Development 121, 1321-1332 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins

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SUMMARY

We identified two cadherins, c-cad6B and c-cad7, expressed by neural crest cells at their premigratory and migratory stages, respectively, in chicken embryos. cDNA transfection experiments showed that both were homophilic adhesion molecules, endowing cells with specific adhesiveness. During development, c-cad6B appeared in the neural fold, localizing at the future neural crest area. This expression was maintained during neural tube closure, but disappeared after neural crest cells had left the neural tube, suggesting its role in neural fold fusion and/or in the formation and maintenance of the presumptive neural crest domain in the neural plate/tube. Crest cells emerging from the neural tube lost c-cad6B, and a subpopulation of them began to express c-cad7. This subpopulation-specific expression of c-cad7 persisted during their migration. The migrating c-cad7-positive cells clustered together, and eventually populated restricted regions including the dorsal and ventral roots but very little ganglia. The latter was populated with N-cadherin-positive crest cells. Migrating neural crest cells expressed α- and β-catenin at cell-cell contacts, indicating that their cadherins are functioning. These results suggest that the migrating crest cells are grouped into subpopulations expressing different cadherins. The cadherin-mediated specific interaction between crest cells likely plays a role in intercellular signaling between homotypic cells as well as in sorting of heterotypic cells.

Key words: cadherins, catenins, neural crest, neural fold, chicken, intracellular signalling

INTRODUCTION

Neural crest cells originate from the neural fold of the ectoderm. They begin emigration, as the neural plate deeply invaginates and fuses to form the neural tube. The crest cells that have left the ectoderm are diversified into subpopulations. They migrate together initially, but eventually colonize distinct places, giving rise to diverse cell types (LeDouarin, 1982). However, little is known about the molecular mechanisms regulating these processes.

Early studies suggest that cell adhesion receptors are involved in the control of neural crest migration, which include integrin, NCAM, and cadherin (reviewed by Erickson and Perris, 1993). The crest cells express integrins, and use them for the attachment to extracellular matrices (Bronner-Fraser, 1985; Lallier and Bronner-Fraser, 1990; Duband et al., 1991; Lallier and Bronner-Fraser, 1993; Delannet et al., 1994). The expression of NCAM shows a correlation with the emigration of neural crest cells (Thiery et al., 1982; Akitaya and Bronner-Fraser, 1992). Concerning the cadherin, two subtypes of this molecular family, an epithelial cadherin (L-CAM in the chicken and E-cadherin in mammals) and N-cadherin, have been implicated in neural crest development. In early embryos, the entire ectoderm expresses L-CAM or E-cadherin, but as the neural plate forms, this cadherin expression is down-regulated in this specific region of the ectoderm, and finally disappears except at local areas of the neural tube (Thiery et al., 1984; Nose and Takeichi, 1986; Shimamura et al., 1992). Coincidentally, N-cadherin appears in the forming neural tube (Hatta and Takeichi, 1986; Hatta et al. 1987; Duband et al., 1988). However, the N-cadherin expression avoids the dorsal midline region of the neural tube, which contains presumptive neural crest cells (Takeichi, 1987; Akitaya and Bronner-Fraser, 1992; Bronner-Fraser et al., 1992). Emigrating neural crest cells do not express N-cadherin either, although its expression is induced after the cessation of their migration in a number of places, including cranial and dorsal root ganglia (Hatta et al., 1987; Akitaya and Bronner-Fraser, 1992). These observations led us to propose that the loss of cadherins may allow neural crest cells to emerge from the epithelial layer (Takeichi, 1987).

However, recent observations indicate that even dispersed or migratory cells express functional cadherins, and in some cases, cadherin expression is vital for their oriented migration. In gastrulating *Xenopus* embryos, mesodermal cells move as a coherent aggregate in a unidirectional pattern, and they express U-cadherin. If the activity of this cadherin is blocked, the cells lose the ability to migrate following guidance cues present on the substrate (Winklbauer et al., 1992). We recently found that loosely associated sclerotome cells strongly express cadherin-11 (Kimura et al., 1995). Primordial germ cells also form clusters during migration (Gomperts et al., 1994). It is therefore possible that migrating neural crest cells are also

interconnected by certain cadherins, and this could be an essential process for control of their migration and homing.

To test the above hypothesis, we sought cadherins expressed in neural crest cells, and identified two cadherins, termed c-cad6B and c-cad7. In situ hybridization analysis showed that c-cad6B is expressed in the neural fold, and c-cad7, in a subpopulation of migrating crest cells. The c-cad7-positive cells colonized only restricted regions. Neural crest cells, thus, express multiple cadherins in a developmental stage- and subpopulation-specific manner. Our detailed analyses of the activity and expression of these cadherins led us to provide a novel model to explain neural crest cell-cell interactions.

MATERIALS AND METHODS

Animals

Fertilized eggs of White Leghorn chickens were incubated at 37˚C. Staging of the embryos was made according to Hamburger and Hamilton (1951).

cDNA cloning

Poly(A)$^+$ RNAs were prepared from 2 to 3 day-old chicken embryos, and cDNAs were synthesized from the obtained mRNAs. Then, PCR was carried out by the method of Suzuki et al. (1991). The nucleotide sequences for the PCR primers were identical to those used by Sano et al. (1993). Amplified 450-bp fragments were subcloned into Bluescript IISK$^+$ (Stratagene) and sequenced. cRNA probes were then synthesized by use of appropriate restriction enzymes and T3/T7 RNA polymerase in order to perform in situ hybridization. Two clones, nk2 and nk9, were thus isolated. A PCR clone of N-cadherin was also obtained during the above procedure, and used for in situ hybridization.

To obtain full-length cDNA clones for each gene, a cDNA library from adult chicken brains (CLONETECH) was screened by use of radiolabelled probes of the above 450-bp fragments. The initial screening gave a few clones for each gene, and these were used for further screening. Finally, multiple overlapping clones to cover a full-length open reading frame were isolated for nk2 and nk9.

cDNA transfection

Expression vectors to be used for transfection of cells were constructed for nk2 and nk9. Phage clones were connected to yield full-length open reading frame sequences, and subcloned in Bluescript IISK$^+$. They were termed pBSSK-c-cad6B and pBSSK-c-cad7 for nk9 and nk2, respectively. After digestion with two flanking restriction enzymes, inserts were cut out and blunt-ended with T4 DNA polymerase. The *Hin*dIII-*Hpa*I fragment encoding the CAT gene of the expression vector pMiwCAT (Kato et al. 1988) was replaced with those fragments to yield pMiwCcad6B and pMiwCcad7.

