trp-Glu-Gln-Ala-Lys 185

 615 AAA GAG GAG GAG TTG CGC AAG GAG GAG GAG AGG AAA AAG GCG TTG GAT GCG CGG CTG CGG TTC GAG CAG GAG CGC
     Lys-Glu-Glu-Glu-Leu-Arg-Lys-Glu-Glu-Glu-Arg-Lys-Lys-Ala-Leu-Asp-Ala-Arg-Leu-Arg-Phe-Glu-Gln-Glu-Arg 210

 690 ATG GAG CAG GAG CGG CTG GAG CAG GAG GAG CGC GAG AGG CGC TAC CGG GAG CGC GAG GAG CAG ATC GAG GAG CAC
     Met-Glu-Gln-Glu-Arg-Leu-Glu-Gln-Glu-Glu-Arg-Glu-Arg-Arg-Tyr-Arg-Glu-Arg-Glu-Glu-Gln-Ile-Glu-Glu-His 235

 765 AGG AGG AAG CAG CAG AGC ATG GAG GCG GAG GAG GCC CGG CAG CGC CTG AAG GAG CAG TCC ATC TTT GGG GAG CAG
     Arg-Arg-Lys-Gln-Gln-Ser-Met-Glu-Ala-Glu-Glu-Ala-Arg-Gln-Arg-Leu-Lys-Glu-Gln-Ser-Ile-Phe-Gly-Glu-Gln 260

 840 CAA GAG GAG GAC GAC AGG CAG CAG CTC CGG AAA TCA GAG TCA GAG GTG GAG GAG GCC GCT GCC ATC ATT GCC CAG
     Gln-Glu-Glu-Asp-Asp-Arg-Gln-Gln-Leu-Arg-Lys-Ser-Glu-Ser-Glu-Val-Glu-Glu-Ala-Ala-Ala-Ile-Ile-Ala-Gln 285

 915 CGA CCT GAC AAC CCC CGG GAG TTC TTC AAG CAG CAG GAG CGG GTG GCA TCG GGC AGC GGC GAC GCC ATC TCG CCG
     Arg-Pro-Asp-Asn-Pro-Arg-Glu-Phe-Phe-Lys-Gln-Gln-Glu-Arg-Val-Ala-Ser-Gly-Ser-Gly-Asp-Ala-Ile-Ser-Pro 310

 990 GGC AGC CAT AGG ACA GGC AGC CAG TCC GAC TAC CGA AAG GTT TCG GCA GCG GGC TGC AGC CCC TGC GAG TCC AGC
     Gly-Ser-His-Arg-Thr Gly-Ser-Gln-Ser-Asp-Tyr-Arg-Lys-Val-Ser-Ala-Ala-Gly-Cys-Ser-Pro-Cys-Glu-Ser-Ser 335

1065 CCG GCC TCC ACG CCG CTG GGC GAG CAG CGC ACC CGC GCC CCG GCC GAA GAG ACG CCG GCA ACG CCC AAA GAC TCC
     Pro-Ala-Ser-Thr-Pro-Leu-Gly-Glu-Gln-Arg-Thr-Arg-Ala-Pro-Ala-Glu-Glu-Thr-Pro-Ala-Thr-Pro-Lys Asp-Ser 360

1140 CCC AGC CCC AGC ACC CAG GTG GCA GAG CCG GCA GCG ACT GAG CAG CAC TGG CCC TTC CCT GGG CCT GAA GAC AAA
     Pro-Ser-Pro-Ser-Thr-Gln-Val-Ala-Glu-Pro-Ala-Ala-Thr-Glu-Gln-His-Trp-Pro-Phe-Pro-Gly-Pro-Glu-Asp-Lys 385

1215 GCT GCA GAG CCG CCG GGG GAC GAG CCC GAC CCC GAC CCC AGG CCG GCG TGG ACA GCG GGG GCT GAC GTG CTG GGG
     Ala-Ala-Glu-Pro-Pro-Gly-Asp-Glu-Pro-Asp-Pro-Asp-Pro-Arg-Pro-Ala-Trp-Thr-Ala-Gly-Ala-Asp-Val-Leu-Gly 410

