Allelic Gene Regulation of Pcdh-α and Pcdh-γ Clusters Involving Both Monoallelic and Biallelic Expression in Single Purkinje Cells*‡

Received for publication, June 14, 2006, and in revised form, July 25, 2006. Published, JBC Papers in Press, August 7, 2006, DOI 10.1074/jbc.M605677200

Ryosuke Kaneko†1, Hiroyuki Kato†1, Yoshimi Kawamura†1, Shigeyuki Esumi§1, Teruyoshi Hirayama†, Takahiro Hirabayashi‡, and Takeshi Yagi‡1,2

From the †KOKORO Biology Group, Laboratories for Integrated Biology, Graduate School of Frontier Biosciences, Osaka University 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan, and the ‡Department of Morphological Neural Science, Graduate School of Medical Science, Kumamoto University, Kumamoto 860-8556, Japan

The molecular basis for providing the identity and diversity of single neurons is a key for realizing the brain system. Diverse protocadherin isoforms encoded by the Pcdh-α and Pcdh-γ gene clusters are expressed in all of the vertebrates studied. For the Pcdh-α isoforms, differential expression patterns have been found in single Purkinje cells by unusual monoallelic and combinatorial types of gene regulation. Here we investigated total allelic gene regulation in the Pcdh-α and -γ clusters, including the C-type variable exons (C1 to C5) and the Pcdh-γA and -γB variable exons in single Purkinje cells. Using split single-cell reverse transcription-PCR analysis, almost all of the Purkinje cells at postnatal day 21 biallelically expressed all the C-type isoforms, whereas the Pcdh-α isoforms showed both monoallelic and combinatorial expression. The Pcdh-γA and -γB isoforms also showed differential regulation in each cell with both monoallelic and combinatorial gene regulation. These data indicated that different types of allelic gene regulation (monoallelic versus biallelic) occurred in the Pcdh-α and -γ clusters, although they were spliced into the same constant exons. It has been reported that each C-type Pcdh-α or -γ transcript has a different expression pattern during brain development, suggesting that the different C-type variable exons may code temporal diversity, although the Pcdh-α, -γA, and -γB isoforms were differential and combinatorial gene regulation within a single cell. Thus, the multiple gene regulations in the Pcdh-α and -γ clusters had a potential mechanism for increasing the diversity of individual neurons in the brain.

The brain contains a huge number of neurons that have diverse characteristics and identities to enable the generation of complex neuronal networks. Therefore, the molecular mechanism for specifying the identity and diversity of single neurons is important for realizing the complexity of the brain. Approximately 100 diverse cadherin superfamily genes are highly expressed in the brain (1, 2). Among them, the CNRs (cadherin-related neuronal receptors) are also diverse and highly expressed in the vertebrate brain (3). The CNRs are Pcdh-α (proto[ coached]erin-α) genes. Their diverse isoforms are generated from gene clusters on chromosomes; these clusters of Pcdh-α and Pcdh-γ contain variable regions (tandemly arrayed diverse variable exons) and constant regions (common, cis-spliced exons of Pcdh-α and Pcdh-γ) (4–12). Their genomic organization is similar to that of immunoglobulin and T-cell receptor genes (3). This diversity of Pcdh cluster genes could be one of the mechanisms by which neurons are distinguished from each other. The single-cell analysis of Purkinje cells using multiple RT-PCRs showed that the expression of each variable exon in the Pcdh-α gene is monoallelic and combinatorial (13). This was a novel type of allelic expression for diversified receptor families in the brain. The differential expression patterns of the Pcdh-α isoforms in single neurons also suggested a mechanism for providing the identity and specificity of single neurons in the brain (13).

