Identification of the Cluster Control Region for the Protocadherin-β Genes Located beyond the Protocadherin-γ Cluster*§

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The clustered protocadherins (Pcdhs), Pcdh-α, -β, and -γ, are transmembrane proteins constituting a subgroup of the cadherin superfamily. Each Pcdh cluster is arranged in tandem on the same chromosome. Each of the three Pcdh clusters shows stochastic and combinatorial expression in individual neurons, thus generating a hugely diverse set of possible cell surface molecules. Therefore, the clustered Pcdhs are candidates for determining neuronal molecular diversity. Here, we showed that the targeted deletion of DNase I hypersensitive (HS) site HS5-1, previously identified as a Pcdh-α regulatory element in vitro, affects especially the expression of specific Pcdh-α isoforms in vivo. We also identified a Pcdh-β cluster control region (CCR) containing six HS sites (HS16, 17, 17′, 18, 19, and 20) downstream of the Pcdh-γ cluster. This CCR comprehensively activates the expression of the Pcdh-β gene cluster in cis, and its deletion dramatically decreases their expression levels. Deleting the CCR non-uniformly down-regulates some Pcdh-γ isoforms and does not affect Pcdh-α expression. Thus, the CCR effect extends beyond the 320-kb region containing the Pcdh-γ cluster to activate the upstream Pcdh-β genes. Thus, we concluded that the CCR is a highly specific regulatory unit for Pcdh-β expression on the clustered Pcdh genomic locus. These findings suggest that each Pcdh cluster is controlled by distinct regulatory elements that activate their expression and that the stochastic gene regulation of the clustered Pcdhs is controlled by the complex chromatin architecture of the clustered Pcdh locus.

The brain contains enormous numbers of neurons, which are assembled into functional neural circuits and express diverse molecules that mediate cell-cell interactions. Identifying the molecular mechanisms underlying neuronal diversity is one of the most basic yet elusive goals for understanding the development of nervous system. Extensive molecular diversity among families of cell surface proteins could contribute to neuronal diversity. The clustered Pcdhs, a subgroup of the cadherin superfamily, encode nearly 60 Pcdh proteins on the same chromosome. The molecular diversity of the clustered Pcdhs makes this family an attractive candidate for generating neuronal diversity in vertebrates (1, 2).

The clustered Pcdhs are classified into three subfamilies, protocadherin-α (Pcdh-α), -β (Pcdh-β), and -γ (Pcdh-γ) (see Fig. 1A) (3–5). Each clustered Pcdh isoform has its own promoter, and the differential promoter activation of the clustered Pcdhs control determines which variable exons are transcribed (6, 7). The Pcdh proteins are located, at least in part, on synapses (3, 8–10). Pcdh-γ-deleted mice die within a few hours of birth (9) and show decreased numbers of synapses in the spinal cord (9, 11). Pcdh-α mutant mice show abnormalities in the axonal sorting of olfactory sensory neurons into glomeruli and in the projections of serotonergic neurons (12, 13). These findings suggest that the clustered Pcdhs may regulate the formation of neural circuits.

Most of the clustered Pcdh isoforms are expressed throughout the developing and adult brain (10, 14, 15). In many regions of the brain, a few scattered neurons are labeled by in situ hybridization of probes for each clustered Pcdh isoform (3, 7, 9, 15–18). By the single-cell RT-PCR analysis of Purkinje cells, a single neuron expresses one or more isoforms from each clustered Pcdh subfamily, with the exception of the Pcdh-α and -γ C-type isoforms (16, 17). Moreover, single neurons express a subset of isoforms from both the Pcdh-α and Pcdh-γ clusters (17). Thus, individual neurons express different combinations of the clustered Pcdhs. On the other hand, the same single-cell RT-PCR analysis also revealed that each Pcdh isoform shows primarily monoallelic expression and rarely biallelic expression, except for the Pcdh-α and -γ C-type isoforms, which show biallelic expression (16, 17). Thus, the stochastic expression...
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and combinatorial expression of the clustered Pcdhs will include the complexity of the gene regulatory mechanisms.