L cells were transfected with pMiwCcad6B or pMiwCcad7 by the calcium phosphate precipitation method, as described (Nagafuchi et al., 1987). Transfectants were screened by RNA blotting for their message expression. Among many transfectant clones isolated, two lines were selected for each cadherin: L-c-cad6B(92) and L-c-cad6B(97) with pMiwCcad6B, and L-c-cad7(23) and L-c-cad7(24) with pMiwCcad7. L cells and their transfectants were cultured in a 1:1 mixture of Dulbecco's modified Eagle's MEM and Ham's F12 medium supplemented with 10% FCS (DH10).

RNA blot analysis

5 μg of poly(A)$^+$ RNA isolated from the brains of just-hatched chicks or 10 μg of total RNA from transfectants was dissolved and electrophoresed. RNA was transferred to Hybond-N (Amersham) and hybridized at 42˚C in a buffer containing 50% formamide with a radiolabeled probe of the 450-bp PCR fragments of nk2 or nk9. After hybridization, the filters were washed at 65˚C in 0.2× SSC and 0.1% SDS, and subjected to autoradiography.

In situ hybridization

Whole-mount in situ hybridization was carried out as described previously (Shimamura et al., 1994). The 450-bp PCR fragment of c-cad6B, c-cad7, or N-cadherin, inserted into a Bluescript vector as described above, was used as a probe for in situ hybridization. Some embryos were subjected to in situ hybridization after slicing by the following procedure. Bleached embryos were rehydrated through a descending series of methanol solutions and embedded in 3% agar in PBS. The samples were then sectioned with a Microslicer (D.S.K. DTK-1000, Kyoto) at a thickness of 250 μm, transferred into 100% methanol, and used for hybridization.

For sectioning embryos prestained by whole-mount in situ hybridization, the samples were immersed in a graded series of sucrose solutions, embedded in Tissue-Tek (Miles) and frozen in liquid nitrogen. Cryostat sections of 10-μm thickness were mounted and dried on slide glasses. They were rehydrated in TBS to remove Tissue-Tek and dehydrated through a graded series of ethanol and xylene. Dehydrated samples were mounted with Entellan (Merck) and examined under a Nomarsky-optics microscope.

For in situ hybridization of cultured neural crest cells, the cells were fixed overnight with 4% paraformaldehyde in PBS. They were then hybridized and washed according to the method for cryosections (Shimamura et al., 1994), except that the acetylation process was omitted.

Immunostaining and immunoblotting

The following antibodies were used: a rabbit antiserum specific for β-catenin (a gift from Dr S. Shibamoto), rat monoclonal antibody α18 specific for αE-catenin (a gift from Dr A. Nagafuchi), a rabbit antiserum against chicken N-cadherin (Matsunaga et al., 1988), HNK-1 antibody (Becton-Dickinson), and CY3-conjugated secondary antibodies (Chemicon).

Cultured cells were fixed in 4% paraformaldehyde for 20 minutes at 4˚C and incubated successively in methanol for 10 minutes at –20˚C, 5% skim milk in TBS-Ca for 30 minutes, primary antibodies, and secondary antibodies. For immunostaining tissue sections, embryos were fixed in 4% paraformaldehyde as above, and cryostat-sectioned at a 10-μm thickness. The samples were rehydrated and stained.

Double-staining for HNK-1 by immunofluorescence and for c-cad7 by in situ hybridization was carried out by the embryos first being subjected to whole-mount in situ hybridization, then sectioned, and collected on cover slips. After rehydration in TBS, they were immunostained. Cell cultures that had been prestained by in situ hybridization were used directly for immunostaining. These samples were incubated in 1% bovine serum albumin in TBS for 30 minutes, and then with a diluted HNK-1 antibody solution for 1 hour. After three washes with TBS for 5 minutes each, they were treated with secondary antibodies.

Immunoblotting was performed by a standard method. Signals were detected by use of HRP-linked secondary antibody and the ECL system (Amersham).

Cell aggregation assay

Untransfected L, L-c-cad6B(97), L-c-cad7(24), and cNLm1, an L cell line expressing the chicken N-cadherin (Fujimori et al. 1990), were used. For short-term aggregation assays, cells in monolayer cultures were treated with 0.01% crystalline trypsin (Sigma) and 10 mM $CaCl_2$ in a Ca^{2+}- and Mg^{2+}-free saline buffered with 10 mM Hepes (HCMF, pH 7.4) (TC-treatment) or with the same concentration of trypsin and 1 mM EDTA in HCMF (TE-treatment) for 30 minutes at 37˚C, and dissociated into single cells, as described previously (Takeichi, 1977). The dispersed cells were suspended in HCMF with 1 mM $CaCl_2$

(HMF) or with 1 mM EGTA at a density of 2×10^5 cells/ml. Twenty four-well plates (CORNING) were coated with 4% bovine serum albumin (Sigma, USA) for 1 hour at room temperature and washed twice with HCMF. Into each well, 0.5 ml of the cell suspension was placed, and the plate was incubated in a humidified incubator for 30 minutes at 37˚C on a gyratory shaker (Marysol KS6320) rotating at 80 r.p.m. The samples were photographed after incubation.

For mixed cell aggregation assays, cells in monolayer cultures were fluorescently labeled by overnight incubation with 15 mg/ml DiO (Molecular Probes, USA) in DH10. The cells were dissociated by TE treatment, and mixed with unlabeled cells in a 1:1 ratio at a total cell density of 2×10^5 cells/ml. The cell suspension (0.5 ml) was placed in a well of a 24-well plate, coated with 0.5 ml of 1% agarose prepared with DH10, and rotated in a 5% CO_2 incubator, as described above. After 3.5 hours, the samples were fixed with 0.5% glutaraldehyde for 5 minutes, placed on glass slides, and observed by fluorescence microscopy. TE treatment allowed us to prepare single cell suspensions more easily than TC-treatment (Nose et al., 1988); this is the reason why we employed TE treatment for this particular assay. Under these culture conditions, the parent L cells aggregated little, but all cadherin transfectants showed significant aggregation, as they recovered cadherins. To avoid the effect of quantitative differences in cadherin expression on the cell mixing pattern, cell lines that aggregated at a similar rate were chosen for these experiments.