The variable exons of the Pcdh-α and Pcdh-γ clusters are classified into several subgroups, Pcdh-α, Pcdh-γA, Pcdh-γB, and C-type (4, 10). The Pcdh-α gene cluster contains the variable exons Pcdh-α1 to Pcdh-α12, and in addition, α7/8 exists in wild mouse strains and two C-type variable exons, C1 and C2 (10, 14, 15). The Pcdh-γ gene cluster contains Pcdh-γA, Pcdh-γB, and three C-type variable exons, C3, C4, and C5 (10). The five C-type variable exons of Pcdh-α and Pcdh-γ are highly homologous to each other and distinct from the other exons in the clusters.

Here we examined the allelic gene regulation of these types of variable exons in the Pcdh-α and Pcdh-γ gene clusters at the single-cell level. Interestingly, the C-type variable exons were constantly expressed in Purkinje cells at postnatal day (P)21 from both alleles, although monoallelic and combinatorial types of expression were commonly found for the Pcdh-α, Pcdh-γA, and Pcdh-γB variable exons. Our results indicated that biallelic and monoallelic gene regulation for variable exons

*This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (to T. Y.), the Uehara Memorial Foundation, the Takeda Foundation, and CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Agency.
† These authors contributed equally to this work.
‡1 The abbreviations used are: Pn, postnatal day; B6, C57BL/6; RT, reverse transcription.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Fig. S1.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Allelic Gene Regulation in Pcdh-α and -γ Clusters

A

Variable region

Constant region

B

C

D

E

FIGURE 1. Expression analysis of Pcdh-α mRNA in the cerebellum of P21 mice. A, structure of the Pcdh-α gene cluster and position of in situ hybridization probes. The Pcdh-α CP3 probe recognized all the Pcdh-α isoforms. B–E, in situ hybridization was performed on sagittal sections of the mouse cerebellum using digoxigenin-labeled RNA probes of Pcdh-α transcripts. The probes used were: Pcdh-α C1 antisense (B), Pcdh-α C2 antisense (C), Pcdh-α CP3 antisense (D), and Pcdh-α-3 antisense (E). B–E are identical magnifications. Scale bars, 100 μm.

coexist for each Pcdh gene cluster. The monoallelic and combinatorial expression of distinct Pcdh-α, Pcdh-γA, and Pcdh-γB variable exons is a potential mechanism for generating distinct identity in single Purkinje cells.

EXPERIMENTAL PROCEDURES

Animals—F1 hybrid offspring were obtained by intercrossing mice of the laboratory strain C57BL/6 (B6) (Purchased from Charles River) with the Japanese wild mouse strain JF1, obtained from the National Institute for Genetics (Mishima, Shizuoka, Japan). The mouse strain JF1 is different from that of our previous report (13) but shares similar polymorphisms in Pcdh-α locus. The JF1 mice show more suitable characteristics for daily care than MSM mice.

In Situ Hybridization—In situ hybridization was performed as described previously (13, 16) on 10-μm-thick frozen sagittal sections prepared from the P21 B6 mouse cerebellum using Pcdh-α3 (nucleotides 379–2362 according to GenBank accession number NM_138662), Pcdh-αC1 (nucleotides 28–2405 according to GenBank accession number NM_001003671), Pcdh-αC2 (nucleotides 63–2516 according to GenBank accession number NM_001003672), and Pcdh-α CP3 (nucleotides 2518–4559 according to GenBank accession number NM_138662), as templates to synthesize digoxigenin-11-UTP-labeled (Roche) cRNA probes. The Pcdh-α CP3 probe bound to the identical 3′-untranslated regions of Pcdh-α1 to 12, C1, C2, and 7/8 (JF1).