Clustered genes consist of two and more genes that encode proteins of similar function and share common cis-regulatory elements. A well known example is the human β-globin gene cluster, which contains six β-like globin genes and their distal regulatory elements, the locus control region (LCR). The β-globin LCR is necessary for the high level expression of all β-like globin genes (19–22). It has been speculated that the Pcdh cluster is controlled by a regulatory element located near the Pcdh cluster locus. Around and within the Pcdh-α cluster, DNase I hypersensitive site (HS) mapping analysis has identified 15 HS sites (HS1–15) (23). Of these, HS5-1 is located 30 kb downstream of the Pcdh-αCR3 exon (Fig. 1A) and has enhancer activity in the central and peripheral nervous systems. Furthermore, Pcdh-α gene expression, except for the Pcdh-αC2 isoform, decreases in HS5-1-deleted ES cells that differentiate into postmitotic neurons (23). However, because the deletion of HS5-1 does not eliminate the expression of the Pcdh-α1–12 isoforms, it has not been clear whether it is a global regulatory element for the Pcdh-α cluster in the mouse nervous system. The regulatory elements for the Pcdh-β and Pcdh-γ genes have not yet been identified.

Here, we identified a Pcdh-β cluster control region (CCR) containing six HS sites (HS16, 17, 17’, 18, 19, and 20) in a region 320 kb downstream of the Pcdh-β22 gene. This CCR comprehensively activated the Pcdh-β genes. We investigated the role of the CCR in several strains of mice in which the regulatory elements were eliminated. First, we showed that the deletion of HS5-1 had strong effects on the expression of the Pcdh-α1–12 isoforms in vivo (23). However, because the deletion of HS5-1 does not eliminate the expression of the Pcdh-α1–12 isoforms, it has not been clear whether it is a global regulatory element for the Pcdh-α cluster in the mouse nervous system. The regulatory elements for the Pcdh-β and Pcdh-γ genes have not yet been identified.

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In Situ Hybridization—In situ hybridization was performed as described previously (18), using 10-μm fresh-frozen cryosections of P21 brains. Briefly, the sections were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by acetylation for 10 min. The sections were hybridized with digoxigenin-labeled antisense probes to Pcdh-αCR, and Pcdh-α isoforms mRNAs (13, 18), at 72 °C. The Pcdh-β isoform-specific probes were designed based on mouse Pcdh-β16 (nucleotides 277–1293; GenBank™ accession number NM_053141) and Pcdh-β22 (nucleotides 213–1146; GenBank™ accession number NM_053147). The signals were detected by alkaline phosphatase-coupled anti-digoxigenin antibodies with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as a chromogenic substrate.

Cytochrome Oxidase Histochemistry—Mice were perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.05% cytochrome oxidase staining, the sections were incubated in a solution containing 0.05% cytochrome c, 0.05% dianinobenzidine, and 4% sucrose for 3 h at 37 °C. The sections were then rinsed with 0.1 M phosphate buffer and mounted onto glass slides.

RESULTS

Strong Effect of HSS-1 Sites on the Pcdh-α10–12 Isoforms—Previous studies reported the enhancer element of the Pcdh-α cluster, HSS-1, which is located 30 kb downstream of the Pcdh-αCR3 exon (Fig. 1A) (23). First, to reveal whether it specifically
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.regulates all of the Pcdh-α1–12 isoforms in vivo, we generated HS5-1-deleted (ΔHS5-1/ΔHS5-1) mice (supplemental Fig. S1) and examined the expression levels of the Pcdh-α isoforms in the P21 brain by qPCR analysis. The expression level of the total Pcdh-α mRNAs (αCR) was decreased in the HS5-1-deleted mice, to 68% of the level in wild-type (+/+ ) mice (Fig. 1B). The expression levels of the Pcdh-α1, α11, and α12 isoforms were markedly decreased, to <10% of the expression levels in the wild-type mice (Fig. 1B), and levels of the Pcdh-α3 to α9 isoforms were also significantly decreased, by 24–61%. The levels of the Pcdh-α1 and α2 isoforms scarcely changed. Thus, for the Pcdh-α1–12 isoforms, the effect of HS5-1 deletion depended on the distance of each isoform from HS5-1. As previously reported (23), the expression level of the Pcdh-αC1 isoform decreased to 29% of the level in wild-type mice, but the level of the Pcdh-αC2 isoform did not change (Fig. 1B). The expression levels of the Pcdh-β genes distal from HS5-1 and the Pcdh-γ genes were unchanged by the HS5-1 deletion (Fig. 1, C and D). However, the expression levels of some Pcdh-β genes, the ones closest to HS5-1, increased significantly in the HS5-1-deleted mice (Fig. 1C). These findings suggested that HS5-1 is necessary for the appropriate expression of the Pcdh-α and -β genes.