In vitro culture of neural crest cells

To culture neural crest cells, we collected 2 day-old embryos in HMF at 4˚C. A block of tissues corresponding to the last 10 somites was dissected. The tissue blocks were treated with 0.25% crude trypsin (Gibco) in HMF for 60 minutes at 4˚C. After addition of an equal volume of DH10, somites, and other adherent tissues were freed from the neural tube. Four to six dissected neural tubes were then placed in a minimum amount of DH10 on a fibronectin-coated culture dish. After a 30-minute incubation, to allow the tubes to attach, the dishes were flooded with DH10 supplemented with 5% chick embryo extract prepared according to Stemple et al. (1992).

RESULTS

Isolation of cadherin cDNAs expressed by neural crest cells

To search for cadherins expressed in neural crest cells, we prepared cDNAs from 2/3-day chicken embryos and carried out PCR using the primers designed by Sano et al. (1993). PCR clones thus obtained were used for pilot in situ hybridization to observe their tissue distribution pattern. Two clones, named nk2 and nk9, were expressed in migrating neural crest cells and the presumptive neural crest, respectively . Further screening of a cDNA library yielded overlapping clones that covered a full-length open reading frame for both nk2 and nk9. They encoded proteins with amino acid sequences typical of classic cadherins (Takeichi, 1991). nk2 and nk9 encoded the putative precursor proteins with 785 and 790 amino acids, and the putative mature proteins with 738 and 737, respectively (Fig. 1A). Among the known cadherins, nk9 showed the highest similarity to rat K-cadherin (cadherin-6) and its putative human homologue (Table 1); it was 88% identical to rat K-cadherin. However, the expression pattern of the chicken nk9 differed from that of mouse or rat cadherin-6 (T. Inoue and M. Takeichi, unpublished). Therefore, it is not certain whether nk9 is the chicken orthologue of the mammalian cadherin 6. For this reason, we designate nk9 as chicken cadherin-6B (c-cad6B).

nk2 exhibited 98% identity to rat cadherin-7 in a 40-amino acid sequence of the cytoplasmic domain (Table 1 and Fig. 1), which is the only published sequence of this cadherin (Suzuki et al., 1991). Assuming that they could be homologues, we designate nk2 as chicken cadherin-7 (c-cad7). c-cad6B and c-cad7 were 60% identical to each other. Northern blot analysis showed that c-cad6B encoded 9.5-kb and 6.0-kb messages, and c-cad7, a 5.6-kb message (Fig. 1B).

Adhesion activity and specificity of c-cad6B and c-cad7

To test whether the cloned c-cad6B and c-cad7 cDNAs encode functional adhesion molecules, we transfected L cells, which have no endogenous cadherins (Nagafuchi et al., 1987), with the cDNAs, and isolated transfectant clones whose exogenous cadherin expression was confirmed by northern blotting analysis (Fig. 1C). Both cadherin cDNAs induced a morphological change in L cell layers; that is, the transfected cells became associated with one another, in contrast with the dispersed pattern of the parent cells (Fig. 2A-C). When the L cells transfected with c-cad6B or c-cad7 cDNA were dispersed by treatment with trypsin in the presence of Ca^{2+} (TC-treatment) and allowed to reaggregate, they quickly aggregated in a Ca^{2+}-dependent manner within 30 minutes (Fig. 2D-G), whereas the transfectants dissociated with trypsin in the presence of EDTA (TE-treatment) did not aggregate during such a short incubation period (data not shown). This profile of Ca^{2+} sensitivity is typical of classic cadherins (Takeichi, 1988). However, the parent L cells aggregated little after either trypsin treatment. These results indicate that c-cad6B and c-cad7 cDNAs encode functional adhesion proteins whose properties are similar to those of other classic cadherins.

We next explored the adhesion specificity of c-cad6B and c-cad7, by mixing their transfectants with each other, or by mixing them with N-cadherin transfectants. Cells of one line were fluorescently labeled with DiO, dissociated, and mixed with cells of another line not labeled. As controls, DiO-labeled cells were mixed with cells of the same line not labeled. In every control combination, cells were randomly intermixed in the aggregates (Fig. 3A,B). When c-cad6B or c-cad7-transfectants were mixed with N-cadherin transfectants, cells of each

Table 1. Percentage amino acid identity between similar or homologous cadherins

	N-ter	CR1	CR2	CR3	CR4	preTM	CP(CP40)	Total
nk9 (c-cad6B)/rK-cadherin	97	94	94	74	90	86	86 (93)	88
nk9 (c-cad6B)/h-cadherin6	–	–	–	–	89	86	93 (93)	–
nk2 (c-cad7)/r-cadherin7	–	–	–	–	–	–	– (98)	–

N-ter, N-terminal domain; CR1-4, cadherin repeat 1-4; preTM, pretransmembrane domain; CP, cytoplasmic domain; CP40, the 40-amino acid region in CP shown in Fig. 1. Minus signs indicate no information available. r and h represent rat and human, respectively.

A

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precursor
c-cad6B  MRTYHCFWLLFWAGQPHQSFLTLLSKRTSGFPEKEKVLVLSGNSRRDLSRSKR
c-cad7   MKLGKVEFCHLL--QIIALFLCLSGMNQAE-PSRSRSKPYFQSGR---TRTKR▲

N-terminal domain
c-cad6B  SWMWNQFFLLEEYTGTDYQYVGKLHSDQD
c-cad7   SWVWNQFFVLEEYMGSDPLYVGKLHSDVD

CR1
c-cad6B  KGDGSLKYILSGDGAGDLFIINENTGDIQATKRLDREEKPVYILRAQAINRRTGRPVEPESEFIIKIHDINDNEPMFTKDVYNASIPEMSDVGTFVVQVTATDAD
c-cad7   KGDGSIKYILSGEGASSIFIIDENTGDIHATKRLDREEQAYYTLRAQAHDRLTNKPVEPESEFVIKIQDINDNEPKFLDGPYTAGVPEMSPVGTSVVQVTATDAD

