Cadherin-6 Expression Transiently Delineates Specific Rhombomeres, Other Neural Tube Subdivisions, and Neural Crest Subpopulations in Mouse Embryos

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Mammalian cadherin-6 (K-cadherin, cad6) was originally identified by means of the polymerase chain reaction, but its biological functions have not yet been determined. We analyzed the expression pattern of the mouse homologue of this cadherin during development and found that it was transiently expressed in restricted rhombomeres and in other subdivisions of the neural plate and tube. In the midbrain and anterior hindbrain of E8.0-8.5 embryos, cad6 was expressed only in neural crest-generating regions. In contrast, in the posterior hindbrain and contiguous spinal cord of these embryos, cad6 occurred throughout the neural plate, forming a sharp anterior limit at the future rhombomere 4 and 5 boundary. Subsequently, this neural plate expression became confined to rhombomere 6, although most of the neural crest-generating areas remained positive throughout the body. Neural crest cells expressing cad6 migrated out of the neural tube, and subsequently accumulated mainly along peripheral nerves. We then studied the effect of Hoxa-1 mutation on the expression of cad6, as their expressions spatiotemporally overlapped with each other in the early posterior hindbrain. In E8.0-8.5 Hoxa-1 mutants, cad6 expression was suppressed in the region of rhombomeres 4 to 6, although that in the other regions was not essentially affected. At later stages, however, cad6-positive crest cells appeared and migrated out of rhombomeres 4 to 6, indicating that the suppression of cad6 expression was transient and restricted to early stages. Importantly, this effect of the Hoxa-1 mutation concurred with the timing of the expression of this gene. We also studied Hoxa-3 mutants, but found no effect of this mutation on the cad6 expression pattern. These findings suggest that cad6 may contribute to the formation of the segmental structure of the early brain through its ability to confer specific adhesiveness on cells and that Hoxa-1 may be required for early cad6 expression in the posterior hindbrain.

INTRODUCTION

Cadherins represent a superfamily of transmembrane or surface-anchored proteins characterized by the presence of unique repeated motifs on their extracellular domain (Ranscht, 1994). Members of one subfamily, called classic cadherins, have been identified as homophilic cell-cell adhesion molecules (Takeichi, 1988). They are subdivided into two groups, types I and II (Suzuki et al., 1991; Takeichi, 1995). The type-I group includes E-, N-, and R-cadherin, and the type-II group includes cadherin-5 to -12. Each of these molecules is expressed in a unique pattern in the body, and dysfunction of them causes defective morphogenesis of tissues, as demonstrated by a number of studies. For example, blocking of E-cadherin disorganizes preimplantation embryos (Larue et al., 1994; Riethmacher et al., 1995) as well as differentiated epithelial layers (Hirai et al., 1989; Hermiston et al., 1995), and that of N-cadherin impairs neural morphogenesis (Matsunaga et al., 1988; Barami et al., 1994). With respect to homophilic interactions between the classic cadherins, like subtypes prefer to bind together (Takeichi, 1995), although heterotypic interactions were observed between certain cadherin subtypes (Murphy-Erdosh et al., 1995). As a result, cells expressing a particular cadherin selectively adhere to those expressing the same cadherin. It was thus proposed that cadherins are essential not only for physical linking of cells but also for selective association of different cell types.

Two kinds of morphogenetic events, likely relevant to the above functions of cadherins in selective cell association, were recently reported. First, we found that two type-II cadherins, cadherin-6B and cadherin-7, were expressed...
during specific steps of neural crest development in the chicken embryo (Nakagawa and Takeichi, 1995). The chicken cadherin-6B (cad6B) is exclusively expressed in the future neural crest regions of the neural fold. When neural crest cells begin migration, they cease the expression of cad6B and instead begin to express cadherin-7 (cad7). This cad7 expression occurs in a subpopulation-specific manner. For example, in the trunk, cad7 is expressed only in crest cells populating the dorsal and ventral roots and spinal nerves, whereas it is never intensely expressed in the cranial and dorsal root ganglia or in the cranial mesenchyme, where neural crest cells are known to populate. Based on these and other observations, we hypothesized that neural crest cells migrate while maintaining their cadherin-mediated associations and that cad7 serves for the sorting of particular subsets of neural crest cells from the other subpopulations.