1290 GAC CTG GTG ACC CTG GAG CCC TCC GAG CCA TCC CCA GCG CCC GCT GCG TCC GAA CCC CAG CCC GTG GAG ACA CCC
     Asp-Leu-Val-Thr-Leu-Glu-Pro-Ser-Glu-Pro-Ser-Pro-Ala-Pro-Ala-Ala-Ser-Glu-Pro-Gln-Pro-Val-Glu-Thr-Pro 435

1365 GGT GTG GCC GAG CCC CTC ATC GAG CTG TGG CAG AGT GAT GGC GCG GCC CCC GCT GCC ACC AGC ACC TGG CCC CTG
     Gly-Val-Ala-Glu-Pro-Leu-Ile-Glu-Leu-Trp-Gln-Ser-Asp-Gly-Ala-Ala-Pro-Ala-Ala-Thr-Ser-Thr-Trp-Pro-Leu 460

1440 CCC GAC ACC CCC GCG GGA CCA CCG GTC CCC CCC GAG GAG GGC ACG CTG CTG GGC CTG GAC GAG CTG CCC GAG CCC
     Pro-Asp-Thr-Pro-Ala-Gly-Pro-Pro-Val-Pro-Pro-Glu-Glu-Gly-Thr-Leu-Leu-Gly-Leu-Asp-Glu-Leu-Pro-Glu-Pro 485

1515 CCC GCC ACC TTC TGT GAC GCG GAG CAG CAC GAG GAG GTG GAA GAA GAA GAA GAG GAG GAG GAG GAG GCC ACA GCG
     Pro-Ala-Thr-Phe-Cys-Asp-Ala-Glu-Gln-His-Glu-Glu-Val-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Ala-Thr-Ala 510

1590 GGT GAG CCC CAT CCC ACA GGG CTG GGC TAC CAG GAG GGC TAC CAA GAA GGC CCC GAG GTG CCC CCC ATC ACC AAT
     Gly-Glu-Pro-His-Pro-Thr-Gly-Leu-Gly-Tyr-Gln-Glu-Gly-Tyr-Gln-Glu-Gly-Pro-Glu-Val-Pro-Pro-Ile-Thr-Asn 535

1665 GGG GAG ATG GGC CCC AAG GAC GGC ACG GCG GGC CGT GGC GAG CAG GCC AGC GAG GGC TAC TTC AGC CAG TCC CAG
     Gly-Glu-Met-Gly-Pro-Lys-Asp-Gly-Thr-Ala-Gly-Arg-Gly Glu-Gln-Ala-Ser-Glu-Gly-Tyr-Phe-Ser-Gln-Ser-Glu 560

1740 GAG GAG GAG GCC CCG CCG CCC GAG GAG CCG TCG GCC AAA GCC CCG CAG CCC GTC TTC TAC AAC AAG CCG CCA GAG
     Glu-Glu-Glu-Ala-Pro-Pro-Pro-Glu-Glu-Pro-Ser-Ala-Lys-Ala-Pro-Gln-Pro-Val-Phe-Tyr-Asn-Lys-Pro-Pro-Glu 585

1815 ATC GAC ATC ACG TGC TGG GAC ACG GAC CCG CTG CCC GAG GAG GAG GAG AGC TTC GGG GGC GGC CTG TAG GCCCAGC
     Ile-Asp-Ile-Thr-Cys-Trp-Asp-Thr-Asp-Pro-Leu-Pro-Glu-Glu-Glu-Glu-Ser-Phe-Gly-Gly-Gly-Leu          607

