Relationship between DNA Methylation States and Transcription of Individual Isoforms Encoded by the Protocadherin-α Gene Cluster*§

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The protocadherin-α (Pcdh-α) gene encodes diverse transmembrane proteins that are differentially expressed in individual neurons in the vertebrate central nervous system. The Pcdh-α genomic structure contains variable first exons, each regulated by its own promoter. Here, we investigated the effect of DNA methylation on gene regulation in the Pcdh-α gene cluster. We studied two mouse cell lines, C1300 and M3, that expressed different combinations of Pcdh-α isoforms and found that 1) the transcription of specific Pcdh-α isoforms correlated significantly with the methylation state of the promoter and the 5′ (but not the 3′) region of the first exon and 2) mosaic or mixed methylation states of the promoters were associated with both active and inactive transcription. Demethylation of C1300 cells up-regulated all of the Pcdh-α isoforms, and, in a promoter assay, hypermethylation of the promoters repressed their transcriptional activity. Cell lines cloned from the demethylated C1300 cells transcribed different combinations of Pcdh-α isoforms than the parental, nondemethylated cells, and the promoters showed differential mosaic or mixed methylation patterns. In vivo, the promoter and 5′-regions of the Pcdh-αC1 and αC2 exons, which are transcribed in all neurons, were extensively hypomethylated. In contrast, the promoters of the Pcdh-αC1 to -αC12 isoforms, which are transcribed differentially by individual Purkinje cells, exhibited mosaic methylation patterns. Overall, our results demonstrated that mosaic or mixed DNA methylation states in the promoter and 5′-region of the first exon may help regulate differential Pcdh-α transcription and that hypermethylation is sufficient to repress transcription.

The brain is a highly organized multicellular system possessing a huge number of diverse neurons that generate a complicated and sophisticated neural network. Individual neurons have distinct identities through their differential expression of several neuronal genes. For example, in the olfactory sensory system, the odorant epithelial cells express thousands of different odorant receptors (ORs) (1), but each cell expresses just one OR protein by allelic exclusion (2, 3). These receptors determine which odor molecules are detected (4) and the trajectory of the neural projection to the glomerulus (5). In Drosophila, Down syndrome cell adhesion molecules regulate neural circuit formation through their diverse mature mRNAs, which are generated by alternative splicing of a pre-mRNA transcribed from a single allele (6). Each variable Down syndrome cell adhesion molecule isoform exhibits isoform-specific homophilic binding, thus guiding appropriate neuronal connections through Down syndrome cell adhesion molecule transcription (7, 8).

Members of the gene-clustered protocadherin (Pcdh) family, a group of diverse cadherin-like transmembrane proteins, are candidates for the individualization of neurons in the vertebrate central nervous system. The Pcdh gene family includes three gene clusters, Pcdh-α, -β, and -γ. Each cluster contains a large region of tandemly arranged, variable exons, each encoding six extracellular (EC) cadherin repeats, a transmembrane domain, and a partial cytoplasmic (CP) domain. In the Pcdh-α and -γ clusters, the rest of the CP domain is encoded by three or four constant region (CR) exons located at the 3′-end of each cluster. Only one of the variable first exons is spliced onto these CR exons (9, 10) (Fig. 1A). RT-PCR analysis of individual Purkinje cells has shown that Purkinje cells differentially express Pcdh-α isoforms by combinatorial transcription, selecting one or two variable first exons for individual cis-splicing from each Pcdh-α allele (11). The Pcdh-αA and -αB genes are regulated similarly (12). Thus, an individual Purkinje cell might be identifiable by its Pcdh-α and -γ transcription patterns. In contrast, Pcdh-αC1, -αC2, -αC3, -γC4, and -γC5, located near the CR exons of Pcdh-α and -γ, are transcribed biallelically in Purkinje cells (12). To elucidate the molecular mechanisms for establishing the identity of each neuron and its specific connections, we need to understand the regulation of gene families, like the Pcdh family, that encode multiple, differentially expressed isoforms.

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1 The abbreviations used are: OR, odorant receptor; Pcdh-α, protocadherin-α; CP, cytoplasmic; CR, constant region; CSE, conserved sequence element; EC, extracellular; FBS, fetal bovine serum; gDNA, genomic DNA; SaC, 5-azacytidine; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BDNF, brain-derived neurotrophic factor.