As for the second morphogenetic event indicating cadherin-dependent selective cell association, R-cadherin was found to be expressed in a restricted fashion in specific rhombomeres of the embryonic chicken or mouse brain (Ganzler and Redies, 1995; Redies, 1995; Matsunami and Takeichi, 1995), some of which corresponded to certain “neuro-meres.” Cell aggregation assays conducted in vitro showed that cells derived from R-cadherin-positive and -negative regions segregated from each other in a cadherin-dependent manner. These findings led us to propose that R-cadherin confers specific aggregating ability on cells belonging to restricted subdivisions of the fetal brain, so as to confine them to the subdivisions. We argued that this mechanism may contribute to the formation or maintenance of the segmented structure of the fetal brain (Matsunami and Takeichi, 1995). It was also reported that a novel cadherin, F-cadherin, is expressed along several boundary regions in the Xenopus embryonic brain (Epeseth et al., 1995).

The above observations on the developing brains suggested that other cadherins may also be expressed in a segment-specific manner in the embryonic brain; this suggestion prompted us to search for such cadherins. It is known that neuroepithelial cells do not cross the boundaries between rhombomeres subdividing the hindbrain (Fraser et al., 1990), although a small percentage of them can (Birgbauer and Fraser, 1994), and that odd and even rhombomeres do not randomly intermix with each other (Clarke and Lumsden, 1993; Guthrie et al., 1993; Graham et al., 1993). These phenomena can be explained by assuming segment-specific adhesiveness of the cells comprising the hindbrain (Lumsden, 1990), and cadherins could be involved in this hypothetical mechanism. In the present study, we analyzed the expression pattern of mouse cadherin-6 (cad6) during embryogenesis, which is the mouse homolog of the previously cloned rat K-cadherin (Xiang et al., 1994) or human cadherin-5 (Shimoyama et al., 1995). While various interesting expression patterns of cad6 were observed, one important finding was that it is transiently and sequentially expressed to delineate the boundaries between prospective rhombomeres 4 and 5, and then 5 and 6, providing the first molecular support of the notion that cadherin may endow hindbrain cells with rhombomere-specific adhesiveness. Because the cad6 expression pattern in the neural plate was reminiscent of that of Hox genes, we tested the possibility that the former may be regulated by these genes and found that in Hoxa-1 mutants, the cad6 expression in the posterior hindbrain was temporarily suppressed.

**MATERIALS AND METHODS**

**Animals**

ICR mice were mated, and embryos at various stages were collected. The precise staging of collected embryos was made according to Theiler (1972). For Hoxa-1 or Hoxa-3 mutants, mice heterozygous for targeted disruptions of the Hoxa-1 or Hoxa-3 gene (Chisaka et al., 1991, 1992) were intercrossed to produce embryos, and their genotypes were determined by polymerase chain reaction (PCR) methods as described previously (Carpenter et al., 1993; Manley et al., 1995). The Splotch heterozygous mice were provided by Dr. S. Aizawa (Kumamoto University), and mated to produce embryos. Their genotypes were determined by their characteristic phenotype of the neural tube (Douglas et al., 1991).

**Cloning of Mouse cad6 cDNA**

To obtain cadherin cDNAs, we carried out PCR using mixed primers which can amplify multiple cadherin subtypes (Suzuki et al., 1991). As templates, cDNAs were prepared from mRNAs isolated from newborn mouse brains. By this PCR, about 160-bp fragments were amplified, and these fragments were subcloned into the pUC18 EcoRI site. To obtain full-length cDNAs, we constructed random primed Ig10 cDNA libraries of postnatal brains derived from ICR mice by use of the cDNA Synthesis Kit (Amersham). We screened these libraries with the above PCR fragments radiolabeled. Many overlapping clones were obtained, and among them one open reading frame (ORF) was found.

**Probes for in Situ Hybridization**

Sense or antisense cad6 RNA probes labeled with digoxigenin (Dig)-UTP (Boehringer-Mannheim) were prepared from pBSI1.0B, which contains the 202–1229 BamHI fragment of cad6 cDNA. For the antisense probe, the EcoRI linearized vector was transcribed with T3 RNA polymerase (Gibco BRL); for the sense probe, the XbaI linearized vector was transcribed with T7 RNA polymerase (Gibco BRL). Hoxa-1 antisense RNA probes labeled with Dig-11-UTP were prepared from a newly constructed plasmid containing the 1233–1950 EcoRI–BamHI fragment of Hoxa-1 cDNA (LaRosa and Gudas, 1988). For its antisense probe, the EcorI linearized vector was transcribed with T7 RNA polymerase. R-cadherin antisense RNA probes labeled with Dig-11-UTP and Kroxx-20 antisense RNA probes labeled with fluorescein (FITC)-12-UTP (Boehringer Mannheim) were prepared, as described previously (Matsunami and Takeichi, 1995; Carpenter et al., 1993). All probes were hybridized to the explants and were hybridized using a protocol that includes a pretreatment of 10 μg/ml of a short RNA fragment, followed by a 30-min prehybridization at 60°C and a washing procedure at 42°C.