1891 GCAGCCTGGAGGCCCCGCCGGCGCGGGGTGTGAGCGGCCGTGGCCGGGGCCCCCTCCCCGCTGGGGCCCCGCAGGATGGGAGCCGGGGGGGCCGCCCCG

1990 CAGCACCCCGGGGTGCCTTTTACAGCCCCCCCATCTCTTTCTCTTTTATAAGTTGTCGGTAGGAGACGTGTCAGCCCTCTCCCCCCTGCCCCCCCCTG

2089 CCCTGTGAACCCGG
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Fig. 1. A: restriction maps of drebrin cDNAs. Restriction sites in gDcw1 of Shirao et al.[23] and other drebrin cDNAs, gDcw6 and gDcw17 are shown for *Apa* I (A), *Hin*fI (H), *Pst* I (P) and *Sma* I (S). The putative initiation codon (☆) and the termination codon (*) are also indicated. B: nucleotide sequence of pDcw17 and deduced amino acid sequence of drebrin. The start and the end of pDcw6 are indicated by the arrows above and below the sequence, respectively. The 129-nucleotide sequence deleted in pDcw6, ins1 is boxed. The underlinings indicate the putative initiation codon as well as the termination codon.

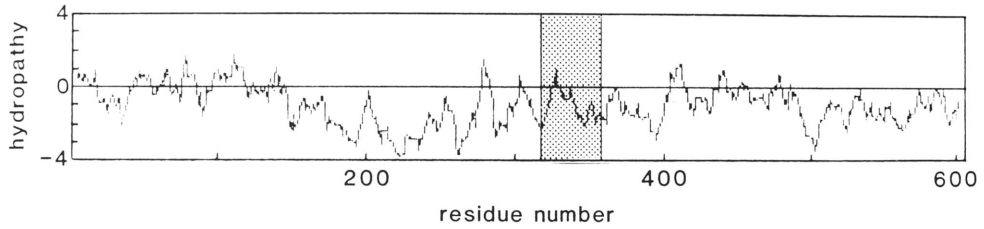

Fig. 2. Hydropathy profile of drebrin. The hydropathy profile was obtained by the method of Kyte and Doolittle[13] with a span setting of 9 residues. The region of the amino acid sequence deduced from ins1 is indicated by the dotted area.

sis and immunoblot analysis (Fig. 3). The product of pDcw17, which was transcribed and translated in *E. coli* JM103, showed reaction with anti-drebrin antibodies and migrated near the spots of drebrins on two-dimensional gel electrophoresis; the estimated molecular weight and isoelectric point were ca. 110 kDa and ca. 4.5, respectively. Therefore, the product of pDcw17 showed similarity to drebrins, although this protein fused with the product including 23 amino acids of β-galactosidase and 21 amino acids translated from the 5′ non-coding region; calculated molecular weight was 4844, at the amino-terminus.