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Each Pcdh variable exon has an exon-specific promoter, located upstream of the exon, that contains a conserved sequence element (CSE) (13, 14) (Fig. 1A). Tasic et al. (14) examined the methylation states of CpG dinucleotides (CpGs) in the promoter and 5′-region of the Pcdh-a6 exon in two human cell lines that differentially transcribe the isoform and found a correlation between DNA methylation of the promoter and the isoform transcribed. However, the correlation between the DNA methylation state and isoform transcription for the other variable-exon promoters of the Pcdh-α gene cluster and whether such a correlation has a role in vivo have not been investigated.

Here we demonstrate that the promoter and 5′-region of the first exon of actively transcribed Pcdh-α isoforms were hypomethylated, but all the 3′-regions of the Pcdh-α1 to -α12 variable exons were hypermethylated. We also show that DNA hypermethylation of Pcdh-α variable-exon promoters was sufficient for transcriptional repression. In subclones derived from demethylated C1300 cells, we found various CpG methylation states in the Pcdh-α promoters, including methylated, unmethylated, mosaic (i.e., methylated and unmethylated CpGs on an individual DNA strand), and mixed methylated and unmethylated DNA strands. The mosaic and mixed methylation states were found in the promoters of both transcribed and untranscribed Pcdh-α isoforms. Finally, in neurons from brain tissue, the differential Pcdh-α transcription patterns in individual neurons correlated with the occurrence of differential mosaic DNA methylation in the Pcdh-α variable-exon promoters, but hypomethylation of the promoters was characteristic of the biallelically expressed Pcdh-αC1 and αC2 mRNA.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—A mouse neuroblastoma cell line, C1300, was obtained from the Imperial Cancer Research Fund Laboratories, Clare Hall, UK. The cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen). Another mouse neuroblastoma cell line, M3, was maintained in RPMI 1640 (Sigma) supplemented with 10% FBS. These cell lines were grown at 37 °C in a humidified atmosphere of 5% CO2. Cells were passaged into 100-mm culture dishes when confluent and used for subcloning, we cultured each subline with medium containing 5aC, the C1300 cells were cultured at a low cell density in normal medium. Each single cell divided individually and formed a colony. For subcloning, we isolated the colonies and cultured them individually. To promote cell survival after subcloning, we cultured each subline with medium containing half Dulbecco’s modified Eagle’s medium-10% FBS and half conditioned medium from normal C1300 cells. First, each subline was cultured in a 96-well plate. When confluent, the cells were transferred to a 24-well plate. Each subline was eventually transferred to a 60-mm dish and finally to a 100-mm dish.

**gDNA Preparation and Bisulfite Genomic Sequencing**—gDNA was isolated from cultured cells and from C57BL/6 mice (Charles River Japan) by standard techniques. All experimental procedures that involved mice were performed in accordance with the 1996 National Institutes of Health guidelines and were approved by the Animal Committee of Osaka University.

gDNA was modified by sodium bisulfite, as described in Paulin et al. (15). The bisulfite-modified DNA was purified by the Wizard DNA Clean-Up System (Promega, Madison, WI) and desulfonated. Following ethanol precipitation, we used 20 ng of the treated DNA for PCR reactions with Ex Taq (Takara, Japan). The primers used for DNA amplification are listed in supplemental Table S1. The first PCR program consisted of 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 54–58 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 3 min. The second PCR was carried out using the same program as the first, except for the annealing temperature, which depended on the primer set. The PCR products were sequenced by standard procedures. The identical procedure was repeated for two independent batches of gDNA and at least two independent PCR reactions for each sample, to minimize PCR artifacts.

To analyze the 3′-region of the Pcdh-α variable exons, we synthesized a set of universal primers for a highly conserved sequence within the Pcdh-a1 to -α12 variable exons (91.8–99.1% identity at the amino acid level). The primers (Table S1) were designed to maximize the highly conserved sequences and amplify all Pcdh-α variable exons simultaneously, except for Pcdh-αC1 and -αC2. The universal primer sequences were 83.3–100% identical to the individual Pcdh-α genes at the

![Diagram of DNA Methylation in Protocadherin-α Cluster](image)
nucleotide level (data not shown). The PCR products were sequenced by standard procedures and assigned to each Pcdh-α10 gene based on sequence comparison.