**Whole-Mount in Situ Hybridization**

Whole-mount in situ hybridization was performed with a single probe and was performed in the manner described previously (Shimamura et al., 1993; Graham et al., 1993; Guthrie et al., 1993).
RESULTS

Isolation of Mouse Cad6 cDNA

We isolated overlapping cDNA clones with a 2373-bp ORF (DDBJ Accession No. D82029). The ORF encoded a protein with 790 amino acids (Fig. 1); it showed 96% identity to each of human cadherin-6 (Xiang et al., 1994) and rat K-cadherin (Shimoyama et al., 1995) and also exhibited 86% identity to chicken cadherin-68 (Nakagawa and Takeichi, 1995). None of the other cadherins thus far identified were more similar to the cloned molecule. We therefore concluded that the isolated molecule is the mouse homologue of cad6.

Cad6 Expression Subdivides the Early Neural Tube

We studied the expression of cad6 mRNA during embryogenesis by in situ hybridization. Its staining signal was first observed at the head process stage in the crescent area located at the anterior end of the embryo (Fig. 2A), that forms the anterior intestinal portal. The cad6 expression in this particular structure of the endoderm was maintained at later stages. Then, cad6 appeared in the neural plate: Its expression was initiated at a portion of the hindbrain at around E7.75 (Fig. 2B, arrows) and subsequently extended posteriorly to the caudal end of the forming spinal cord by E8.0 (Fig. 2D). The rostral end of this cad6 expression exhibited a sharp limit in the hindbrain region (Fig. 2D, arrow). In those positive regions of the neural plate, cad6 expression seemed to occur in all neuroepithelial cells, as revealed by sectioning of the samples (Fig. 2F). Soon after the hindbrain expression, the entire forebrain also began cad6 expression, which bordered the midbrain (Fig. 2C, arrow). At the same time, the midbrain and the anterior hindbrain also expressed cad6, but their cad6 expression was confined to the ridge of the neural fold (Fig. 2D).

At the subsequent stages, the above pattern changed dramatically in the posterior hindbrain and spinal cord. In these regions, cad6 expression was downregulated in the neural plate and closing neural tube, leaving only the neural crest zones positive. Exceptionally, however, the cad6 expression persisted in a band-like unit of the hindbrain (Figs. 2G and 2I). To identify this portion as well as the initial cad6 positive area in the hindbrain, we used Krox-20 transcript as a marker, which is expressed only in rhombomeres 3 (r3) and 5 (r5) (Wilkinson et al., 1989). By double in situ hybridization for Krox-20 and cad6 messages, we found that the anterior limit of the initial cad6 expression delineated the future r4/r5 boundary, as it coincided with the anterior limit of the Krox-20 signals in r5 (Fig. 3A). When cad6 expression was confined to the band, its anterior limit bordered the r5 Krox-20 signals (Fig. 3B), suggesting that the cad6-positive band corresponds to r6. This r6-specific expression of cad6 persisted until the time of neural tube closure and thereafter diminished (Figs. 4A and 4B). To summarize, cad6 expression in the neural plate occurred initially from the future r4/r5 boundary to the caudal end throughout and then was confined to r6.

Cad6-Positive Neural Crest Cells Populate Peripheral Nerves

The expression of cad6 along the ridge of the neural fold suggested that cad6 is expressed in neural crest cells or their...
FIG. 1. The alignment of cadherin-6 amino acid sequences from different species. The netted amino acids are conserved among the species. K-cadherin is the rat homologue of cadherin-6. m, mouse; h, human; c, chicken; a.a., amino acids. PRE, precursor; EC, extracellular domain; TM, transmembrane domain; CP, cytoplasmic domain.

precursors, although r3 and r5 were virtually negative for cad6 expression (Fig. 3). As expected from these observations, cells with intense cad6 expression migrated out of the neural plate or tube. The first migration occurred in the midbrain (Fig. 2C), and then in the other regions; cells in r6 migrated out last. Neural crest cells expressing cad6 and derived from r1/2 became arranged into the form of the trigeminal ganglion (Fig. 4C), but these expressions were transient, as described below.