In many regions of the brain, the spliced CR transcripts (CR1–CR3 exons) signals, which are common to all the Pcdh-α genes, were detected, whereas a few scattered neurons are labeled by in situ hybridization of probes for each Pcdh-α isoform (12, 13, 16–18). The signals of the Pcdh-αCR transcripts detected by in situ hybridization were decreased, consistent with the qPCR results (supplemental Fig. S2). Clear, scattered signals of the Pcdh-α12 transcripts were not detected in the HS5-1-deleted brain by in situ hybridization. The scattered expression of the Pcdh-α4 transcript was detected in these mice, although it was less obvious than in wild type. Thus, HS5-1 had a graded effect on the expression of the Pcdh-α3-12 isoforms, except for the Pcdh-αC type isoforms; in particular, HS5-1 was an essential regulatory element for the expression of Pcdh-α10–12, but its effect on the expression of Pcdh-α3–9 was much less strong. On the other hand, these results also suggested that isoform expression in each Pcdh cluster is regulated by specific regulatory elements.

Identification of Novel HS Sites in the Downstream Region of the Pcdh-γ Locus—We next sought to reveal comprehensively the regulatory elements in the clustered Pcdh locus. The total length of the Pcdh-β and Pcdh-γ clusters is nearly 600 kb in the mouse genome. To identify candidates for cis-regulatory elements in the Pcdh-β and Pcdh-γ loci, we compared the mouse genomic sequence from 5 kb upstream of the Pcdh-β1 to 40 kb downstream of the Pcdh-γCR3 exon with the corresponding human and opossum genomic sequences. We found 19 highly conserved elements in the intergenic and intronic regions among the three species (Fig. 2, supplemental Tables S3 and S4). Six highly conserved elements were located in the intronic regions between the Pcdh-γA12 and Pcdh-γCR3 exons, and three of these were in the 5′-UTR of the Pcdh-γ C-type exons. In addition, 13 highly conserved elements lay within the 40-kb region downstream of the Pcdh-γCR3 exon. Eight of the 13 were also located in the intronic regions of Diap1, a ubiquitously expressed noncatalytic gene that encodes a Rho effector protein (24). Diap1 knock-out mice show no apparent abnormality, except in the immune system (25).

To analyze whether the identified conserved elements included functional regulatory elements, we first performed DNase I hypersensitivity assays in mouse neuroblastoma C1300 cells, which express the Pcdh-β and Pcdh-γ isoforms. A schematic diagram of the Southern probes and restriction enzyme sites is shown in Fig. 3A. We detected one HS site upstream of the Pcdh-γC3 exon in the C1300 cells (Fig. 3B). This HS site corresponded to the Pcdh-γC3 proximal promoter region, which includes a conserved sequence element (7). Because Pcdh-γC3 mRNA was detected in the C1300 cells (data not shown) and the DNase I hypersensitivity assay can detect active promoters, we excluded this HS site from the candidates for regulatory elements of the clustered Pcdhs. We did not detect any other HS sites among the highly conserved elements within the Pcdh-γ locus. Downstream of Pcdh-γCR3, we found 5 HS sites: two at 7–10 kb downstream and three at 35–39 kb downstream (Fig. 3B). Because a previous study reported 15 HS sites (HS1–15) in the Pcdh-α locus (23), we called these new HS sites HS16, 17, 18, 19, and 20. To determine whether these HS sites were present at a similar position in another cultured cell line expressing the clustered Pcdhs, we performed a DNase I hypersensitivity assay using M3 cells (Fig. 3C). We confirmed that all five HS sites observed in the C1300 cells were also present in the M3 cells. However, the M3 cells contained an additional HS site next to HS17, which we termed HS17* (Fig. 3C). A schematic summary of the six HS sites is shown in Fig. 3D.
Because HS17 and HS17' were close to one another on the genomic locus, as were HS19 and HS20, we treated each pair as a single element, HS17–17' and HS19–20, in the following experiments. HS19–20 Has Enhancer Activity in Vitro and in Vivo—To evaluate whether these HS sites had enhancer activity, we performed transient luciferase reporter assays. To drive the luciferase gene, the respective 1-kb upstream regions that included the Pcdh-γ6 or -γ1 elements were used as the promoter. The HS19–20 element strongly increased the reporter activity, by 5–30-fold, in both cell lines (Fig. 4A).