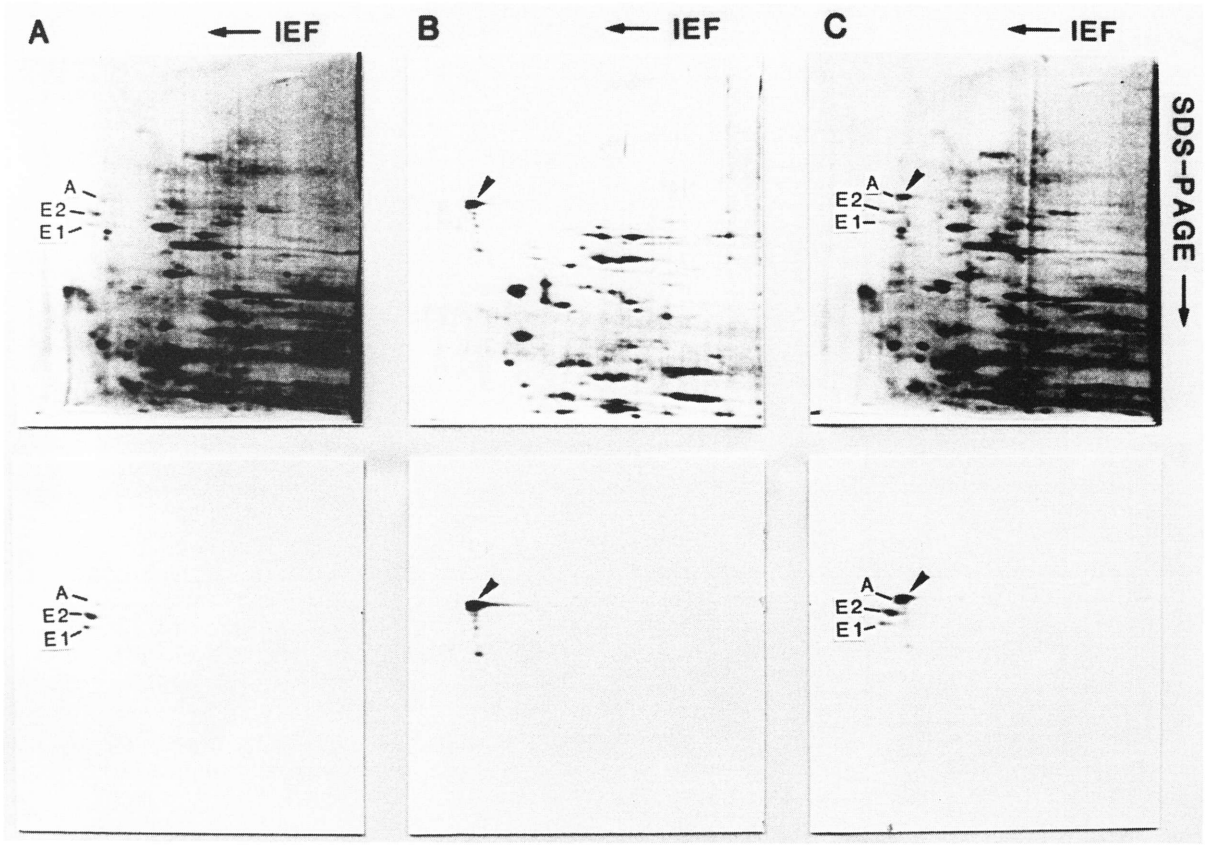

Fig. 3. Identification of the fusion protein of drebrin cDNA on two-dimensional gel electrophoresis. Homogenate of the brain of a 12-day chick embryo (A) and extract of the transformant of pDcw17 after induction with isopropyl-β-D-thiogalactopyranoside (10 mM) (B) were electrophoresed by the method of O'Farrell[18]. In C, the homogenate shown in A was coelectrophoresed with the extract shown in B. One (upper panel) was stained with silver and the other (lower panel) was blotted for staining with anti-drebrin monoclonal antibody, M2F6 (ref. 21). The arrow heads indicate the fusion protein of pDcw17.

Immunoblot analysis of antiserum against the synthetic peptide

In order to determine the drebrin to which each cDNA corresponded, we raised an antiserum against the synthetic peptide, which covered part of the deduced amino acid sequence (residues 334–358) of ins1, designated pep1, and performed immunoblot analysis of this antiserum. As shown in Fig. 4, anti-pep1 antiserum specifically reacted with drebrins E2 and A, but not E1. This finding suggested that drebrins E2 and A had the sequence of pep1, whereas E1 did not. Therefore, pDcw6, which did not contain the sequence of ins1, seemed to correspond to drebrin E1.

Southern blot analysis of the drebrin gene

In order to determine the organization of the drebrin gene in the chicken genome, genomic DNA from chicken blood corpuscles was digested with several restriction endonucleases and analyzed by Southern blot hybridization using drebrin cDNAs as probes. One strongly hybridized band and one additional weak band were detected with the gDcw1 insert in each digest (Fig. 5a). These hybridization patterns were the same irrespective of whether pDcw6 or pDcw17 were used as probes. When the 5′ *Pst* I fragments of gDcw 1 were used as probes, only one *Bam*HI DNA fragment (6.0 kb) could be detected (Fig. 5b). Furthermore, only one *Hin*dIII DNA fragment (10.5 kb) was hybridized with the 3′ *Pst* I fragment of the cDNA (Fig. 5c). These results suggested that each drebrin was not a member of a subfamily of closely related genes, but a product from a single gene. Furthermore, the intensities of the hybridized bands were consistent with a single drebrin gene per haploid chicken genome when compared with the cDNA used as a standard (data not shown).