Sections of the whole-mount stained samples revealed the following profiles of the generation and migration of cad6-positive neural crest cells: In the midbrain and anterior hindbrain, cad6 expression was observed only at the ridge of the neural fold (Fig. 2E); the positive cells then migrated out (Fig. 2H). In the other regions, however, all neural plate cells initially expressed cad6 (Figs. 2F and 2I). Then positive cells progressively became restricted to the ridge of the neural fold. Finally, the cad6-positive crest cells left the fold (Figs. 2J and 2L). Forming cranial ganglia were positive for cad6 expression, especially at their outer peripheral zones (Fig. 3A). In sections of E12.0 embryos, the most intense cad6 signals were detected along peripheral nerves (Fig. 5A). The signals were also located beneath the epidermis, exhibiting nerve-like networks (Fig. 5G). When these samples were...
FIG. 2. Cad6 mRNA expression in early embryos. Embryos were stained by in situ hybridization as whole-mount samples (A–D, G), and some of them were subsequently sectioned (E, F, H–J). (A) E7.5. The anterior crescent area (arrowheads) expresses cad6. (B) Anterolateral view of an E7.75 embryo. The neural plate has begun cad6 expression at regions indicated by the arrows. The arrowhead indicates the anterior intestinal portal. (C, D) Lateral (C) and dorsal (D) views of an E8.0 embryo. In the neural plate, two borders of cad6-positive areas are observed, at the forebrain/midbrain boundary and in the hindbrain (arrows). Arrowheads point to migrating neural crest cells in the midbrain. (E, F) Frontal sections of the embryo in (D), at the levels of "e" (E) and "f" (F). (G) Dorsolateral view of an E8.5 embryo. A rhombomere-like band in the hindbrain at the level "i," and streams of neural crest cells (arrowheads) are positive for cad6 expression. (H, I, J) Frontal sections of the embryo in panel G, at the levels of "h" (H), "i" (I), and "j" (J). Arrowheads denote cad6-positive migrating neural crest cells. hf, head fold; fb, forebrain; fp, floor plate; mb, midbrain; hb, hindbrain. Bars, 200 μm for (A–D, G), 100 μm for (E, F, H, I), and 10 μm for (J).

double-stained for cad6 mRNA and neurofilament proteins, most cad6-positive cells were found to be associated with the latter signals (Figs. 5B and 5C), suggesting them to be a group of Schwann cells. For further confirmation of this identification, we examined cad6 expression in Splotch mutant mice. In this mutant, Schwann cells migrate into the forelimbs but not into the hindlimbs (Franz et al., 1990). We found that cad6-positive cells were present in the fore-
FIG. 3. Cad6 mRNA expression sequentially defines prospective rhombomere boundaries. Double in situ hybridization for cad6 (blue) and Krox-20 (red) messages. (A) E8.0. The anterior limit of Krox-20 signals in rhombomere 5 (r5) coincides with that of cad6 signals. (B) E8.5. Krox-20 signals in r5 posteriorly border the cad6 band. Rhombomere numbers are shown. Short lines indicate prospective rhombomere boundaries. Bars, 200 μm.