The HS17–17' and HS18 elements caused slight increases in the reporter activity. In contrast, although the HS16 element did not show enhancer activity compared with base line in the C1300 cells, it caused a 19–50-fold enhancement in the M3 cells. Because the HS19–20 element showed strong enhancer activity in both cell lines, we analyzed its enhancer function in vivo, by generating HS19–20 transgenic mice. The founder animals were then stained for lacZ expression on E14.5, when the Pcdh-γ6 and -γ1 genes are known to be expressed in the CNS (10, 26). Of five independent transgenic mouse lines, four showed strong -gal signals in the CNS (Fig. 4B). These results indicated that HS19–20 was a likely candidate for an enhancer element of the clustered Pcdhs.

Absence of Cortical Barrels in HS16–20-deleted Mice—To reveal whether the total set of sites HS16–20 contains enhancing regulatory elements for the clustered Pcdhs, we generated HS16–20-deleted mice, which lacked a 39-kb fragment that included elements HS16–20. We obtained an HS16–20-deleted (ΔHS16–20) allele after recombination between the loxP

FIGURE 3. Identification of novel HS sites by DNase I hypersensitivity assay. A, schematic diagram of the DNase I hypersensitivity assay of the Pcdh-γ6 gene. Upper part, the large colored boxes, Pcdh-γ6 or Diap1 exons. The small red boxes indicate the 19 identified highly conserved elements. Lower part, restriction fragments used in the DNase I hypersensitive assay (black lines) and the Southern probes (black boxes). The lowercase letters in parentheses under the restriction fragments correspond to Fig. 3, B and C. 3, BamHI; X, XbaI; N, NheI; S, SacI; A, ApaI; H, HindIII; Bg, BglI; K, KpnI. 3, DNase I hypersensitivity assay. Nuclei were prepared from C1300 cells and treated with DNase I. The purified DNA was analyzed by Southern blotting. Black triangles indicate increasing concentrations of DNase I. The approximate size of each fragment (kb) and the position of each HS site are indicated. Asterisks indicate nonspecific signals. 3, DNase I hypersensitivity assay in M3 cells. 3, schematic showing the locations of the six identified HS sites. Red arrows indicate the position of each HS site.
sites of the γLacZ allele and the HS19–20-deleted (ΔHS19–20) allele by the targeted meiotic recombination (TAMERE) system (supplemental Fig. S3–S5) (27). In the process of generating the HS16–20-deleted allele, some Diap1 exons were also deleted. No visible abnormalities were observed in the HS16–20-deleted mice at birth. However, approximately 50% of them died within the first 3 days (P0–P3). The other mice survived weaning. The HS16–20-deleted mice could suck milk, but some of them lacked a milk spot at P0, suggesting that they might be out-competed by their littermates. In fact, the body weight of the HS16–20-deleted male mice was 66% ($p < 0.01$; $n = 8–9$) of that of their male littermates at 3 weeks.

To reveal whether the deletion of HS16–20 affected brain development, we examined the development of the barrel cortex in the HS16–20-deleted mice at P7 or P8. A comparison of the Nissl and cytochrome oxidase staining of the barrel cortex in wild-type and the HS16–20-deleted mice revealed that the organization of the barrel field was absent in the HS16–20-deleted mice (Fig. 5, A–D). On the other hand, the brainstem barrellettes and thalamic barreloids were normal even in the HS16–20-deleted mice (Fig. 5, E–H). These results indicated that the fragment containing HS16–20 is required for the proper segregation of barrels in the somatosensory cortex.

**FIGURE 4.** The HS19–20 element has enhancer activity in vitro and in vivo. A, the identified HS sites were introduced into luciferase vectors containing a Pcdh-γA6 or γB1 promoter, which were then transiently transfected into C1300 or M3 cells. The HS19–20 element had enhancer activity in both cell lines. The HS16 element had enhancer activity in only the M3 cells. Results are the mean ± S.D. (error bars). B, schematic represents the construct used for the HS19–20 enhancer assay. X-gal was used to stain the sagittal section of an E14.5 embryo harboring the HS19–20 transgene. Enhancer activity was detected in the central nervous system. Scale bar, 1 mm.