CR2
c-cad6B  DPTYGNSAKVVYSILQGQPYFSVESETGIIKTALLNMDRENREQYQVVIQAKDMGGQMGGLSGTTTVNITLTDVNDNPPRFPQSTYQFRAPESTPPDSPIGRIKANDAD
c-cad7   DPTYGNSARVVYSILQGQPYFSVEPKTGIIKTALPNMDREAKDQYLLVIQAKDMVGQNGGLSGTTSVTVTLTDVNDNPPRFPRRSYQYNVPESLPLASVVARIKAADAD

CR3
c-cad6B  VDENAEIEYSITEGDGYDMFGITTDKDTQEGIITVKKALDFENKNLYILKVEATNTHVDPRFLYLGPFKDSATIRIQVEDVDEPPVFSRPAYIIEVKEDVPINSVIGTVTAQDPD
c-cad7   VGPNAEMEYKIVDGDGLGVFKISVDKDTQEGIITIQKELDFEAKTSYTLRIEAANMHVDPRFLSLGPFSDMTTVKIIVEDVDEPPVFTSRLYSMVVSEAAKVGTIIGTVAAHDPD

CR4
c-cad6B  AAKNPVKYSVDRHTDMDRVFNINSGNGSIFTSKTLDRETLLWHNITVIAAEINNPKQSSRVPVFIKVLDVNDNAPEFAMFYETFVCENAKAEQLIQTLSAVDKD
c-cad7   ASNSPVRYSIDRNTDLERYFNIDANSGVITTAKSLDRETNAVHNITVLAMESQNPAQIGRGYVAITILDINDNAPEFAMEYETTVCENAQPGQIIQKISAIDKD

premembrane domain
c-cad6B  DSYSGHQFSFSIAPEAASSSNFTLQDNRDNTAGIFTRKIRYNRHEMSTYLLPVVISDNDYPIQSSTETVTIRVCACDHRGKMLSCNAEALIHPTGLST
c-cad7   DPPNGHQFYFSLTAEAANNHNFTLQDNKDNTATVLTRRNGFRRQEQSVFYLPIFIVDSGSPSLSSTNTLTIRVCDCDADGIAQTCNAEAYILPAGLST

transmembrane domain
c-cad6B  GALIAILLCIIILLVTVVLFAAL
c-cad7   GALIAILACVLTLLVLVLLIVTM

cytoplasmic domain
c-cad6B  RRQRKKEPLII-SKEDIRDNIVSYNDEGGGEEDTQAFDIGTLRNPEAIDDNKLRRDIVPETLFMPR-RTATARDNTDVRDFINQRLKEND
c-cad7   RR-RKKEPLIFDEERDIRENIVRYDDEGGGEEDTEAFDMAALRNLNIIRDTKTRRDVTPEIQFLSRPTFKSIPDNVIFREFIWERLKEAD

c-cad6B  TDPAAPPYDSLATYAYEGNGSVAESLSSLESVTTDGDQDYDYLSDWGPRFKKLADMYGSMDSDKDS
c-cad7   VDPCAPPYDSLQTYAFEGNGSVAESLSSLDSISSNSDQNYDYLSDWGPRFKRLADMYGSGPDCLYS
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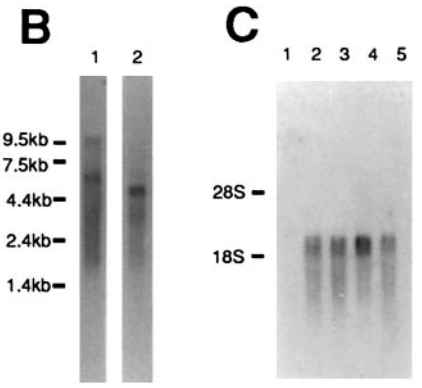


Fig. 1. Predicted amino acid sequences of c-cad6B and c-cad7, and northern blot analyses. (A) The predicted amino acid sequences of nk9 (c-cad6B) and nk2 (c-cad7) are aligned. The triangle indicates the putative cleavage site for protein maturation, inferred from other cadherin data. Arrows show the positions for PCR primers used for amplifying 450-bp DNA fragments. The underlined cytoplasmic 40-amino acid sequence corresponds to CP40 in Table 1. CR, cadherin repeat. The accession numbers for the nucleotide sequences are D42149 and D42150 for c-cad6B and c-cad7, respectively. (B,C) RNA blot analyses. (B) Expression of c-cad6B (lane 1) and c-cad7 (lane 2) transcripts in the chick brain. (C) Expression of c-cad6B/c-cad7 in the parent L cell line (lane 1), of c-cad6B in two lines, clone nos. 92 and 97, of its transfectants (lanes 2, 3), and of c-cad7 in two lines, clone nos. 23 and 24, of its transfectants (lanes 4, 5). For lane 1, a mixture of the probes for c-cad6B/c-cad7 were used for detection. Transcripts of 3.5 kb, corresponding to the expression vectors in size, are detected in the transfectants but not in the parental L cells.

line independently aggregated, forming few intermixed aggregates (Fig. 3D,E). Thus, c-cad6B or c-cad7 essentially does not crossinteract with N-cadherin. However, in mixtures of c-cad6B and c-cad7 transfectants, most of them formed chimeric aggregates, although in these aggregates, they never randomly intermixed; cells of the same line tended to cluster together and segregate from those of the other line within the same aggregate (Fig. 3C). Occasionally, even in this combination, aggregates composed of only one cell line were observed. This suggests that, while c-cad6B and c-cad7 can crossinteract with each other, they prefer like molecules.

We also studied expression of αE-catenin, a subtype of α-catenin (Nagafuchi et al., 1991; Herrenknecht et al., 1991), and β-catenin (McCrea et al., 1991), that are cadherin-associated proteins, in the above transfectant lines. The parent L cells expressed a certain level of αE-catenin, but little β-catenin (Fig. 4A). In both c-cad6B and c-cad7 transfectants, the expression level of αE-catenin was increased, and also high β-catenin expressions were induced (Fig. 4A). Immunofluorescence studies showed that these catenins were concentrated at cell-cell contact sites, mostly on fine filopodia bridging cells (Fig. 4B-E). Such localization of catenins at cell-cell contacts never occurred in the parental L cells (data not shown). These observations suggest that c-cad6B and c-cad7 function in adhesion through the association with the two catenins, as found with other classic cadherins (reviewed by Kemler, 1993).