FIG. 4. Cad6 mRNA expression in migrating neural crest cells and in brains. (A) E9.0. Cad6-positive neural crest cells migrate into the mandibular arch. (B) E9.5. Cad6 signals are observed in forming cranial ganglia and in nerves, as well as in migrating crest cells in the trunk. In the brain, the signals are seen at the anterior border of the dorsal hindbrain (arrow) and in a dorsal portion of the midbrain (arrowhead). (C) A longitudinal section of the embryo in (B), which was cut along level “c” depicted in (B). Asterisk, anterior hindbrain signals; arrows, migrating cad6-positive crest cells in the trunk. (D) A dissected brain of E12.5 embryo. The brain was longitudinally cut into half and observed from the ventricular side. The cerebral hemisphere was removed in this sample. The arrow indicates the alar/basal boundary, and arrowheads point out the dorsal/ventral thalamus boundary. (E, F) Ventricular view of an isolated E12.5 telencephalon, hybridized for cad6 (E) and R-cadherin (F). White arrowheads mark the border of the cortex to the lateral ganglionic eminence. md, mandibular arch; v, trigeminal ganglion; vii, viii, facial/acoustic ganglion; ix, x, glossopharyngeal/vagus nerves; dt, dorsal thalamus; vt, ventral thalamus; hy, hypothalamus; me, mesencephalon; mt, metencephalon; my, myelencephalon; ctx, cerebral cortex; ge, ganglionic eminence. Bars, 0.5 mm for (A, B, D±F) and 200 μm for (C).
FIG. 5. Cad6 mRNA expression in peripheral nerves and other tissues. (A) Slice of an E12.0 embryo at the level of "a" in (D). Positive regions include nerve tracts, a peripheral zone of the sympathetic ganglia, roof plate, floor plate, and a portion of the motor column (mc). Neural crest-derived cells around the trachea, artery, and esophagus (arrowheads) also express cad6 mRNA. (B) Double-staining for cad6 mRNA (blue) and neurofilament proteins (brown) on a slice of an E12.0 embryo at the level of "b" in (D). Dorsal root and its ganglia (asterisk) are negative for cad6 expression, whereas other peripheral nerve fibers show both signals (arrowheads). (C) High magnification of a peripheral nerve stained as in (B). Note that cad6-positive cells (arrowheads, blue) are associated with neurofilament-positive fibers (brown). (D) Drawing of an E12.0 embryo, showing the levels of sections. (E, F) Cad6 expression in E12.0 Splotch+/− (E) and Splotch−/− (F) embryos at the level of "e/f" in (D). Cad6-positive nerves are absent in the hindlimbs of the homozygous embryo. Arrowheads indicate cad6 signals at the edge of the open neural tube in the mutant. (G) A hindlimb of an E12.0 embryo. Interdigital portions and nerve fibers running underneath the epidermis are positive. (H) A slice of E15.0 embryo showing absence of cad6 signals in the peripheral nerves. fp, floor plate; mc, motor column; rp, roof plate; sg, sympathetic ganglion. Bars, 400 μm for (A, B, E–H) and 20 μm for (C).

limbs but not in the hindlimbs (Figs. 5E and 5F), consistent with the above identification. In the caudal spinal cord of the Splotch mice, which fails to close, cad6 signals were detected at the edges of the open neural plate. This suggests that cad6-positive neural crest precursors formed but did not migrate out from these positions in the neural plate. Not all of the peripheral nerves were positive for cad6 expression; e.g., the dorsal root was negative. Also, cranial ganglia, dorsal root ganglia, and sympathetic ganglia were negative at least in their central portions when their differentiation had been completed, although some peripheral signals were observed. Most of these nerve-associated expressions of cad6 were no longer evident in E15.0 embryos (Fig. 5H). In addition, we found that, in the limbs, interdigital mesenchyme expressed cad6 in a characteristic pattern (Fig. 5G).

Cad6 Expression Delineates Fetal Brain Subdivisions

The expression pattern of cad6 in the brain dynamically changed during development. By E9.5, the cad6 expression
in the forebrain had become regional (Fig. 4B); positive regions included the dorsal midline, the future neural retina and optic stalk, and portions of the diencephalon. It also occurred in a dorsal area of the midbrain and dorsally at the rostral limit of the hindbrain (Fig. 4B). In the brain of E12.5 embryos, cad6 expression was found in the dorsal portion of the ganglionic eminence, and this signal sharply bordered the lateral cortex (Fig. 4E). The latter expresses R-cadherin (Fig. 4F), as was noted earlier (Matsumani and Takeichi, 1995); therefore, these two cadherins showed a complementary pattern at the boundary of the two contiguous cerebral compartments. Besides, a faint continuous line of cad6 signals occurred at the zona limitans infratemporalis along the boundary between the dorsal and ventral thalamus and along the alar/basal boundary in the diencephalon and midbrain (Fig. 4D). Hypothalamic compartments were also positive. All these staining signals emanated from the ventricular neuroepithelial cells. In the spinal cord, a certain portion of motor columns as well as the roof and floor plates expressed this cadherin (Figs. 5A, 5B, and 5E); the floor plate signals extended to the brain. In the eye, the future iris and optic stalk were positive (data not shown). At later developmental stages, cad6 expression in the brain underwent further complex changes following differentiation of brain nuclei, and these will be reported elsewhere (S. C. Suzuki, T. Inoue, and M. Takeichi, in preparation).

Cad6 Expression in Hoxa-1/− Mice

Since the cad6 expression pattern in the early hindbrain was in part reminiscent of the well-known patterns of Hox gene expressions, we considered the possibility that the former might be regulated by the latter genes. As an initial step for testing this possibility, we examined the effect of Hoxa-1 knockout on the expression of cad6.