**FIGURE 5.** Disruption of the cortical barrel fields in ΔHS16–20 homozygous mice. A and B, cortical sections in control and HS16–20-deleted (ΔHS16–20/ΔHS16–20) mice were stained with cytochrome oxidase to visualize the barrel fields. Coronal sections revealed that the organization of barrels in the HS16–20-deleted brains was completely disrupted. C and D, Nissl staining of cortical sections showed defective organization in the HS16–20-deleted brains. E–H, coronal sections were cut through the thalamus (E and F) and brainstem (G and H), and stained with cytochrome oxidase. The barreloid and barrelette organization was similar in each genotype. Scale bars, 200 μm (A–F); 400 μm (G and H).
Pcdh-\(\gamma\) isoforms were also significantly decreased, to 30–69\% of the level in the \(\gamma\) LacZ-homozygous littermates. There was no specificity for the Pcdh-\(\gamma(A, -B, or -C) isoforms. However, the level of the Pcdh-\(\gamma\)A7 isoform increased to 172\% of the control level. These results indicated that, although the fragment including HS16–20 may contain regulatory elements activating the Pcdh-\(\gamma\) expression, the major regulatory elements for Pcdh-\(\gamma\) are at a different genomic locus.

Next, we investigated the expression of Pcdh-\(\beta\) in the HS16–20-deleted mice, using specific primers for each Pcdh-\(\beta\) isoform. Surprisingly, qPCR revealed that the expression levels of all 12 Pcdh-\(\beta\) genes tested in the HS16–20-deleted mice were...
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greatly decreased, to <10% of the levels in γLacZ-homozygous mice (Fig. 6C). Even though HS16 is located nearly 320 kb downstream of the Pcdh-β22 gene, its loss led to decreased expression of the Pcdh-β gene cluster. The 39-kb fragment including HS16–20 therefore included essential regulatory elements for the Pcdh-β gene cluster. In contrast, the HS16–20 deletion had no effect on the expression of the Pcdh-α, Taf7, and Slc25a2 genes (Fig. 6, D–F). Thus, the HS16–20 fragment is an important regulatory region for the Pcdh-β gene cluster and acts despite its location on the far side of the Pcdh-γ locus. Thus, we refer to the fragment including HS16–20 as a Pcdh-β cluster control region (CCR).

In the γLacZ/ΔHS16–20 mice, the expression levels of all 12 tested Pcdh-β genes were intermediate between those of the γLacZ/γLacZ and ΔHS16–20/ΔHS16–20 mice (Fig. 6C). If the CCR were a trans-acting regulatory element, the expression of Pcdh-β in the ΔHS16–20-heterozygous mice would have been derived from both alleles. We therefore carried out a cis-trans test. To distinguish from which allele the expression of the Pcdh-β genes in the +/ΔHS16–20 mice was derived, we performed direct sequencing to detect polymorphisms, using the brain of F1 mice obtained by crossing JF1 mice with +/ΔHS16–20 mice, in which the B6 allele was mutated. We then amplified the cDNA fragments of five Pcdh-β genes using specific primer combinations. We obtained F1 sequences from the +/ΔHS16–20 mice, whereas both B6 and JF1 sequences were obtained from the +/+ littermates (Fig. 6G). Thus, we concluded that the CCR is a cis-acting regulatory element specific for the Pcdh-β gene cluster.

To reveal whether deletion of the CCR would alter the sparse expression patterns of the Pcdh-β genes, we performed in situ hybridization in P21 γLacZ-homozygous and HS16–20-deteted mice. In the HS16–20-deteted Purkinje cells, we did not detect the normal strong, scattered signals of the Pcdh-β16 and β22 transcripts by in situ hybridization, although in the γLacZ-homozygous controls, the signals were easily detected in a normal scattered pattern (Fig. 7A). This was also true in the hippocampal CA3 region and the cerebral cortex (Fig. 7, B and C). Thus, for the Pcdh-β genes, the CCR in the HS16–20 region was essential to drive high enough levels of expression to detect the normal, scattered patterns. The CCR Requires Not Only HS19–20 but Also HS16–18—Finally, to evaluate the role of the only HS19–20 element in the mouse brain directly, the expression levels of the Pcdh-β genes in mouse whole brain at E18.5 were analyzed by RT-qPCR. The expression levels of the five tested Pcdh-β genes significantly decreased in the HS19–20-deleted (ΔHS19–20/ΔHS19–20) mice, to 39–63% of the level in wild-type mice (supplemental Fig. S7). Thus, the overall expression levels of the Pcdh-β genes in the HS19–20-deleted mice decreased significantly, by about half. These results suggested that the CCR required not only HS19–20 but also HS16–18 to regulate the Pcdh-β gene expression.