Methylation-sensitive Restriction Enzyme Cleavage and Southern Blots—gDNA was digested by general restriction enzymes (BglII for Pcdh-α10, KpnI for Pcdh-α1, or HindIII for Pcdh-α2) and subsequently by MspI or the CpG methylation-sensitive isoschizomer HpaII.

The probes for Southern blots to recognize the Pcdh-α10 upstream, Pcdh-α1 upstream and downstream, or Pcdh-α2 upstream regions were amplified by KOD-plus (Toyobo, Japan) PCR with the primers listed in supplemental Table S2. The PCR template was the Mus musculus genomic BAC clone RP23–303I8 or RP24–318M13. The PCR products were ligated into pCR-Blunt vectors (Invitrogen) and subcloned. The vectors were amplified, and then the probe fragments were cut out of the vectors. For random primer labeling, a fluorescein-conjugated nucleotide mix (GE Healthcare UK, Ltd.), NEB Klenow (New England Biolabs, Beverly, MA), and a random 9-mer mix (Hokkaido System Science, Japan) were used. The probe fragments were recognized by an anti-fluorescein alkaline phosphatase-conjugated antibody (GE Healthcare UK Ltd., diluted 5000 times) and detected by using CDP-Star detection reagents (GE Healthcare UK Ltd.).

RNA Preparation and RT-PCR—Total cellular RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Three micrograms of RNA were reverse-transcribed using SuperScript III RT (Invitrogen) and an oligo(dT)20 primer. Each Pcdh-α isoform amplification was performed with LA Taq (Takara) for 30 cycles at 96 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. One specific primer and one universal primer (Table supplemental S3) were used to amplify each Pcdh-α cDNA.

Quantitative Real-time RT-PCR—The quantification of Pcdh-α mRNAs and the control, murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, was carried out by a real-time fluorescence detection method. The RNA samples (5 μg in an 80-μl reaction volume or 2.5 μg in a 40-μl reaction volume), which had been treated with DNase I (Takara), were reverse-transcribed using SuperScript III RT and random hexamers. PCR amplifications were carried out with the gene-specific primers summarized in supplemental Table S4 using the 7900HT Sequence Detection System and SYBR Green according to the manufacturer’s instructions (Applied Biosystems). The Pcdh-α mRNA levels were normalized for each well to the GAPDH mRNA level or to the entire amount of Pcdh-α CR. At least four independent analyses were performed for each gene. Statistical significance was calculated using StatViews, and analysis of variance was performed with Student’s t test as a multiple comparison test.

Plasmid Construction and DNA Methylation for Luciferase Assay—Upstream fragments of Pcdh-α9 and -α10 were isolated by PCR using the set of primers shown in supplemental Table
S5. The template for PCR was the *M. musculus* genomic BAC clone RP23–303I8. Each blunt-ended PCR product was cloned into the SmaI site of the pGL3-Basic vector (Promega, Madison, WI), a promoterless luciferase vector.

To make methylated or unmethylated vectors for the luciferase assay, *Pcdh*-α9 and -α10 vectors were methylated by SssI CpG methyltransferase (M. SssI, New England Biolabs) in the presence (methylated) or absence (mock-methylated) of 160 mM S-adenosyl-L-methionine. The completion of the methylation reaction was confirmed by digestion with HpaII, HhaI, ClaI, Nael, or AccII. The vectors were then treated with phenol-chloroform-isooamyl alcohol and precipitated with ethanol. Both the methylated and unmethylated *Pcdh*-α9 and -α10 vectors were diluted in Tris-EDTA buffer and quantified by their absorbance at 260 nm.