Developmental change in the level of drebrin mRNAs

Previously we analyzed proteins in the chick optic tectum by two-dimensional gel electrophoresis and

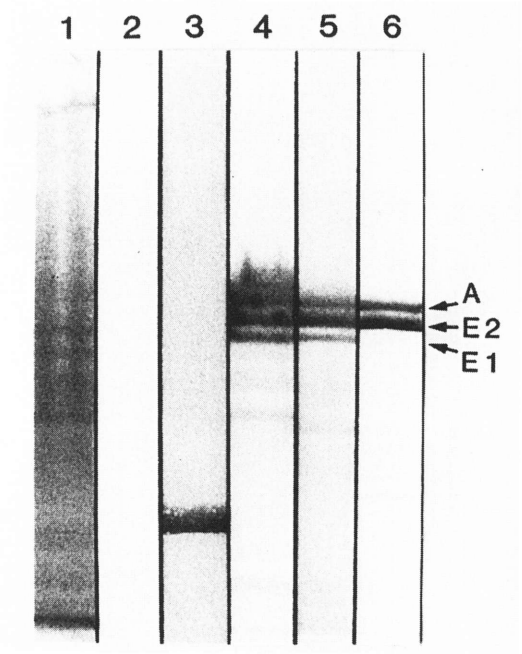

Fig. 4. Immunoblot analysis of anti-pep1 antiserum. Homogenate of brain of a 12-day chick embryo was loaded onto SDS polyacrylamide gel and blotted for staining with several antibodies. Lanes: 1, protein staining with amide black; 2, rabbit preimmune serum; 3, anti-β-tubulin monoclonal antibody (152H6; Obata, unpublished); 4, anti-drebrin antiserum (RS6, ref. 21); 5, anti-drebrin monoclonal antibody (M2F6, ref. 21); 6, anti-pep1 antiserum.

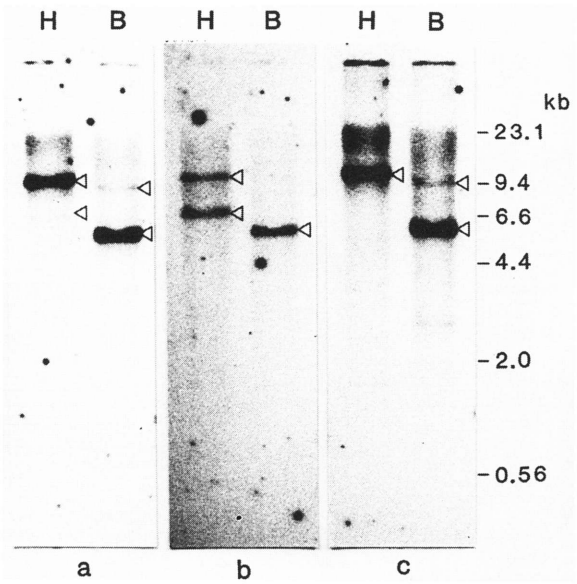

Fig. 5. Southern blot analysis of the drebrin gene. Genomic DNA prepared from chicken blood corpuscles was digested with restriction endonuclease *Hin*dIII (H) or *Bam*HI (B) and electrophoresed on 0.8% agarose gel, followed by blotting onto nylon membrane. The blotted membrane was separately hybridized with ^{32}P-labeled gDcw1 insert (a), 3′ *Pst* I fragment of gDcw1 (c) and the remaining 5′ *Pst* I fragments of gDcw1 (b). Conditions of hybridization are described in Materials and Methods. The hybridized bands are indicated by the clear triangles.