Expression pattern of c-cad6B in early embryos

The expression patterns of c-cad6B and c-cad7 in embryos were analyzed by whole-mount in situ hybridization. c-cad6B transcripts were first detected in the splanchnic mesoderm in primitive streak-stage (stage 4) embryos (Fig. 5A). Then, a strong expression of c-cad6B appeared along the neural fold in embryos at stage 6 (Fig. 5B-D). Sections of the stained embryos revealed that the hybridization signals were exclusively localized to the neural fold corresponding to the future neural crest (Fig. 5E). In this c-cad6B-positive zone, all cells

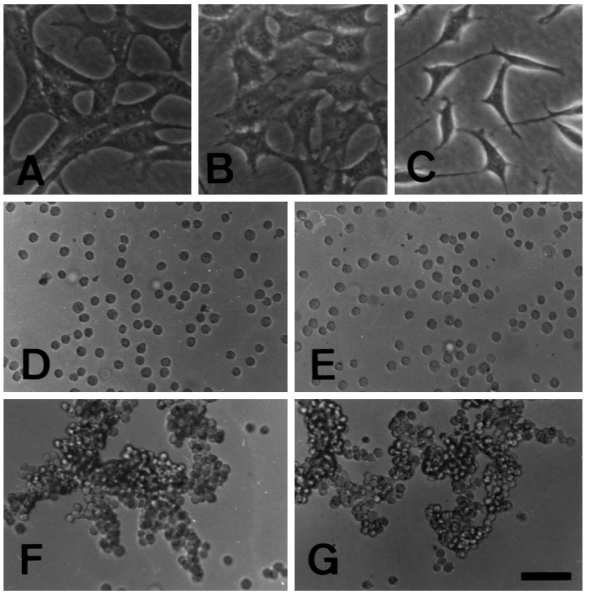


Fig. 2. Adhesion activity of c-cad6B and c-cad7 transfectants. (A-C) Cells in monolayer cultures. Phase-contrast photomicrographs of a c-cad6B transfectant line, L-c-cad6B(92) (A), a c-cad7 transfectant line, L-c-cad7(23) (B), and the untransfected L (C). (D-G) Cells in aggregation cultures. TC-treated L-c-cad6B(92) (D,F) and L-c-cad7(23) (E,G) were incubated with 1 mM EGTA (D,E) or with 1 mM Ca^{2+} (F,G) for 30 minutes. Bar, 50 μm.

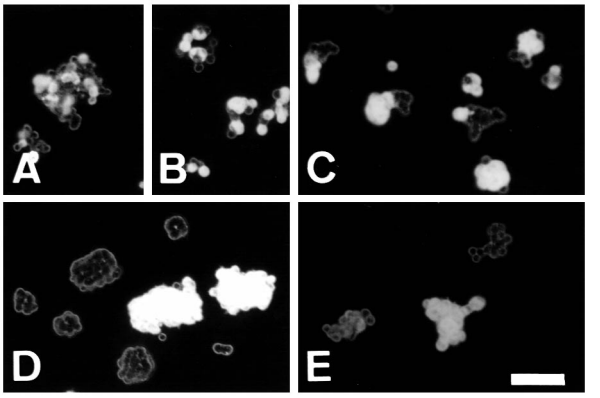


Fig. 3. Comparison of adhesion specificity among c-cad6B, c-cad7 and N-cadherin. Cells labeled with DiO and dissociated by TE-treatment were mixed in a 1:1 ratio with unlabeled cells dissociated by the same treatment, and incubated for 3.5 hours. (A,B) Control combinations: L-c-cad6B(97) + L-c-cad6B(97) (A), and L-c-cad7(24) + L-c-cad7(24) (B). (C) L-c-cad6B(97) + L-c-cad7(24). (D,E) Mixing with the N-cadherin transfectant line cNLm1: cNLm1 + L-c-cad6B(97) (D), and cNLm1 + L-c-cad7(24) (E). In all these combinations, the former was DiO-labeled. Note the random intermixing of cells in the controls, a partial segregation between c-cad6B and c-cad7 transfectants, and their almost complete segregation from the N-cadherin transfectants. Bar, 50 μm.