**Transfection and Luciferase Reporter Assay—**C1300 cells were plated in 24-well plates at 1 x 10⁶ cells per well 1 day prior to transfection. All luciferase vectors (100 ng) were introduced into C1300 cells by using Lipofectamine 2000 reagent (Invitrogen) with pRL-TK (2 ng). Luciferase activities were determined 40 h after the transfection, using a Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions. The promoter activity was normalized via the Renilla luciferase activity. Assays were performed three times, each time in triplicate.
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DNA Methylation Analysis of Pcdh-α in Purkinje Cell Populations—Purkinje cells were isolated as described by Esumi et al. (16). The cerebellum of C57BL/6 mice at postnatal day 21 was dissociated by a brief treatment with papain. The debris was removed by filtration and FBS-density slope centrifugation. Following resuspension in Dulbecco’s modified Eagle’s medium, the Purkinje cells were collected using a glass capillary under an optical microscope. All the Purkinje cells obtained from a single cerebellum were gathered in a tube containing 10 μl of phosphate-buffered saline/10 mM EDTA.

gDNA was extracted from 500 Purkinje cells with the QIAamp DNA Micro Kit (Qiagen). Bisulfite treatment was performed on the gDNA with the Epitect Bisulfite Kit (Qiagen) according to the manufacturer’s instructions. The treated gDNA was eluted twice with a 10-μl volume of elution buffer and divided into two 10-μl aliquots for use as individual PCR samples. PCR conditions were the same as for the bisulfite genomic sequencing described above.

RESULTS

Comparison of the Pcdh-α Isoform mRNA Levels and Genomic Methylation in C1300 and M3 Cell Lines—To explore the Pcdh-α gene regulation mechanism, we searched cultured cell lines for those that consistently expressed a given combination of Pcdh-α isoforms. Using RT-PCR to detect each Pcdh-α isoform, we found that neuroblastoma C1300 cells and melanoma M3 cells showed different Pcdh-α transcription patterns: C1300 transcribed the α1, α8, α9, αC1, and αC2 isoforms, and M3 transcribed the α8, α9, α10, and αC2 isoforms (Fig. 1B).

To determine the methylation states of individual CpGs in the Pcdh-α isoforms, we carried out bisulfite genomic sequencing of the Pcdh-α10 and -αC1 promoter regions and of the 5′-region of the Pcdh-αC2 exon in both cell lines. The Pcdh-α isoforms showing the highest mRNA levels (αC1 and αC2 in C1300, and α10 and αC2 in M3) were hypomethylated at the promoter and the 5′-regions of the first exon, whereas the promoter regions of the Pcdh-α isoforms with low mRNA levels (α10 in C1300 and αC1 in M3) were hypermethylated at the CpGs (Fig. 2, A and B). In addition, a methylation-sensitive Southern blot for the Pcdh-α10 locus (supplemental Fig. S1A) showed that the CCGG located at the Pcdh-α10 promoter was highly methylated in C1300 cells but showed virtually no methylation in M3 cells (supplemental Fig. S1B). These data are consistent with a report on human cell lines (14).

Surprisingly, the bisulfite sequencing did not show hypermethylation of the promoter region of the silent Pcdh-α1 in M3 cells, unlike the other silent promoter regions. Instead, we found a mosaic pattern of methylated and unmethylated CpGs in the Pcdh-α10 promoter (supplemental Fig. S1A). In addition, the promoter region of Pcdh-α9, which is transcribed in M3 cells, also showed varying methylation patterns, including unmethylated, hypermethylated, and mosaic methylated DNA strands, but not hypomethylation, unlike the other active Pcdh-α promoter regions (Fig. 2A).

DNA Methylation Patterns of the Promoter and Exon 5′ Region but Not 3′ Region Correlate with the Selective Transcription of Pcdh-α Isoforms—To determine if CpG methylation patterns in other regions of the exons might also affect selective isoform transcription in the Pcdh-α cluster, we examined the
DNA methylation in the CpG-rich 5′ and 3′ domains (Fig. 3A) as well as the promoter, for each variable exon in the Pcdh-α cluster of C1300 cells.

We found that the Pcdh-α4 and -α10 isoforms, which were transcribed at low levels, were hypermethylated in the promoter and 5′-regions of the exons. In contrast, the Pcdh-α1, -α8, and -α9 isoforms, which were transcribed at higher levels, were hypomethylated in these regions (Fig. 3B). These data corresponded with those of Tasic et al. (14). However, the 3′-regions of the variable exons were heavily methylated in all the Pcdh-α1 to -α12 isoforms, including Pcdh-α1, -α8, and -α9 (Fig. 3B). A methylation-sensitive Southern blot for the downstream region of the Pcdh-α1 variable exon (Fig. 4A) revealed highly methylated CCGG sites in the 3′-region in the gDNA of both C1300 and M3 cells (Fig. 4B). On the other hand, a Southern blot for the 5′-region of the Pcdh-α1 exon (supplemental Fig. S1C) showed that the C1300 gDNA was unmethylated, but that of the M3 cells was methylated (supplemental Fig. S1D).