Split Single-cell RT-PCR of Purkinje Cells—Split single-cell RT-PCR of Purkinje cells was carried out as described previously (13). In brief, single Purkinje cells were prepared from the cerebellum of the F1 mice from B6 female × JF1 male, at P21. The tissue was dissected and subjected to enzymatic digestion at 37 °C for 30 min in 10 ml of dissociation solution consisting of 90 units of papain (Worthington), 0.002% dL-cysteine HCl (Sigma), 0.05% DNase I (Sigma), 0.1% bovine serum albumin (Sigma), and 0.05% glucose (Nacalai Tesque). The digested tissue was spun for 8 min at 300 × g, and the pellet was resuspended in Dulbecco’s modified Eagle’s medium (Sigma). To remove debris, the cells were filtered through a 100-μm cell strainer (Falcon). Single Purkinje cells were picked up by glass capillary and placed in thin, 200-μl PCR tubes with 6 μl of RNAse-free water in each tube. Complementary DNA was synthesized from single-cell samples after being primed with 40 pmol/reaction of each primer in a total volume of 10 μl, using 7.5 units of Thermoscript reverse transcriptase (Invitrogen) according to the manufacturer’s protocol, at 55 °C for 60 min. The reaction was stopped by heating at 85 °C for 5 min. The cDNAs derived from a single Purkinje cell were split into three PCR tubes as 3.3-μl aliquots and used as a template for the multiplex first PCR. The first multiplex PCR was performed using 3.3 μl of single-cell cDNA, 5 μl of 10× LA PCR buffer, 3.9 μl of 25 mM MgCl2, 4 μl of 2.5 mM each dNTP mix, 0.3 μl of 10 mM each primer set, 0.5 μl of LA Taq polymerase (TAKARA), and 31.5 μl of water, and the PCR conditions of 1 cycle of 5 min at 96 °C and then 30 cycles of 20 s at 96 °C and 10 min at 65 °C. The primer sequences are shown in supplementary Table S1.

The 5′ PCR primer was based on a consensus sequence contained in all of the variable exons and located in their EC5 regions. The primers for RT-PCR were located in different exons to rule out, by size differences, the detection of amplified genomic DNA. The second round of each PCR was then performed using 0.1 μl of the first PCR product as a template (final volume, 20 μl). In this second PCR, each cDNA was amplified individually using its specific primer pair by performing semi-nested PCR: 1 cycle of 5 min, 95 °C; 35 cycles of 30 s, 95 °C; 30 s,
60 °C; 1 min, 72 °C. The PCR-amplified products were purified by polyethylene glycol precipitation, subjected to direct sequencing using a BigDye® DNA sequencing kit (version 3.1) (ABI), and analyzed on an ABI Prism 3100 or an ABI Prism 3730 Genetic Analyzer. The direct sequencing permitted us to identify the allele from which each purified PCR product was derived. The discrimination of monoallelic expression from biallelic expression was accomplished by using SeqScape software (ABI).

RESULTS

In Situ Hybridization Analysis of the C-type Variable Exons in the Pcdh-α Cluster—We previously examined the expression levels of the Pcdh-α variable exons in Purkinje cells (13). The Pcdh-α cluster contains two of the C-type of variable exons, C1 and C2 (14, 15). Therefore, we examined the expression levels of the C1 and C2 mRNAs in individual Purkinje cells of the B6 mouse cerebellum using in situ hybridization (Fig. 1). Although the RNA probes specific for the C1 and C2 variable exons stained all of the Purkinje cells uniformly, the signal intensities, which may reflect expression level, were different: faint for the C1 probe (Fig. 1B) and strong for the C2 probe (Fig. 1C), similar to that of the constant region (CP) antisense probe (Fig. 1D). The expression pattern of Pcdh-α3 was different from that of the C1 and C2 variable exons (Fig. 1E); the signal for Pcdh-α3 was not detected uniformly but rather was detected in sub-populations of Purkinje cells. These results suggested that every Purkinje cell expressed C1 and C2 transcripts, in contrast to the expression of the Pcdh-α variable exons.