We first carried out whole-mount in situ hybridization for Hoxa-1 expression in wild-type E8.0 embryos to confirm the previous observation that this gene is expressed posteriorly to the presumptive r3/4 boundary in the hindbrain (Murphy and Hill, 1991). As reported, the Hoxa-1 expression exhibited a sharp anterior limit within the hindbrain. In four-somite stage embryos, however, the limit was located more posteriorly to the r3/4 boundary, although it covered a posterior portion of r4 (Fig. 6A, 4s). In nine-somite stage embryos, the expression was diminishing, but it was still positive to a certain degree (Fig. 6A, 9s).

We then examined cad6 expression in E8.0 (2- to 4-somite stage) Hoxa-1/− embryos (Chisaka et al., 1992) and found that they expressed this molecule in a pattern similar to that in wild-type embryos. However, close comparisons of the mutant and wild-type samples revealed that the staining intensity for cad6 messages was considerably reduced in the posterior hindbrain of the mutants (Fig. 6B). Moreover, the anterior limit of the expression was less sharp and located more posteriorly in the mutants than in the wild-type, as estimated by distances from the position of the anteriormost somite and from a sulcus corresponding to the presumptive r2/3 boundary. The latter distance was larger in the mutants, despite their shorter hindbrain due to the absence of r5 (Carpenter et al., 1993). At the 9-somite stage when the discrete r6 band of cad6 appears in the wild-type embryos, the corresponding band was dim in the mutants (Figs. 6C – 6F). Furthermore, cad6 expression at the neural fold ridge of the putative r4 region was faint (Figs. 6C – 6F). The other cad6 expressions were, however, basically normal. At the 11-somite stage, cad6 appeared at the neural fold ridge of the putative r4 and r6 regions (Figs. 6G and 6H), although the main portion of the neural plate remained essentially negative. These cad6 signals were contiguous to each other on the ridge, consistent with the previous finding that r5 is missing in Hoxa-1/− embryos (Carpenter et al., 1993; Mark et al., 1993; Döllé et al., 1993). Curiously, cad6 signals in r2 extended more caudally in the mutant embryos than in normal embryos (Figs. 6F and 6H), although no such effect of Hoxa-1 mutations has been reported. In E9.5 homozygous mutant embryos, neural crest cells expressing cad6 migrated out of the putative r4 and r6 positions (Fig. 6J). The two clusters of cad6-positive migrating crest cells fused to each other over the dorsal portion of the otic vesicle, as reported previously (Mark et al., 1993). Thus, the suppression of cad6 expression in the posterior hindbrain by Hoxa-1 mutation was limited to earlier developmental stages. Importantly, the observed effect of Hoxa-1 mutation coincides with the period of the expression of this gene. We performed similar analysis for Hoxa-3 mutants, but found no effect on cad6 expression in them (data not shown).

DISCUSSION

Recently, we reported the cloning and expression of chicken cadherin-6B (cad6B; Nakagawa and Takeichi, 1995). The mouse cad6, studied here, showed 86% identity in amino acid sequence to the cad6B, so they can be considered to be homologs of each other. Despite this high similarity of the two molecules, their expression patterns were not identical, raising a question about their relationship. The chicken cad6B was expressed only in the neural fold, whereas in the mouse cad6 was expressed in both the neural fold and migrating neural crest cells; this was the reason why the chicken molecule was termed cad6B instead of cad6. We found another chicken cadherin, cadherin-7 (cad7); this cadherin is expressed in migrating crest cells but not in the early neural plate and tube (Nakagawa and Takeichi, 1995). Therefore, in a sense, the expression pattern of cad6 represents a mixture of those of cad6B and cad7. However, the cad6 expression pattern was not a simple sum of the two chicken cadherin patterns; it was different in certain details. For example, neither of these chicken cadherins displayed a metameric expression in the hindbrain neural plate. In the chicken, other unidentified cadherins might be present to subdivide the hindbrain.

Possible Roles for cad6 in Neural Tube Segmentation

It was shown earlier that neuroepithelial cells in chicken embryos cannot cross the boundaries between rhombomeres
after a certain developmental stage (Fraser et al., 1990), except that a minor population of them can do (Birgbauer and Fraser, 1994). A hypothesis to explain this phenomenon was that the restriction is created by immiscibility of cells belonging to contiguous rhombomeres (Lumsden, 1990; Wingate and Lumsden, 1996). Our present finding supports this hypothesis. After dynamic changes in expression pattern, the cad6 signals became restricted to rhombomere 6 (r6), although temporarily. We recently showed that cells expressing cad6 segregate from cells expressing other cadherins, such as N-cadherin and cad7, upon their aggregation in vitro (Nakagawa and Takeichi, 1995). Such adhesion specificity was also found for mammalian cad6 (S. C. Suzuki and M. Takeichi, unpublished). Based on these observations, we can assume a process such that r6 cells coaggregate by use of cad6, and segregate from cells of contiguous rhombomeres without this cadherin; the latter probably express other unidentified cadherins. This is a likely mechanism for prohibiting r6 cells from free migration to the contiguous rhombomeres. Grafting experiments suggested that cells derived from different odd-number rhombomeres are miscible with one another but not with those from even ones (Guthrie et al., 1993). To be consistent with this observation, odd or even rhombomeres should express the same cadherins, but this was not the case in the present observation. Other cadherins that can crossinteract with cad6 might be expressed in even rhombomeres. A combinatory action of