These data showed differential states of DNA methylation in the promoter and the 5′, but not the 3′-region of each Pcdh-α variable exon. Therefore, methylation of the promoter and/or the 5′-region might control the selective transcriptional activation of each Pcdh-α isoform.

Alteration of the Pcdh-α Isoform Transcription Patterns in C1300 Cells by Treatment with the Demethylating Reagent 5aC—To examine directly the effects of CpG methylation on the transcription levels of Pcdh-α mRNAs, we blocked the activity of DNA methyltransferase I (Dnmt1) with the demethylating reagent 5aC to obtain unmethylated CpGs. We treated C1300 cells with different concentrations of 5aC and extracted both the total RNA and the gDNA and compared the total Pcdh-α mRNA levels (Pcdh-α CR) in normal and 5aC-treated C1300 cells by quantitative real-time RT-PCR. The overall transcription levels of the Pcdh-α isoforms began to rise even at the lowest dosage (1 μM) of 5aC and increased dose dependently, saturating at 5 μM 5aC (Fig. 5A), when all of the isoforms were
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expressed. In contrast, and as expected, the untreated C1300 cells transcribed only the α1, α8, and α9 isoforms (Fig. 5B).

We next focused on the effects of 5aC on the mRNA levels of the Pcdh-α4, -α9, and -α10 isoforms in a detailed analysis. Quantitative real-time RT-PCR showed that the mRNA level of Pcdh-α10 (which is untranscribed in untreated C1300 cells) was detectable in C1300 cells treated with 5 or 10 μM 5aC, but not 1 μM (Fig. 6A). Treatment with 5 μM 5aC induced the transcription of Pcdh-α10 mRNA to 8.2 times its level in the untreated cells (Fig. 6A). The DNA methylation patterns of the Pcdh-α10 promoter region in the 5aC-treated C1300 cells showed heterogeneous demethylation (Fig. 6B), compared with untreated C1300 cells (Fig. 3B). Treatment with 1 μM 5aC did not lead to significant CpG demethylation (Fig. 6B), and the level of the Pcdh-α10 mRNA did not change (Fig. 6A). Treatment with 5 and 10 μM 5aC increased the frequency of demethylated CpGs (Fig. 6B), and the level of Pcdh-α10 mRNA increased significantly in parallel (Fig. 6A). Thus, demethylation of the Pcdh-α10 promoter region may be sufficient to up-regulate its transcription. A parallel increase in mRNA level with DNA demethylation was also seen for the Pcdh-α4 isoform following treatment with 5 and 10 μM 5aC, although the increase in mRNA was smaller than for Pcdh-α10 (Fig. 6, C and D). On the other hand, Pcdh-α9, which was already transcribed at a high level and extensively hypomethylated in the untreated C1300 cells, was transcribed at even higher levels with 1 μM 5aC treatment, although the methylation pattern was unchanged (Fig. 6, E and F).

Differential Transcription Patterns and DNA Methylation of the Promoter Regions of Pcdh-α Isoforms in Cell Lines Subcloned from 5aC-treated C1300 Cells—To examine the relationship between differential transcription patterns and the methylation states of the Pcdh-α genes, we isolated nine independent sublines from the 5aC-treated C1300 cells. Each subline showed increases in the total Pcdh-α mRNA levels compared with the untreated C1300 cells (Fig. 7A). RT-PCR for the Pcdh-α1 to -α12 isoforms showed similar transcription patterns to the untreated C1300 cells in five of the sublines. The other four (Clones 1, 2, 5, and 7) showed different transcription patterns (Fig. 7B). These sublines maintained high levels of Pcdh-α4, -α8, and -α9 mRNA and additionally transcribed Pcdh-α10 and -α12 (Clone 1), -α5, -α6, and -α7 (Clone 2), -α2, -α4, -α5, -α7, -α10, -α11, and -α12 (Clone 5), or Pcdh-α2, -α5, -α7, -α10, -α11, and -α12 (Clone 7). Thus, subcloning from the demethylated C1300 cells yielded novel cell lines that transcribed different combinations of Pcdh-α isoforms as well as lines exhibiting the original C1300 transcription pattern.