Distinct Expression Patterns of Pcdh-α C1 and C2 from Those of Pcdh-α1 to α12 in Single Purkinje Cells—We next examined the expression profiles of the C1 and C2 variable exons in the Pcdh-α cluster. We first sequenced the C1 and C2 variable exons of the JF1 mouse strain and detected polymorphisms against the B6 strain (supplemental Fig. S1). Using the cerebellar Purkinje cells of P21 F1 mice from a B6 × JF1 cross, we performed split single-cell RT-PCR analyses (described under “Experimental Procedures”). Single Purkinje cells were picked up by glass capillary. Complementary DNA of Pcdh-α, Pcp-2, and β-actin was synthesized from the single-cell samples in a same tube, and the resulting cDNA was then divided into three tubes and subjected to separate, first round multiplex PCR analysis. The second round of PCR amplification was carried out individually for each tube and used nested primers for each Pcdh-α variable exon, the Pcdh-α consensus region, Pcp-2 (a marker for Purkinje cells) (17), and β-actin (Fig. 2).

Of the 14 single Purkinje cells analyzed, all had a Pcdh-α variable exon consensus PCR product in at least one of the three tubes. Neither sample 1-8 nor sample 1-16, which were subjected to the entire procedure without Purkinje cells, gave any PCR products, confirming the reliability of the method. When the cells were subjected to exon-specific second round PCR (Fig. 2), all 14 were positive for β-actin and Pcp-2, confirming that they were differentiated Purkinje cells. All 14 Purkinje cells had three of three specific PCR amplifications for at least one of the 13 Pcdh-α variable exons (α1 to α12), consistent with our previous results (13). In addition to three of three PCR amplifications, some of the cell showed one of three or two of three PCR amplifications (for example, α1 in cell 1-2); it may suggest the low amount of the corresponding mRNAs in that cell. All 14 cells were positive for C1 and C2 (Fig. 2), supporting our in situ hybridization results (Fig. 1, B and C). Although C2 showed specific PCR amplification in all the divided tubes, C1 was not always amplified in all three divided tubes (cells 1-1, 1-2, 1-3, 1-6, 1-14, and 1-15). These results indicated that most of the Purkinje cells uniformly expressed the C1 and C2 variable exons, which is different from the other Pcdh-α variable exons.

Next, each PCR product was subjected to direct sequencing to distinguish three possible patterns of expression: monoallelic expression of the maternal allele, monoallelic expression of the paternal allele, and biallelic expression. A monoallelic expression pattern was found for 14 of the 21 three of three PCR-positive products (66.7%) for the Pcdh-α1 to α12 variable exons (Table 1). The monoallelic expression pattern was found in the following 14 isoforms; α2 in cell 1-1; α4 in cell 1-4; α2, α5, and α10 in cell 1-5; α2 and α3 in cell 1-6; α2 and α4 in cell 1-9; α7 and α10 in cell...
1-10; α7 in cell 1-11; and α4 and α12 in cell 1-14. In the cases of some isoforms (for example, α2 in cell 1-3), each PCR product showed different results, suggesting that the isoform was expressed biallelically and at a low abundance. If this assay could detect a single cDNA molecule even though biallelic products were present in one PCR tube, complete three of three monoallelic expression would be theoretically observed at 25% (18). Clearly, at 66.7% versus 25%, there was a highly significant difference by χ² analysis between monoallelic and biallelic expression (p < 10⁻²). Of the 14 monoallelically expressed isoforms, nine were derived from the B6 allele and five from the JF1 allele. Thus, no allelic distortion from the paternal or maternal allele was found in this assay. These findings supported previous results showing monoallelic and combinatorial expression of the Pcdh-α variable exons in Purkinje cells (13). —In the same cells, we analyzed the allelic expression patterns of the C1 and C2 variable exons. Biallelic expression patterns were found for 14 of the 14 three of three PCR-positive products (100%) for the C2 variable exon. For the C1 variable exon, biallelic expression patterns were found for eight of the nine three of three PCR-positive products (89%). One cell (11%) (cell 1-9) showed a monoallelic expression pattern, but the value was almost similar to the theoretical threshold, 25%, indicating biallelic expression of the C1 variable exon. These results demonstrated that, in contrast to the Pcdh-α1 to α12 variable exons, the C-type variable exons in the Pcdh-α cluster were expressed biallelically in Purkinje cells.