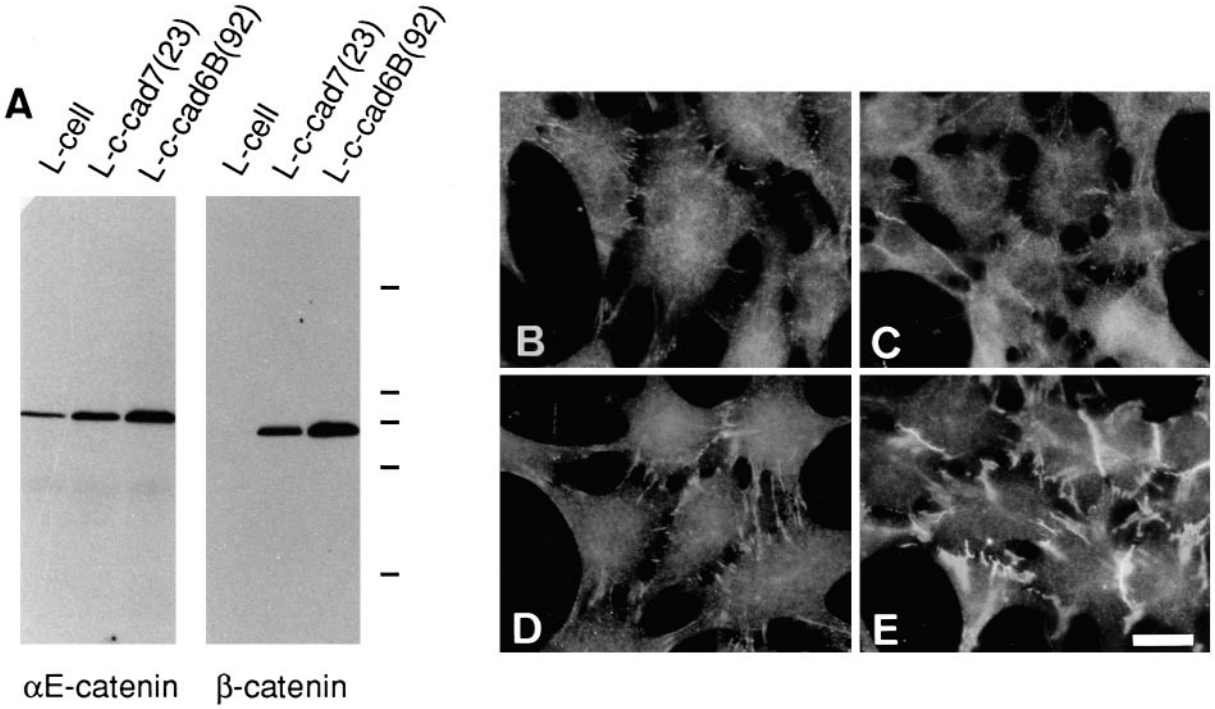


Fig. 4. Expression of catenins in c-cad6B and c-cad7 transfectants. (A) Immunoblot analysis for αE-catenin and β-catenin in the parent L, L-c-cad7(23) and L-c-cad6B(92) cells. Lysates obtained from the same number of cells were applied to each lane. (B-E) Immunofluorescence staining for αE-catenin (B,C), and β-catenin (D,E) in L-c-cad7(23) (B,D) or L-c-cad6B(92) (C,E). Bar, 20 μm.

stained evenly. The expression of c-cad6B in the neural fold was maintained during the closure of the neural tube (Fig. 5F), resulting in positive signals at the dorsal midline of the closed tube (Fig. 5G). At this stage, neural crest cells emerge from the neural tube, and we found that all crest cells that had left the neural tube no longer expressed c-cad6B (Fig. 5F,G). The c-

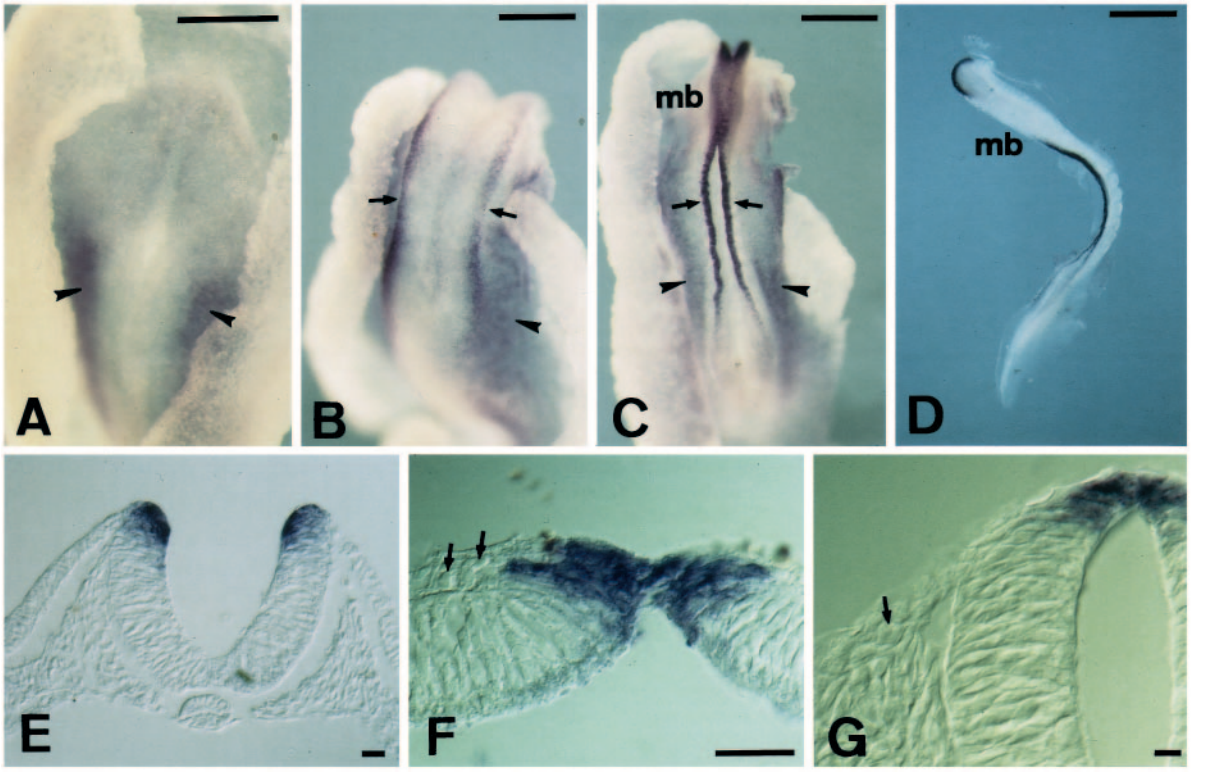


Fig. 5. c-cad6B expression during early development. (A-D) Whole-mount in situ hybridization for c-cad6B at stage 4 (A), stage 6 (B), stage 8 (C), and stage 9 (D). Arrows and arrowheads in (A-C) indicate c-cad6B signals at the neural folds and splanchnic mesoderm, respectively. c-cad6B expression is first turned off in the midbrain (mb) after the neural fold fusion. (E-G) Embryos prestained by whole-mount in situ hybridization were sectioned at the hindbrain level at stage 8 (E), at the forebrain level at stage 9 (F), and at the trunk level at embryonic day 3 (E3) (G). Arrows in F and G indicate c-cad6B-negative migrating neural crest cells. Bars, 100 μm for (A-D), and 10 μm for (E-G).

cad6B signals in the dorsal neural tube diminished as neural crest cells left; this first occurred in the midbrain (Fig. 5C,D) and then extended to other regions.

c-cad7 expression in neural crest cells and its relation to HNK-1 and N-cadherin expressions

In whole-mount stained samples, c-cad7 transcripts were first observed in a disperse cell population localized on the dorsal midbrain in embryos at stage 10 (Fig. 6A). Sectioning these samples revealed that the signals were present on a subset of cells in the neural crest (Fig. 6E). According to a rough estimate, less than 50% of the neural crest cells exhibited the hybridization signals. Subsequently, similar signals appeared in other parts of the head and also in the trunk, which were again localized on migrating neural crest cells (see below).