To compare the DNA methylation patterns with the transcription levels of Pcdh-α isoforms in each subline, we performed quantitative real-time RT-PCR and bisulfite genomic sequencing analysis for the Pcdh-α4 and -α10 isoforms. In untreated C1300 cells, the Pcdh-α4 and -α10 isoforms were not transcribed or were transcribed at undetectable levels (Fig. 5B). However, Clones 1, 5, and 7 accumulated high levels of Pcdh-α10 mRNA (Fig. 8A). Clone 5 also transcribed high levels of Pcdh-α4 mRNA (Fig. 8C). In the Pcdh-α10 promoter, Clones 5 and 7 contained both unmethylated and hypermethylated DNA strands. In contrast, the Pcdh-α10 promoter of Clone 1 showed demethylation at only one CpG among the eight in the promoter region (Fig. 8B). A similar pattern was observed in the Pcdh-α4 promoter of Clone 5 (Fig. 8D). In contrast, several sublines that showed mosaic demethylation or a mixture of unmethylated and hypermethylated DNA strands in Pcdh-α promoters also showed low transcription levels of these isoforms (Pcdh-α10, Clones 9 and 10; Pcdh-α4 Clones 2 and 7; Fig. 8). These data showed that a mosaic of methylated and demethylated CpGs or a mixture pattern in the promoter regions of Pcdh-α genes could either activate or inactivate transcription.

The CSE core sequence in each Pcdh promoter contains a CpG (14). To clarify the role of the methylation of this CpG, we investigated the relationship between its methylation and the corresponding transcription level. The promoters containing a methylated CpG in the CSE core sequence (Pcdh-α10 of Clone 1 and Pcdh-α4 of Clone 5) showed high transcription levels (Fig. 8). In contrast, the promoters with a demethylated CpG in the CSE core sequence (Pcdh-α10 of Clone 10 and Pcdh-α4 of Clones 2 and 7) showed low transcription levels (Fig. 8). These data suggested that the methylation state of the CpG in the CSE core sequence would not affect the transcription levels of Pcdh-α isoforms.
DNA Hypermethylation Represses the Activity of the Pcdh-α Promoters—Because mosaic methylation patterns in the Pcdh-α promoters were associated with both active and inactive transcriptional states, and because the hypermethylated Pcdh-α promoters seemed not to drive transcription, we investigated the effect of promoter demethylation or hypermethylation using a promoter assay. To do this, we constructed several Pcdh-α promoter-luciferase vectors with or without CpG methylation in the promoter region. We used a fragment of ~500 bp upstream from the ATG of the Pcdh-α isoform to drive the luciferase vector, and performed reporter assays in C1300 cells. The unmethylated Pcdh-α promoter-luciferase vectors showed high levels of transcription (Fig. 9A), even though the Pcdh-α isoform was expressed at low levels in normal C1300 cells (Fig. 1B), consistent with an inhibitory role for CpG methylation in the Pcdh-α promoter. On the other hand, when the Pcdh-α promoter-luciferase vectors were artificially methylated by M. SssI, both promoters drove very low levels of luciferase transcription compared with the unmethylated vectors (Fig. 9B and C), even though the Pcdh-α isoform was transcribed at a high level in normal C1300 cells (Fig. 1B). These results support the hypothesis that DNA hypermethylation in the Pcdh-α promoter represses transcription.

DNA Methylation of Specific Pcdh-α Promoter Regions in the Central Nervous System—To explore DNA methylation patterns in the Pcdh-α promoter region in vivo, we analyzed total gDNA extracted from the cerebral cortex of adult C57BL/6 mice. The DNA methylation patterns of the Pcdh-α1, -α4, and -α10 promoters showed hypermethylation, hypomethylation, and mixed methylation (Fig. 10A). In contrast, the Pcdh-αC1 promoter and the 5′-region of the Pcdh-αC2 exon showed extensive hypomethylation (Fig. 10B).