### TABLE 1

Distribution of the B6 and JF1 allele as the source of each Pcdh-α variable exon revealed in the split single-cell RT-PCR analysis in Purkinje cells

To determine whether the amplified PCR products were from the B6 or JF1 allele, we performed direct sequencing of the products in three tubes for 14 Purkinje cells. This PCR analysis could not distinguish between B6 and JF1 α6. The samples that gave second round PCR product but were not sequenced are indicated with a closed circle, and the samples that gave an insufficient quantity of product for sequencing are indicated with an ×.

| cell | tube | α1 | α2 | α3 | α4 | α5 | α6 | α7 | α8 | α9 | α10 | α11 | α12 | α7/8 | c1 | c2 | Pcdh-α consensus | Pcp2 | β-actin |
|------|------|----|----|----|----|----|----|----|----|----|----|----|----|------|----|----|------------------|------|---------|
| #1-1 | 1    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-2 | 1    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-3 | 1    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-4 | 1    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-5 | 1    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-6 | 1    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-7 | 1    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-9 | 1    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-10| 1    | x  | B6 |     |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | x  | B6 |     |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | x  | B6 |     |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-11| 1    | x  | JF1|     |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | x  | JF1|     |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | x  | JF1|     |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-12| 1    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-13| 1    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-14| 1    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
| #1-15| 1    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 2    | JF1|     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
|      | 3    | B6 |     |    |    |    |    |    |    |    |    |    |    |      |     |    |                   |      |         |
Uniform and Biallelic Expression of the C-type Variable Exons in the Pcdh-α and Pcdh-γ Clusters—To extend the results obtained for the C1 and C2 variable exons, the expression patterns of all five C-type variable exons in the Pcdh-α and Pcdh-γ clusters were analyzed in single Purkinje cells by split single-cell RT-PCR analysis (Fig. 3). The C-type variable exons in the Pcdh-α and Pcdh-γ clusters were expressed relatively uniformly; 19 of 21 cells analyzed showed the C1 variable exon, and all 21 cells showed the C2 to C5 variable exons. Next, the allelic expression patterns were analyzed by sequencing the PCR products (Table 2). Biallelic expression patterns were commonly found for all five of the C-type variable exons in the Pcdh-α and Pcdh-γ clusters; biallelic expression was observed in 13 of the 13 three of three PCR-positive products (100%) for the αC1 variable exon, 18 of 18 (100%) for the αC2 variable exon, 21 of 21 (100%) for the γC3 variable exon, 19 of 19 (100%) for the γC4 variable exon, and 15 of 16 (94%) for the γC5 variable exon. These results demonstrated that the C-type variable exons in the Pcdh-α and Pcdh-γ clusters were relatively uniformly expressed and biallelically gene-regulated in Purkinje cells.

Monoallelic and Combinatorial Expression of the Pcdh-α, Pcdh-γA, and Pcdh-γB Variable Exons in Purkinje Cells—The allelic expression patterns of the Pcdh-γA and -γB variable exons in the Pcdh-γ cluster were examined. Prior to this, we identified polymorphisms of the Pcdh-γ variable exons between the B6 and JF1 strains. First, certain Pcdh-γ exons (Pcdh-γA7 and -γA10 of the 12 γA variable exons and Pcdh-γB1and -γB7 of the 7 γB variable exons) were chosen, and their expression patterns were analyzed using split single-cell RT-PCR analysis in single Purkinje cells (Table 3 and Fig. 4). Among 32 Purkinje cells (Pcp-2 positive), 14 showed the PCR amplification of Pcdh-γA7, -γA10, -γB1, or -γB7 in all three divided tubes (Fig. 4). These cells were used for allelic expression analysis. The other 19 cells did not show PCR amplification in all three of the divided tubes: 8 cells showed no PCR amplification, and the other 11 cells showed PCR amplification in one or two tubes. Monoallelic expression patterns were found for all 18 of the three of three PCR-positive products (100%) for the Pcdh-γA and Pcdh-γB variable exons (Table 3). Of the 18 monoallelically expressed isofoms, 9 were derived from the B6 allele, and 9 were derived from the JF1 allele. No allelic distortion was found for the Pcdh-γA or Pcdh-γB variable exons. These results demonstrated that the Pcdh-γA and Pcdh-γB variable exons were each regulated monoallelically in single Purkinje cells. Monoallelic and combinatorial expression patterns of distinct variable exons were also found for the Pcdh-γA and Pcdh-γB variable exons. Taken together with our previous findings, these results indicated that each Purkinje cell coexpressed both Pcdh-α and Pcdh-γ variable exons.