demonstrated that the amounts of drebrins E1, E2 and A changed during development[20]. In order to determine whether such changes in the amounts of drebrins were regulated at the mRNA levels, we made a RNA dot-blot analysis of the developmental changes in drebrin mRNA levels in the optic tectum using the pDcw17 insert as a probe. The pDcw17 insert did not differentiate between mRNAs for 3 drebrin molecules and the hybridized signal would represent the sum of drebrin mRNAs. The densitometric quantification is graphically represented in Fig. 6. No noticeable change in the level of RNA hybridized with the β-actin probe was observed throughout the developmental stages we investigated. On the other hand, when the pDcw17 insert was used as a probe, the level of hybridized RNA changed significantly during development. Transcripts of the drebrin gene could be detected on day 4 and slightly increased until day 6. Thereafter, the level of hybridized signal gradually decreased and finally became uniformly low from day 16 up through and including the postnatal period (postnatal day 7). The levels on day 6 were about 7-fold higher than those on day 18. The developmental change in the level of drebrin mRNAs was consistent with that in the amounts of the 3 drebrins; the abundance of drebrins is high in the early embryonic brain and is gradually reduced as development proceeds[21]. It is therefore suggested that developmental changes in the amounts of drebrins are controlled largely at the levels of their mRNAs.

DISCUSSION

We have investigated drebrins, proteins which appear transiently at certain stages of development in the chicken brain, using biochemical[20] and immunochemical[21,22] techniques. In the present study, cDNA clones encoding 2 of the 3 drebrins, were isolated and sequenced to show the primary structures of drebrins and to understand the difference between their sequences. We confirmed that two forms of drebrins were entirely identical except for the insertion or deletion of a 43-amino acid sequence translated from ins1. Immunoblot analysis with the antiserum against pep1, the sequence of which was deduced from the appropriate part of ins1, showed that the pDcw6 insert corresponds to mRNA of drebrin E1. Both drebrins E2 and A have the sequence of pep1, and it is not yet determined to which mRNA of drebrin E2 or A the pDcw17 insert corresponds. However, it is reasonable to assume that pDcw17 encoded the mRNA of drebrin E2, because the amount of drebrin A was much lower than those of the two embryonic forms in 10-day chick embryo[20,21], and only two kinds

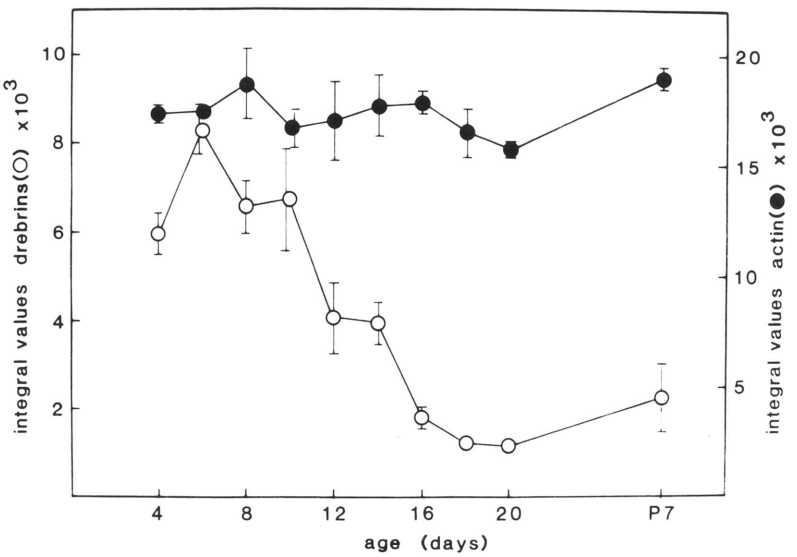

Fig. 6. Expression of drebrin mRNAs during development. Total RNA was extracted from chick optic tecta at the indicated embryonic and postnatal ages. Ten micrograms of total RNA was probed with β-actin cDNA (Oncor; solid circle) or pDcw17 (clear circle). The relative intensities of the hybridized signals were quantified by densitometry and graphically represented as means ± S.E.M. (n = 3).

of cDNA were isolated from a cDNA library of 10-day chick embryo. If the individual mRNA of drebrin A exists, the cDNA encoding drebrin A possibly has another sequence inserted to pDcw17, because the molecular weight of drebrin A is 10 kDa higher than that of drebrin E2.