It is known that Purkinje cells transcribe the Pcdh-α1 to -α12 isoforms with a monoallelic pattern that is variable and stochastic (11) but transcribe the Pcdh-αC1 and -αC2 isoforms with a biallelic pattern that is constant (12). To explore the relationship between DNA methylation in the Pcdh-α promoters and their transcriptional activity in neurons, we analyzed the DNA methylation patterns of cerebellar Purkinje cells. As shown in
Tomikawa et al. (17), the amount of gDNA extracted from 200–500 cells was sufficient to analyze the DNA methylation states. First, we verified that we could determine the CpG methylation patterns of the Pcdh-α10 promoter in gDNA derived from 500 C1300 or M3 cells (data not shown). Next, we analyzed the methylation states of the Pcdh-α10 promoter in 500 Purkinje cells from the cerebellum of C57BL/6 mice at postnatal day 21. Several different patterns, including hypermethylated and hypomethylated DNA strands, were found (Fig. 10C). These data suggested that individual Purkinje cells had individual DNA methylation patterns in the Pcdh-α10 promoter region, a region that is activated stochastically.

In contrast, methylation-sensitive Southern blots for the Pcdh-α2C locus using gDNA from the whole cerebellum (supplemental Fig. S2A) revealed that the CCGG sites in the Pcdh-α2C promoter region were hypomethylated (supplemental Fig. S2B). A similar result was obtained from M3 cells (supplemental Fig. S2B), in agreement with the bisulfite genomic sequencing data (Fig. 2B). Purkinje cells were among the cerebellar cells loaded on the blot, so these data are consistent with the Purkinje cells containing an extensively hypomethylated Pcdh-α2C promoter region and with the known biallelic transcription of the Pcdh-α2C isoform by Purkinje cells. These data indicated that the monoallelic or biallelic transcription patterns of Pcdh-α isoforms are correlated with the DNA methylation patterns of their promoter and 5′ variable exons, in individual neurons.

**DISCUSSION**

In this study, we showed that DNA methylation in the promoter and 5′-regions, but not the 3′-regions, of the Pcdh-α-variable exons correlates with the differential transcription patterns of the Pcdh-α isoforms in C1300 and M3 cells. Treatment with 5aC, a demethylating reagent, induced the transcription of all the Pcdh-α isoforms in C1300 cells. Subcloning of the 5aC-treated C1300 cells yielded sublines showing differential transcription of the Pcdh-α isoforms and corresponding differential methylation patterns. These results indicated that CpG hypermethylation in the Pcdh-α promoter region is necessary to repress the transcription of the corresponding isoform and that hypomethylation or mosaic methylation states could result in active or inactive transcriptional states. In addition, in the central nervous system, extensive hypomethylation was found in the Pcdh-αC1 and -αC2 isoforms, which are transcribed biallelically in neurons. In contrast, various patterns of DNA methylation were found in the promoters of the Pcdh-α1 to -α12 isoforms, which transcribed differentially in individual neurons. These results indicated that the differential transcription patterns of individual Pcdh-α isoforms in neurons may be regulated in part by DNA methylation.

Tasic et al. (14) previously showed a close correlation between Pcdh-α6 isoform transcription and DNA methylation in two human cell lines. Here, using two mouse cell lines (C1300 and M3) and mouse cerebral and cerebellar tissue, we confirmed the correlation between transcription and DNA methylation for the Pcdh-α10, -αC1, and -αC2 isoforms. We also investigated the relationship between the transcription and methylation states of the Pcdh-α genes in C1300 cells. Extensive DNA methylation was found in the 3′-regions of all the Pcdh-α1 to -α12 variable exons, whereas little DNA hypermethylation was seen in the promoter and 5′-region of actively transcribed isoforms (Pcdh-α1, -α8, and -α9). Thus, DNA hypomethylation in the promoter and 5′-regions of the variable exons correlated with transcriptional activation, but that of the 3′-region did not, despite its high CpG content. A similar result was reported for the rat sphingosine kinase-1 gene (18). The multiple first exons of sphingosine kinase-1 are transcribed tissue specifically, and this specificity is regulated by the DNA methylation of specific CpGs in certain CpG islands. Similar tissue-dependent regulation of transcription by differential methylation in CpG islands has been reported for E-cadherin (19), endothelin receptor B (20), and proopiromelanocortin (21).