FIG. 6. Effects of Hoxa-1 mutation on cad6 mRNA expression. Dorsal views for A–D, G, and H and lateral views for E, F, I, and J. (A) Hoxa-1 expression in 4-somite (4s) and 9-somite (9s) embryos. Intense signals in the 9s hindbrain are mostly derived from mesenchymal tissues. (B) Cad6 expression in Hoxa-1−/− (left) and Hoxa-1+/− (right) embryos at the 4s stage. Triangles indicate the gap between the first and second somite blocks. Arrows show the anterior limit of cad6 expression. Arrowheads point to the sulcus corresponding to the future r2/3 boundary in both panels of (A) and (B). Note the posterior retreatment of the cad6-positive region in the mutant hindbrain. In these samples, cad6 expression had not fully developed in the r4 and more anterior portions. (C–F) E8.5 (9-somite stage). (C, E) Hoxa-1−/− and (D, F) Hoxa-1+/−. In the mutant, cad6 signals in r4 and r6 are greatly reduced; positive signals visible at r4 in B are mostly derived from ventral tissues. (G, H) E8.5 (11-somite stage). (G) Hoxa-1−/− and (H) Hoxa-1+/−. Some cad6 signals appeared in the putative r4/r6 region on the ridge of the neural plate; however, the signals in the main portion of the r6 neural plate remain very faint or negative. (I, J) E9.5. (I) Hoxa-1−/− and (J) Hoxa-1+/−. Two streams of cad6-positive cranial ganglion cells, derived from r4 (arrowheads) and r6 (arrow), have fused with each other over the otic vesicle (o). v, trigeminal ganglion. Prospective rhombomere numbers are shown. Bars, 200 μm.
multiple cadherins is perhaps important for determining the overall adhesive behavior of rhombomere cells. For testing these hypotheses, it would be important to identify other cadherins expressed in the early hindbrain. N-cadherin is known to be expressed in the neural tube, but its expression in the hindbrain is not so intense yet at the stage of the uncleared neural plate (Kimura et al., 1995).

We should point out that cad6 expression in the hindbrain dynamically changes. Its anterior limit initially delineated the prospective r4/5 boundary and subsequently the r5/6 boundary. This pattern implies that cad6 could be involved in determining both boundaries in a sequential manner. An important question is whether these cad6 expressions indeed correlate with cell migration restriction at these boundaries. No precise cell lineage analysis has been reported for the early mouse hindbrain except for neural crest origin (Osumi-Yamashita et al., 1996), so that this question remains to be answered.

In brains at more advanced developmental stages, we found other correlations between cad6 expression pattern and cell migration restriction. An example was seen in the developing telencephalon. Cad6 expression delineated the dorsal border of the ganglionic eminence, which is contiguous to the R-cadherin-expressing lateral cortex (Matsunami and Takeichi, 1995). These two cadherins showed a complementary distribution pattern at the junction of the ganglionic eminence and cortex. It was previously shown that cells in the ganglionic eminence do not migrate into the cortex (Fishell et al., 1993), and we also demonstrated that R-cadherin-positive and -negative fetal brain cells segregate from one another in an in vitro aggregation system (Matsunami and Takeichi, 1995). Furthermore, a weak cad6 expression occurred along the boundaries between the dorsal and ventral thalamus; it was demonstrated that cells do not cross the corresponding boundary in the chicken embryo (Figdor and Stern, 1993). A similar type of expression pattern defining boundaries in the embryonic brain were also recently reported for Xenopus F-cadherin (Espeseth et al., 1995). These findings support the hypothesis that regional expressions of multiple cadherins in the fetal brain play a role in the maintenance of its segmental structure (Ganzler and Redies, 1995; Matsunami and Takeichi, 1995).