The amino acid sequences presented in this study support our earlier conclusion that drebrins are soluble proteins rich in negatively charged residues. The calculated molecular weights of the drebrins (62 and 67 kDa) are about two-thirds of the molecular weights obtained by SDS–PAGE (ca. 100 kDa). Although the initiation at the ATG selected here has not been confirmed experimentally, the product of pDcw17 which fused with only 23 amino acids of β-galactosidase at the amino-terminus, closely resembled drebrins with regard to mobility on SDS polyacrylamide gel. Therefore, the discrepancy between the molecular weights of drebrins predicted from the cDNA sequences and the apparent molecular weights estimated by SDS–PAGE seems to have resulted from the anomalous migration of drebrins on SDS polyacrylamide gel, i.e., drebrins seem not to bind a constant weight ratio of SDS due to the unusual features of their amino acid sequences, such as a paucity of hydrophobic regions and an abundance of negatively charged residues. A similar discrepancy in the molecular weights of other proteins has also been reported. For example, the molecular weight of GAP-43 calculated from the cDNA sequence was about half of the molecular weight estimated from SDS–PAGE, due to the lack of a hydrophobic region in GAP-43[1]. Also, Napolitano et al.[16] have reported that over-estimation of the molecular weight of the middle subunit of neurofilament by SDS–PAGE was due to the presence of long stretches of glutamate in the carboxyl-terminal extension.

Elucidation of the cDNA sequence for drebrins allowed comparison of the amino acid sequences of drebrins with those of other known proteins (NBRF protein sequence data bank). Using Kanehisa's program[9], drebrins showed significant similarity only with the collagen α1(I) chain[8] (3.18 S.D. units). Several identical sequences of 2 or 3 residues can be found, such as Pro-Pro, Gly-Glu, Pro-Gly-Pro and Gly-Pro-Pro. Nevertheless, it is clear that these proteins are quite distinct from each other. The presence of glycine is essential in every third position in the collagen molecule in order to maintain the triple helical structure. This arrangement is not found anywhere in the drebrins.

We have previously demonstrated that the time courses of appearance of drebrins were distinct among the three forms, and that the tissue distribution of each drebrin was also different from that of the others[20,21]. In immunohistochemistry, the premigratory granule cells in the cerebellum and the immature tectobulbar tract neurons in the optic tectum were stained with anti-drebrin antibodies but not stained with anti-pep1 antiserum (data not shown). Therefore, the differences in the primary structures of the drebrins among the three molecules should reflect the differences in their manner of expression and biological functions. Southern blot analysis showed that two types of drebrin mRNA, corresponding to pDcw6 and pDcw17, were transcribed from a single gene. This finding suggests that these two drebrin mRNAs would result from alternative splicing of the drebrin gene. This splicing site selection seems to be regulated developmentally and spatially. It is possible that *trans* factors required for alternative splice site selection[2,3,15] may regulate the expression of each drebrin molecule. Isolation of the drebrin gene and investigation of its expression in cultured cells or in vivo would lead to an understanding of the mechanism regulating the expression of each drebrin during development.

Multiple substances reacting with anti-drebrin antibody were also detected in the brains of mammals, including rat, guinea pig[21] and rabbit (Onoda, unpublished data). A preliminary study has shown that the drebrin-like substances in the rat were slightly larger than drebrins in the chicken, and that the heterogeneity of the molecules was developmentally regulated, as was the case in the chicken. Although the functions of drebrins in the nervous system are still not understood, their wide interspecific distribution suggests that these molecules play a general role in development of the nervous system.

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