Next, we directly examined the coexpression of the Pcdh-α and Pcdh-γ variable exons in single Purkinje cells. The split single-cell RT-PCR analysis clearly showed that each single Purkinje cell coexpressed both Pcdh-α and Pcdh-γ variable exons (data not shown). We further analyzed four Purkinje cells; two of these cells expressed the Pcdh-γB1 variable exon from the B6 allele, and two expressed Pcdh-γB7 from the JF1 allele. These four Purkinje cells expressed different combinations of Pcdh-α. One Pcdh-γB1-positive cell showed two of three PCR-positive results for the α5 product from the B6 allele and three of three for the α6 product (the alleles could not be distinguished because there are no polymorphisms), another Pcdh-γB1-positive cell had two of three positive PCR results for α6 expression and three of three for α12 from the B6 allele. In addition, one Pcdh-γB7-positive cell shows two of three positive PCR results for α5 from the B6 allele, two of three for α9 from the JF1 allele, and two of three for α12 from the B6 allele. Another showed three of three positive PCR results for α4 from the B6 allele and three of three for α6 (Fig. 5 and Table 4). Thus, an individual Purkinje cell could express various combinations of the Pcdh-α, Pcdh-γA,
and Pcdh-γB variable exons from different alleles, suggesting that more combinatorial diversity is provided by such differential expression.

**DISCUSSION**

Here we showed that the mouse Pcdh gene clusters contain both monoallelically and biallelically expressed variable exons and that the Pcdh genes are expressed combinatorially and differentially within a single Purkinje cell. Specifically, the Pcdh-α and Pcdh-γA and -γB variable exons were monoallelically regulated, whereas the C-type variable exons in the Pcdh-α and Pcdh-γ clusters were biallelically regulated. All of the C-type variable exons were relatively uniformly expressed in all Purkinje cells of the P21 mouse cerebellum. This finding is consistent with the in situ hybridization results reported previously (19). On the other hand, the Pcdh-α and Pcdh-γA and -γB variable exons were expressed monoallelically and combinatorially in single Purkinje cells of the P21 mouse cerebellum. The expression patterns of the Pcdh-α and Pcdh-γ gene clusters revealed in the present study are shown in Fig. 6.

Various combinations of the Pcdh-α and Pcdh-γ isoforms were expressed in individual Purkinje cells at P21, whereas all of the C-type variable exons in the Pcdh-α and Pcdh-γ gene clusters were constitutively expressed. Our previous report and this study have shown that two species of Pcdh-α

---

**TABLE 2**

Distribution of the B6 and JF1 allele as the source of each Pcdh-α C1, -αC2, and Pcdh-γ C3, -γC4, and -γC5 exons revealed in the split single-cell RT-PCR analysis in Purkinje cells.

To determine whether the amplified PCR products were from the B6 or JF1 allele, we performed direct sequencing of the products in three tubes for 21 Purkinje cells. The samples that gave an insufficient quantity of product for sequencing are indicated with an X. The samples that gave second round PCR product but were not sequenced are indicated with a closed circle.