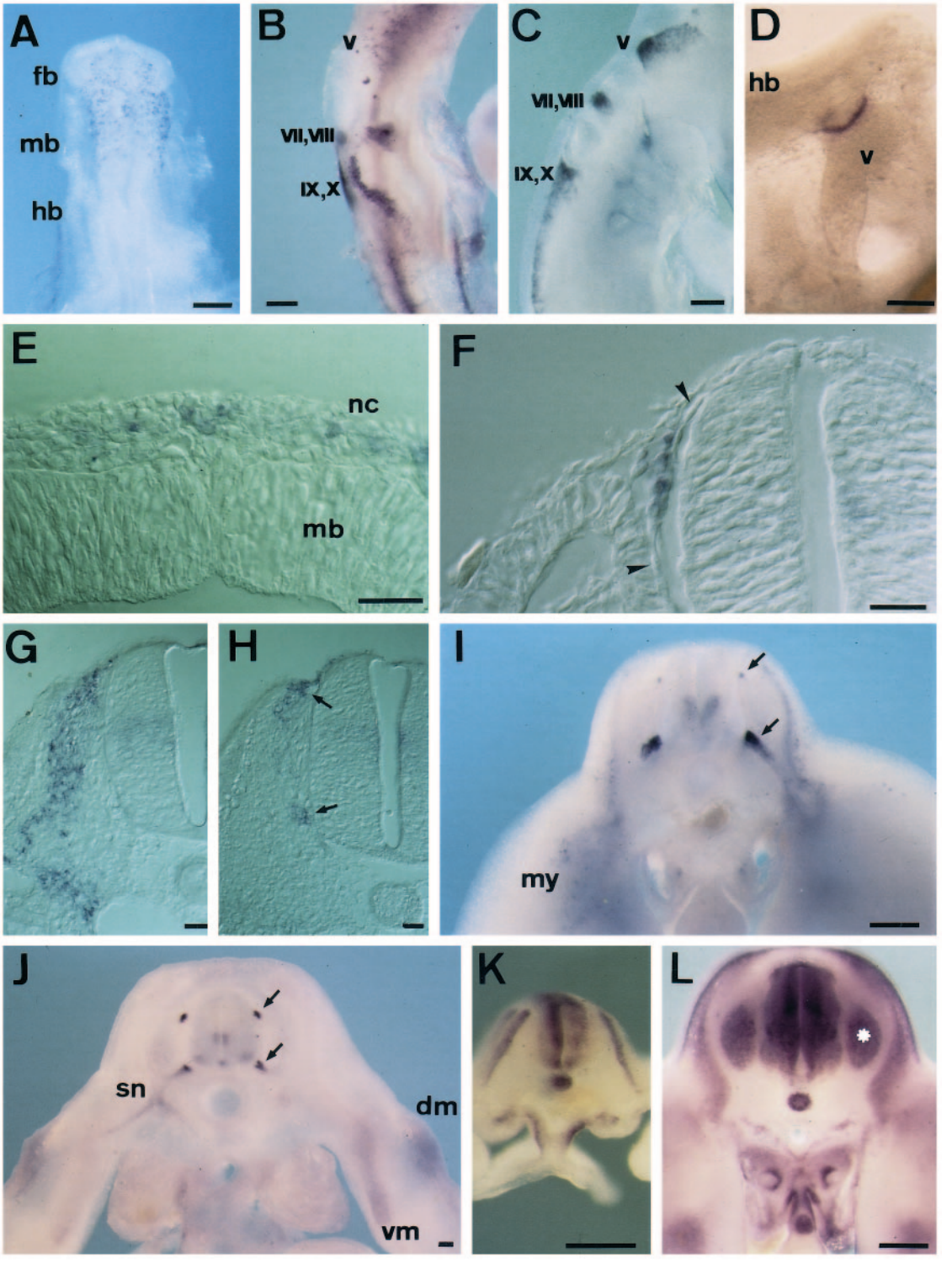
In the hindbrain region at subsequent developmental stages, the c-cad7 hybridization signals became clustered into three distinctive areas where the trigeminal (V) ganglion, the facial/acoustic (VII/VIII) complex, and the glossopharyngeal/vagus (IX/X) nerves were developing (Fig. 6B). Weak c-cad7 signals further descended along the nerves. At the next stage, intense c-cad7 signals remained only at the proximal portion of each ganglion or nerve (Fig. 6C). These signals were eventually arranged into a sharp, ring-shaped structure surrounding the nerve (Fig. 6D). In more anterior portions of the brain, similar c-cad7 signals associated with cranial nerve differentiation were observed (data not shown). However, we did not observe any strong c-cad7 signals in the head mesenchymal cells, many of which are known to be derived from the neural crest.

In the trunk of embryos at stage 15, a cluster of c-cad7-positive neural crest cells was located between the dorsal edge of the dermamyotome and the neural tube (Fig. 6F). In the same samples, we could also observe c-cad7-negative migrating crest cells (Fig. 6F). At stage 19, c-cad7-positive cells migrated down into various regions lateral to the neural tube, forming a stream of cells (Fig. 6G). In addition to this stream, certain populations of c-cad7-positive cells accumulated in the proximal ventral root, and perhaps also in the

Fig. 6. c-cad7 and N-cadherin expression during development. (A-J) In situ hybridization for c-cad7. (K,L) In situ hybridization for N-cadherin. (A-D) Whole-mount samples in the head region at stage 10 (A), and in the hindbrain region at stage 13 (B), stage 18 (C), and E4 (D). Signals observed around branchial arches in C are non-specific stainings. (E-H) Thin sections of the midbrain at stage 10 (E), and of the trunk at stage 15 (F) and stage 19 (G,H). G and H are adjacent sections of the same embryo. (I,J) Thick slices of embryos at the wing bud level at E4 (I) and E6 (J). Arrowheads in F indicate c-cad7-negative neural crest cells, and arrows in H, I, J, indicate the proximal dorsal and ventral roots. (K, L) N-cadherin expression in thick slices at the wing bud level at stage 19 (K) and E4 (L). Asterisk indicates a dorsal root ganglion. fb, forebrain; mb, midbrain; hb, hindbrain; V, trigeminal ganglion; VII,VIII, facial/acoustic complex; IX,X, glossopharyngeal/vagus nerves; nc, neural crest; my, migrating myoblasts; sn, spinal nerve; dm, dorsal muscle mass; vm, ventral muscle mass. Bars, 200 μm (A-D, I-L), and 20 μm (E-H).