**Subpopulation-Specific Expression of cad6 in Neural Crest Cells**

We found that cad6 is expressed along the ridge of the neural fold and subsequently in migrating neural crest cells. The cad6-positive cells in the ridge are most likely neural crest precursors. It thus seems that neural crest cells begin cad6 expression when they are still part of the neural plate, and subsequently they migrate out. Most of the cad6-positive crest cells selectively accumulated along nerve fibers, suggesting that they differentiated into certain types of Schwann cells. It remains to be investigated whether this subpopulation-specific cad6 expression is established from the beginning of neural crest generation or during their migration. Whatever the case is, cad6 may contribute to the clustering of migrating Schwann cell precursors and/or their sorting from other cells, as we proposed recently (Nakagawa and Takeichi, 1995). Since motor neurons also express cad6, this cadherin could also be used for the association of Schwann cells with motor axons.

It is interesting to note that cad6 expression was virtually negative in r3 and r5 of E8.5 embryos. These odd rhombomeres are known to produce neural crest cells, which migrate together with others from even rhombomeres (Sechrist et al., 1993; Birgauer et al., 1995). It remains to be resolved whether these r3/r5-derived crest cells express cad6 when joining those derived from even rhombomeres. In case they never express cad6, migrating crest cells are assumed to be a mixture of cad6-positive and negative cells; if so, this situation could be effective for segregating subpopulations of migrating crest cells from one another, as discussed above and before (Nakagawa and Takeichi, 1995). In addition, it is worthy of note that cad6 can be used as a marker for neural crest cells, at least for their subpopulations, in the future studies; for the mouse system, such markers have been lacking.

**Hoxa-1 Mutation Affects cad6 Expression**

As is well known, Hox genes are expressed in the neural tube along its anteroposterior axis, and many of them exhibit their anterior limit at specific rhombomere boundaries (Wilkinson, 1993). The early pattern of cad6 expression in the neural plate and tube is reminiscent of those Hox expression patterns. This correlation prompted us to test the possibility that some of the Hox genes may be regulating cad6 expression. We examined the effect of Hoxa-1 and Hoxa-3 mutations on cad6 expression, and found that, in Hoxa-1 mutants, the cad6 expression pattern was altered in a developmental stage-specific manner. In these mutants at earlier developmental stages, cad6 expression was suppressed at r4 and r6. The cad6 expression in these rhombomeres was, however, upregulated afterward, and crest cells migrating from these regions expressed a normal level of cad6. According to previous (Murphy and Hill, 1991) and present observations, Hoxa-1 is expressed in E8.0 hindbrains, posteriorly to the r3/r4 boundary. The Hoxa-1 message then diminish from the developing brain, and migrating neural crest cells do not express Hoxa-1. Thus, both positionally and temporally, the Hoxa-1 expression pattern in normal embryos correlates with the observed major effects of the Hoxa-1 mutation on cad6 expression; that is, the effects were limited to rhombomeres posterior to the r3/r4 boundary and up to the stage E8.5. It should be stressed that, in the Hoxa-1 mutant strain used in the present study, r4 and r6 are present, as revealed by use of specific regional markers, although r4 seems to be reduced in size (Carpenter et al., 1993). It is therefore most likely that the downregulation of cad6 is not due to a simple loss of these rhombomeres. These findings support the idea that Hoxa-1 might regulate cad6 expression at restricted portions and stages. Other genes perhaps take over Hoxa-1 to sustain the later cad6 expressions, when the Hoxa-1 expression is turned off.
However, the present study cannot eliminate the possibility that loss of Hoxa-1 was not directly linked with the changes in the cad6 expression pattern. Gross morphological changes or some noncharacterized phenotypic transformation of rhombomeres in the mutant hindbrain could have indirectly led to the alterations in cad6 expression. Thus, whether Hoxa-1 activity directly regulates cad6 expression remains to be further investigated. Obviously, Hoxa-1 cannot be the sole regulator for cad6 expression, since the expression pattern of the latter is much more complex than that of the former, and also the spinal cord expression of cad6 was not affected by Hoxa-1 mutation. Multiple positive and negative regulators should be involved in the transcription of the cad6 gene in a position-specific manner. Another Hox gene, Hoxd-9, was found to regulate the expression of a cadherin, L-CAM, in an in vitro system (Goozer et al., 1994).

In summary, cad6 is expressed in various local regions of the developing mouse brain and in subpopulations of neural crest cells. These regional cad6 expressions likely serve for sorting of heterogeneous cells to establish and maintain the regionalized structure of the body. Hoxa-1 gene could be one of the regulators for the spatiotemporal expression of cad6 at early developmental stages.

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