| cell | tube | α C1 | α C2 | γ C3 | γ C4 | γ C5 | Pcd-2 |
|------|------|------|------|------|------|------|-------|
| 2-1  | 1    | B6   | JF1  | B6   | JF1  | B6   | JF1   |
|      | 2    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 3    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
| 2-2  | 1    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 2    | JF1  | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 3    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
| 2-3  | 1    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 2    | JF1  | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 3    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
| 2-4  | 1    | B6   | JF1  | B6   | JF1  | B6   | JF1   |
|      | 2    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 3    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
| 2-5  | 1    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 2    | JF1  | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 3    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
| 2-6  | 1    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 2    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 3    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
| 2-7  | 1    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 2    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 3    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
| 2-9  | 1    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 2    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 3    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
| 2-10 | 1    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 2    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 3    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
| 2-11 | 1    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 2    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
|      | 3    | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | B6/JF1 | ●     |
| 2-12 | 1    | B6   | B6/JF1 | B6   | B6/JF1 | B6   | B6/JF1 | ●     |
|      | 2    | B6   | B6/JF1 | B6   | B6/JF1 | B6   | B6/JF1 | ●     |
|      | 3    | JF1  | B6/JF1 | B6   | B6/JF1 | B6   | B6/JF1 | ●     |
transcripts were combinatorially expressed from the Pcdh-α cluster, and all five of the C-type exons in Pcdh-α and Pcdh-γ were expressed uniformly. The variety of Pcdh-γA and -γB species can be extrapolated from the present results. Among the 32 Purkinje cells analyzed, a clear band representing the PCR product in all three split tubes was found in eight cells for the Pcdh-γA7/A10 genes and in 10 cells for the Pcdh-γB1/B7 genes (Fig. 3). That is, 0.25 (8/32) Pcdh-γA7/A10 and 0.31 (10/32) Pcdh-γB1/B7 exons were expressed per Purkinje cell. There are 12 Pcdh-γA and 7 Pcdh-γB variable exons in the gene cluster. Therefore, assuming there is no distortion of choice for expression, we can estimate that 1.5 kinds of Pcdh-γA (0.25 multiplied by 12/2) and 1.09 kinds of Pcdh-γB (0.31 multiplied by 7/2) should be expressed per individual Purkinje cell. There are 12 Pcdh-γA and 7 Pcdh-γB variable exons in the gene cluster. Therefore, assuming there is no distortion of choice for expression, we can estimate that 1.5 kinds of Pcdh-γA (0.25 multiplied by 12/2) and 1.09 kinds of Pcdh-γB (0.31 multiplied by 7/2) should be expressed per individual Purkinje cell. In theory, the Pcdh-α and Pcdh-γ transcripts can produce 109,200 \((26\times 25)/2\times 24\times 14\) possible combinations (2 from 26 Pcdh-α (13 from each allele), one from 24 Pcdh-γA (12 from each allele), and one from 14 Pcdh-γB (7 from each allele)) in a single Purkinje cell. Therefore, these variable exons may provide diversity sufficient to represent 10^5 different individual Purkinje cells. The present study showed that both the Pcdh-α and Pcdh-γ transcripts were expressed in the same Purkinje cell. It was previously reported that Pcdh-α protein and Pcdh-γ protein can form a heterodimer, which translocates to the cell surface. Although Pcdh-α is hardly expressed on the cell surface of HEK293 cells transfected with Pcdh-α alone (20), it is easily detected when HEK293 cells are transfected with both Pcdh-α and Pcdh-γ (21). It suggests that this heterodimeric complex of Pcdh-α and Pcdh-γ may be functional on the cell surface of the Purkinje cells. Although it remains to be shown whether the ligand specificity of this protein complex is different from that of the corresponding monomeric proteins, the formation of the heterodimeric complex on the cell surface may provide molecular diversity to guide specific cell-to-cell interactions and recognition in the brain. It was previously shown that the expression patterns of the C-type Pcdh-α and Pcdh-γ transcripts are different from those of the other variable exons during mouse brain development. The transcription levels of Pcdh-α C1 and C2 peak at P10, whereas those of the other Pcdh-α exons peak at P0 (15). Recently, Frank et al. (19) reported that the Pcdh-γ transcripts of γA, γB, and γC3 are expressed before birth and peak at P9. The γC4 transcript expression peaks at P9 but is down-regul-
lated strongly thereafter; in contrast, the γC5 transcript is detected around the end of the first postnatal week and then increases (19). Notably, the study by Frank et al. revealed that the Pcdh-γC3 variable exon was more prominently expressed than the others. This is consistent with our present results and supports our interpretation that the number of PCR products in the split single-cell RT-PCR analysis reflects the expression level. Given that the combinations of C-type Pcdh-α and Pcdh-γ transcripts vary at different developmental stages, it can be postulated that the C-type variable exons confer temporal diversity, and the Pcdh-γA and Pcdh-γB variable exons confer spatial diversity for neuronal cell identity in the developing mouse brain.