As described above, HS19–20 regulates the expression of the Pcdh-β cluster, even though it lies beyond the entire Pcdh-γ cluster, suggesting that the clustered Pcdh locus is organized into a complex chromatin architecture. We found potential CTCF binding sites in the HS16, HS17–17’, HS18, and HS19–20 elements by bioinformatics analysis (data not shown). In addition, a ChIP-chip analysis has shown that CTCF binds to human genomic sequences corresponding to the mouse genomic sequences identified as HS17–17’, HS18, and HS19–20 (28). These findings imply that each identified HS site regulates the expression of Pcdh-β and/or -γ by cooperatively forming CTCF-mediated changes in the chromatin architecture. DISCUSSION

Our present study demonstrated that the CCR contains cis-acting regulatory elements specific for the Pcdh-β cluster and that HS5–1 regulates the Pcdh-α gene, particularly the Pcdh-α10–12 isoforms. The deletion of HS5–1 had a relative, graded effect on the expression of the Pcdh-α3–12 isoforms and almost abolished the expression of the Pcdh-α10–12 isoforms. We also
identified six novel HS sites (HS16−20), downstream of the Pcdh-γ cluster, by homology analysis and DNase I hypersensitivity assays (supplemental Fig. S8). HS19−20 had enhancer activity in vitro and in vivo. Targeting analyses revealed that deletion of the CCR nearly abolished the expression of the Pcdh-γ genes, but not that of the Pcdh-α genes. Full Pcdh-β expression required not only HS19−20 but also the region containing HS16−18. These results suggested that each Pcdh-α, -β, and -γ gene is regulated by distinct cluster control regions, which have differential effects on each cluster (Fig. 8).

The CCR was specifically required for the full expression of the Pcdh-β cluster; that is, for the stochastic promoter choice of the Pcdh-β genes. Stochastic mechanisms use cis-regulatory elements to control the expression of multiple genes from one genomic locus (29). We suggest a model for the regulation of Pcdh-β cluster genes by CCR. The individual HS sites within the CCR may cooperatively regulate the expression of the Pcdh-β genes. In the β-globin locus, six hypersensitive sites (HS1−6) of the LCR, the upstream 5′ HS-60/-62, and the downstream 3′ HS1 together form an active chromatin hub (30), where it is widely thought that the LCR and promoters associate with each other. In the β-globin LCR, the deletion of HS2, which acts as an enhancer in transient reporter assays, leads to a slight decrease in the expression of the Bmajor and Bminor genes in the fetal liver and adult peripheral blood definitive erythroid cells (31, 32). In the present study, we identified six HS sites within the CCR. The deletion of HS19−20 decreased the expression of the Pcdh-β genes by approximately half, but did not abrogate it. HS16−18 were also necessary for the full expression of the Pcdh-β genes. In some single Purkinje cells of wild-type mice, plural Pcdh-β genes are expressed from one chromosome.3 We surmise that HS16−20 within the CCR might have the ability to regulate multiple promoters of Pcdh-β genes concomitantly, or that the expression of one Pcdh-β gene might temporarily alter the promoter use for other Pcdh-β genes, due to a change in the interaction between the CCR chromatin complex and the individual promoters of the Pcdh-β genes. Thus, the ability of complex chromatin architecture to form among the HS sites within the CCR might determine the number of Pcdh-β genes expressed in a single neuron.

Previous studies did not clarify how HS5−1 affects the expression of each Pcdh-α1–12 isoform. In this study, we revealed that HS5−1 had a large effect on the expression of the Pcdh-α10–12 isoforms, but did not control the expression of all the Pcdh-α isoforms. Because the deletion of HS5−1 had only a weak effect on the upstream Pcdh-α variable isoforms, we speculate that the upstream isoforms are regulated by a different element. Fifteen HS sites are located at the 3′ end of the Pcdh-α cluster (23). These HS sites are important candidate elements for regulating the remaining expression of the Pcdh-α isoforms. However, these HS sites are located at a distance from the 5′ end Pcdh-α isoforms, and regulatory elements for the Pcdh-αC2 isoform, which shows a constitutive and biallelic expression, have not been identified. Thus, we expect that there are other enhancer elements for the Pcdh-α isoforms at the 5′ end. In the MOR28 cluster of the mouse odorant receptor gene cluster, there is an H element with a similar graded effect on promoter activations (33, 34) as HS5−1 has in the Pcdh-α cluster. These studies proposed that there are other regulatory elements located at the opposite side of the gene cluster from the H element. In fact, two enhancers have been identified in the zebrafish odorant receptor gene cluster (34). We speculate that the situation is similar for the Pcdh-α genes, and that other regulatory elements, which we refer to as Xα element(s), located upstream of the Pcdh-α genes, regulate the upstream variable exons of the cluster (Fig. 8). A previous homology analysis revealed no highly conserved sites in the upstream region between the Pcdh-α1 exon and the Zmat2 gene (23). Therefore, the Xα element(s) may be quite far from the Pcdh-α locus, or they may lie within a nearer, but low homology, region. In any case, we suggest that the Pcdh-α1–12 isoforms are regulated by HS5−1 and by unidentified elements in an overlapping manner (Fig. 8).