In addition, the DNA demethylation induced by 5aC treatment resulted in the transcription of Pcdh-α isoforms that were unexpressed in untreated C1300 cells. Similarly, Pcdh-γA11 transcripts, which are repressed in astrocytic gliomas, are upregulated by demethylation (22). We examined the correlation between DNA methylation at the Pcdh-α promoters and their transcriptional regulation in the subclones derived from the C1300 cells treated with 5aC. Demethylation of the promoter region of the Pcdh-α4 or -α10 exon correlated with an increased transcription level, but the transcription also increased in some cases when the DNA showed mosaic or
mixed demethylation. Although Pcdh-α10 in Clone 1 and Pcdh-α4 in Clone 5 were transcribed at higher levels, only one unmethylated CpG site (not in the CSE core sequence) was found in the promoter regions. Furthermore, several clones that showed demethylation at the CSE core sequence showed low levels of transcription. These results suggest that demethylation of even one CpG can affect the level of Pcdh-α transcription, and that these functional CpGs are not necessarily located within the CSE core sequence.

Tasic et al. (14) have suggested that the CSE core sequence is essential for transcriptional activity of the Pcdh-α promoters, because promoter regions lacking this sequence drive lower transcription levels. However, our finding that the methylation state of the CpG in the CSE core sequence was independent of transcriptional activity does not support this interpretation. We obtained similar results for Pcdh-α1 in M3 cells, where the CpG of the CSE in the Pcdh-α1 promoter showed mosaic methylation and hypomethylation, but its overall transcription level was low. These findings suggest that there are several transcription activation domains in the Pcdh-α promoter region: the CSE core sequence, which is not repressed by CpG methylation, and other domains that are repressed by it. In contrast, the Pcdh-α10 promoter in Clone 5 contained two different DNA-strand types: the CpGs in the promoter region were either all unmethylated or all methylated. A similar observation was made for Pcdh-α9 in M3 cells. Pcdh-α9 was transcribed in M3 cells, and the promoter region contained unmethylated and methylated DNA strands. Thus, each allele of the Pcdh-α promoter region in individual cells could have a different CpG methylation pattern, which would regulate which isoforms were expressed and from which allele.

We also demonstrated the effects of DNA hypermethylation in luciferase assays. The Pcdh-α10 promoter was inactive in C1300 cells, but a luciferase expression vector inserted behind the unmethylated α10 promoter showed transcriptional activity as strong as that of the unmethylated α9 promoter, which is
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normally active in C1300 cells. The transcriptional activities of the recombinant Pcdh-α9 and -α10 promoters were inhibited by DNA hypermethylation. These results suggest that every Pcdh-α promoter is potentially active for transcription, but DNA hypermethylation is sufficient to repress the activity. These data also suggested that binding proteins or accessory regulators for the Pcdh-α promoters might be sensitive to DNA methylation. A recent study of a large portion of the genome in primary human fibroblasts revealed that multiple binding sites for CCCTC-binding factor are localized to Pcdh gene clusters (23). CCCTC-binding factor is sensitive to DNA methylation (29–31). Thus, the regulation of DNA methylation also contributes to the regulation of differential expression (26) and CpG-specific demethylation (27). In addition, maternal behavior alters the methylation state of a CpG site in the promoter (28) and increases the transcriptional activity. Interestingly, sublines of 5aC-treated C1300 cells transcribed additional Pcdh-α isoforms, and which isoforms were expressed varied among the sublines, indicating that demethylation of the parent cells resulted in the derepression of some isoforms. Furthermore, all the sublines showed a decrease in the transcription levels of the Pcdh-α9 isoform, whose promoter region is extensively hypomethylated in normal C1300 cells (supplemental Fig. S3). In particular, Clone 5, which additionally transcribed Pcdh-α2, -α6, -α5, -α7, -α10, -α11, and -α12, expressed much lower Pcdh-α9 mRNA levels than the untreated C1300 cells. These results suggest that the number of demethylated Pcdh-α promoters might influence the transcription level for each Pcdh-α isoform. To reveal the molecular mechanism for the regulation of Pcdh-α promoters, we need to explore further the relationship between cis-regulatory elements and methylated or hypomethylated promoters in the Pcdh-α gene cluster.

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