Monoallelic expression has been observed for some genes. It is categorized into five types: (i) X inactivation (22, 23), (ii) genomic imprinting (24, 25), (iii) allelic exclusion (26–28), (iv) random monoallelic expression, and (v) random yet combinatorial and differential monoallelic expression. The fourth group includes IL-2 (29), IL-4, IL-5, IL-13 (29–31), Pax5 (32, 33), Nubp2, Igals, and Jasp1 (34). The fifth group contains Pcdh-α (13). The present study indicates that the Pcdh-γA and -γB variable exons also belong in the fifth group, whereas the C-type Pcdh-α and Pcdh-γ exons are not regulated monoallelically and do not belong in any of these groups. Almost all of the random monoallelically expressed genes (groups iv and v) exist as a cluster, with the genes in the same cluster all showing monoallelic expression (31, 34). A well-known exception is the Rad50 gene in the interleukin gene cluster, in which the interleukin genes are expressed monoallelically, whereas the Rad50 gene is expressed biallelically (31). Notably, although the Rad50 gene has a different promoter and different exons from the interleukin genes, the C-type Pcdh-α and Pcdh-γ variable exons have a distinct but related promoter and share common exons with other protocadherin genes in the same cluster (35, 36). It will be interesting to investigate the regulatory mechanism(s) of protocadherin expression, which may uncover a novel mechanism for the regulation of monoallelic gene expression.

The present study showed that both the Pcdh-α and Pcdh-γ gene clusters contain monoallelically and biallelically expressed genes. Our results indicate that the Pcdh-α and Pcdh-γ gene clusters were generated by the duplication of a common ancestor gene and that the ancestral gene cluster already contained both monoallelically and biallelically expressed variable exons. Further studies aimed at revealing the mechanisms of expression of the protocadherin gene cluster will be particularly valuable.
for understanding the molecular mechanisms involved in generating the diversity of individual neurons. The creation of neuronal cell diversity by the repertoire of\textsubscript{\textit{Pcdh}}-\textsubscript{H9251} and \textsubscript{\textit{Pcdh}}-\textsubscript{H9253} is expected to be revealed as a significant molecular mechanism by which functional neuronal circuits are acquired in the vertebrate brain.

Acknowledgments—We thank T. Shiroishi for providing the JF1 strain and members of the Yagi laboratory for suggestions and discussion during the course of this work.

**REFERENCES**

1. Yagi, T., and Takeichi, M. (2000) *Genes Dev.* 14, 1169–1180
2. Hamada, S., and Yagi, T. (2001) *Neurosci. Res.* 41, 207–215
3. Kohmura, N., Senzaki, K., Hamada, S., Kai, N., Yasuda, R., Watanabe, M., Ishii, H., Yasuda, M., Mishina, M., and Yagi, T. (1998) *Neuron* 20, 1137–1151
4. Wu, Q., and Maniatis, T. (1999) *Cell* 97, 779–790
5. Wu, Q., and Maniatis, T. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 3124–3129
6. Sugino, H., Hamada, S., Yasuda, R., Tuji, A., Matsuda, Y., Fujita, M., and Yagi, T. (2000) *Genomics* 63, 75–87
7. Sugino, H., Yanase, H., Hamada, S., Kurokawa, K., Asakawa, S., Shimizu,
Allelic Gene Regulation in Pcdh-α and -γ Clusters