The up-regulation of some Pcdh-β transcripts in the absence of HS5−1 was interesting because HS5−1 can act as an enhancer, but not a suppressor (23). We speculate that the deletion of HS5−1 alters the chromatin architecture of the clustered Pcdh locus, thereby inducing the up-regulation of Pcdh-β gene expression. This is one of the next questions to be addressed in revealing the comprehensive regulatory mechanisms of the Pcdh cluster.

The location and identity of the regulatory elements for the Pcdh-γ gene remain a mystery. Although there are HS sites within the Pcdh-α locus (23), we did not detect any within the Pcdh-γ locus by the DNase I hypersensitive assay, and we did not find any highly conserved elements between the Pcdh-B22

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3 Keizo Hirano, Ryosuke Komeko, and Takeshi Yagi, unpublished data.
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and -γA12 exons. The expression level of the Pcdh-γCR exon was only slightly decreased in the HS16–20-deleted mice; therefore, the CCR has only a minor role in regulated Pcdh-γ expression. We suggest that the critical regulatory elements for Pcdh-γ may not be located within the clustered Pcdh locus and CCR, but at another genomic locus. We postulate that the Xγ element(s) that regulate the Pcdh-γA and -γB variable exons exist downstream of the clustered Pcdh locus (Fig. 8). Further studies will be required to identify the regulatory elements for Pcdh-γ.

In this study, we found that the CCR predominantly affected the Pcdh-β cluster, and HS5-1 affected the Pcdh-α cluster in vivo. However, the identified HS sites up-regulated the activity of the Pcdh-γ promoter (Fig. 4A), and HS5-1 also up-regulated transcription activity of the SV40 promoter (23). These results suggest that the individual HS sites do not have specificity for the each clustered Pcdh promoter, although each regulatory element has specificity for individual Pcdh cluster in vivo. These findings led us to speculate that the clustered Pcdh locus forms a complex chromatin architecture to regulate each Pcdh cluster in vivo. We suggest that CTCF is a key molecule for determining the specific regulatory targets of CCR and HS5-1. CTCF is a major protein with insulator activity (34). However, recent studies suggest that CTCF can establish contacts between CTCF-binding sites, to stabilize long range interactions and promote the global organization of the chromatin architecture (35, 36). There are many CTCF-binding sites within the Pcdh-γ locus and at each identified HS site (28, 37). A bioinformatics analysis revealed that HS5-1 also contained potential CTCF-binding sites (data not shown). If all of the CTCF molecules that bind within Pcdh-γ had insulator activity, the CCR would have no effect on the Pcdh-β gene expression, because it is physically separated from the clustered Pcdhs. This is also true of the relationship between the Pcdh-α cluster and HS5-1. Thus, we speculate that the CTCF-mediated chromatin architecture determines the specificity of each regulatory element and that CTCF-mediated long range interactions between promoters and enhancers enable the stochastic promoter choice for each clustered Pcdh subfamily. In fact, the deletion of CCR or HS5-1 also disturbed the expression of other proximal clustered Pcdh genes. These misregulations may result from disturbances of the chromatin architecture in the clustered Pcdh locus.

The clustered Pcdh families do not cause mutual allelic exclusions because the same Pcdh isoform can be expressed from both chromosomes (16, 17). In this study, we showed that the CCR act in cis. Thus, in the Pcdh-β gene cluster, the independent regulation of genes on each chromosome could generate monoallelic expression, with the same Pcdh-β gene being stochastically expressed from both alleles in a single cell. A previous study showed that the deletion of HS5-1 has a strong effect on expression from the cis-allele (23). We observed that in the +/ΔHS5-1 mice, the expression levels of the Pcdh-α10–12 isoforms were intermediate between those of the +/+ and ΔHS5-1/ΔHS5-1 mice. These results suggested that the chromatin structure of the clustered Pcdh locus on each allele forms independently, and independent stochastic gene regulation between the two alleles might increase the number of isoforms expressed by the clustered Pcdh subfamilies within a single neuron.

Our study revealed that CCR is a regulatory unit for the Pcdh-β gene cluster and that HS5-1 is a regulatory element for the Pcdh-α gene cluster, indicating that each clustered Pcdh subfamily uses distinct cluster control regions. These results imply that clustered Pcdh isoforms are independently and randomly expressed by distinct regulatory mechanisms for each clustered Pcdh subfamily. These distinct control mechanisms for the clustered Pcdhs would increase the molecular diversity within a single neuron. Clarification of the cluster-wide regulatory mechanisms for the Pcdh gene expressions will improve our understanding of how neural diversity is generated through stochastic gene regulation.

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REFERENCES

1. Yagi, T. (2008) Dev. Growth Diff 50, S131–140
2. Zipursky, S. L., and Sanes, J. R. (2010) Cell 143, 343–353
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., Zhang, T., Cheng, J. F., Kim, Y., Grimwood, J., Schmutz, J., Dickson, M., Noonan, J. P., Zhang, M. Q., Myers, R. M., and Maniatis, T. (2001) Genome Res. 11, 389–404
6. Tasic, B., Nabholz, C. E., Baldwin, K. K., Richter, E. H., Ribich, S. A., Cramer, P., Wu, Q., Axel, R., and Maniatis, T. (2002) Mol. Cell 10, 21–33
7. Wang, X., Su, H., and Bradley, A. (2002) Genes Dev. 16, 1890–1905
8. Phillips, G. R., Tanaka, H., Frank, M., Elste, A., Figler, L., Benson, D. L., and Colman, D. R. (2003) J. Neurosci. 23, 5096–5104
9. Wang, X., Weiner, J. A., Levi, S., Craig, A. M., Bradley, A., and Sanes, J. R. (2002) Neuron 36, 843–854
10. Junghans, H., Heidenreich, M., Hack, I., Taylor, V., Frotscher, M., and Kemler, R. (2008) Eur. J. Neurosci. 27, 559–571
11. Weiner, J. A., Wang, X., Tapia, J. C., and Sanes, J. R. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 8–14
12. Hasegawa, S., Hamada, S., Kumode, Y., Esumi, S., Katori, S., Fukuda, E., Uchiyama, Y., Hirabayashi, T., Mombaerts, P., and Yagi, T. (2008) Mol. Cell. Neurosci. 38, 66–79
13. Katori, S., Hamada, S., Noguchi, Y., Fukuda, E., Yamamoto, T., Yamamoto, H., Hasegawa, S., and Yagi, T. (2009) J. Neurosci. 29, 9137–9147
14. Takei, Y., Hamada, S., Senzaki, K., Mutoh, T., Sugino, H., and Yagi, T. (2001) Genomics 72, 321–330
15. Frank, M., Ebert, M., Shan, W., Phillips, G. R., Arndt, K., Colman, D. R., and Kemler, R. (2005) Mol. Cell. Neurosci. 29, 603–616
16. Esumi, S., Kakazu, N., Taguchi, T., Hirayama, T., Sasaki, A., Hirabayashi, T., Koide, T., Kitsukawa, T., Hamada, S., and Yagi, T. (2005) Nat. Genet. 37, 171–176
17. Kaneko, R., Kato, H., Kawamura, Y., Esumi, S., Hirayama, T., Hirabayashi, T., and Yagi, T. (2006) J. Biol. Chem. 281, 30551–30560
18. Noguchi, Y., Hirabayashi, T., Katori, S., Kawamura, Y., Sanbo, M., Hirabayashi, M., Kiyonari, H., Nakao, K., Uchiyama, A., and Yagi, T. (2009) J. Biol. Chem. 284, 32002–32014
19. Tuan, D., Solomon, W., Li, Q., and London, I. M. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 6384–6388
20. Forrester, W. C., Thompson, C., Elder, J. T., and Groudine, M. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 1359–1363
21. Grosfled, F., van Assendelft, G. B., Greaves, D. R., and Kollas, G. (1987) Cell 51, 975–985
22. Bender, M. A., Bulger, M., Close, J., and Groudine, M. (2000) Mol. Cell 5,
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