proximal dorsal root (Fig. 6H), as observed in sections adjacent to the one shown in Fig. 6G. Soon after this stage, the strongest c-cad7 signals became confined to the proximal dorsal root and proximal ventral root, although faint signals were also apparent along the spinal nerves, and even fainter ones on the dorsal root ganglia (Fig. 6I,J). The c-cad7 signals in the dorsolateral trunk were continuous with those in the proximal glossopharyngeal/vagus nerves (Fig. 6C), suggesting that cells strongly expressing c-cad7 in the trunk and head regions are of the same type. However, c-cad7-positive cells did not migrate between the somite and overlying ectoderm.

The above observations suggest that only a subpopulation of neural crest cells expresses c-cad7. To verify this idea, we double-stained sections of stage 16 embryos for c-cad7 transcripts and the HNK-1 antigen, a neural crest marker (Vincent et al., 1983; Bronner-Fraser, 1986). At this stage, most c-cad7-positive cells were still located around the level of the dorsal edge of the dermamyotome (Fig. 7A). Among these cells,



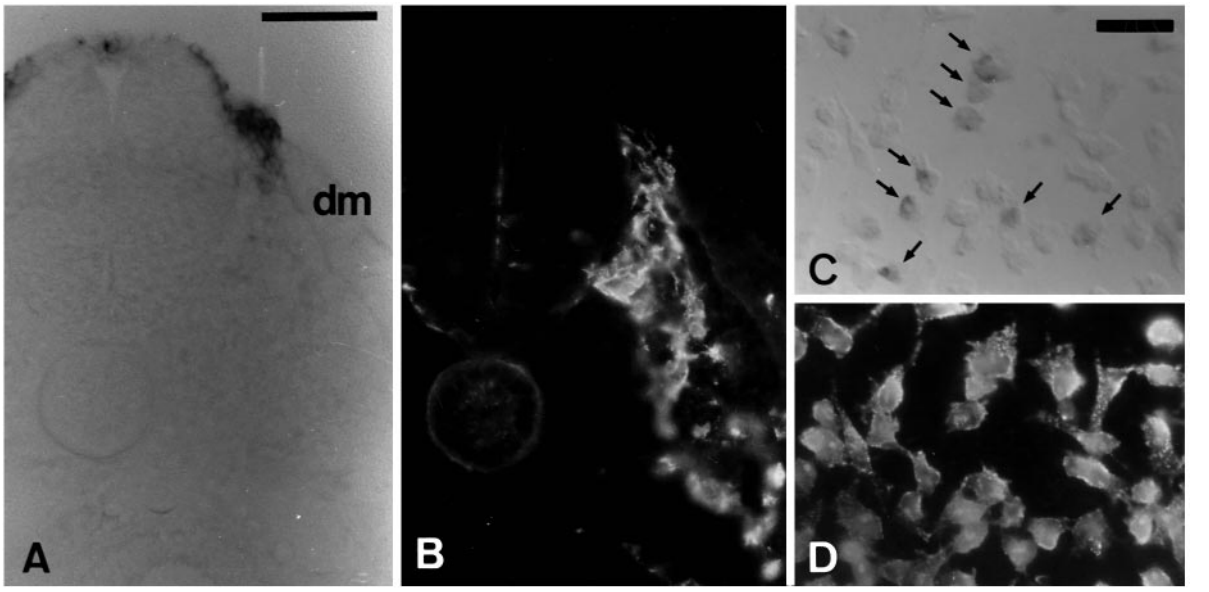


Fig. 7. Double-staining for c-cad7 and HNK-1 in neural crest cells. (A,B) A cryostat section of a stage 16 embryo at the trunk region, double-stained for c-cad7 by in situ hybridization (A) and for HNK-1 by immunofluorescence (B). Note the presence of HNK-1-positive, c-cad7-negative neural crest cells that migrate faster than c-cad7-positive cells. (C,D) Neural crest cells double-stained for c-cad7 (C) and for HNK-1 (D) in a neural tube explant culture incubated for 24 hours. Arrows show c-cad7-positive cells. dm, dermamyotome. Bar, 50 μm.

HNK-1 expression was observed only in those ventrally located (Fig. 7B), suggesting the onset of HNK-1 expression begins at this level. Other HNK-1-positive cells were already migrating further down through the sclerotome without expression of c-cad7. This observation confirmed the idea of a subpopulation-specific expression of c-cad7, and also indicated that the c-cad7-positive cells form clusters segregated from the negative populations, migrating more slowly than the latter. For a similar analysis at the individual cellular level, we explanted neural tubes in vitro and stained neural crest cells migrating out of the explants for HNK-1 and c-cad7. As found in vivo, only a subset of HNK-1 positive cells expressed c-cad7, and they often formed small clusters (Fig. 7C,D).

We also examined N-cadherin expression by in situ hybridization during the neural crest migration process. While the transcripts of this cadherin occurred in the neural tube, dermamyotome, and notochord, they were not detected in migrating crest cells, at least at the present level of sensitivity (Fig. 6K). At later stages, intense N-cadherin signals appeared in forming dorsal root ganglia (Fig. 6L), and later, in many other regions as described previously (Hatta et al. 1987; Duband et al., 1988).

Expression of c-cad7 in non-neural crest cells

c-cad7 signals were also detected within the neural tube and in other tissues of the trunk. In the spinal cord at stage 19, ventricular cells at the sulcus limitans began to display a weak c-cad7 expression (Fig. 6F,G), and subsequently clusters of motor neurons and the floor plate faintly expressed it (Fig. 6J). The myotome weakly expressed c-cad7. Furthermore, a group of cells migrating from the ventral dermamyotome into the limb buds expressed c-cad7 (Fig. 6I). This cell group subsequently split into the ventral and dorsal portions of the limb bud, and finally clustered at each position (Fig. 6J). The localization pattern of these cells is reminiscent of that of *Pax-3*-positive myoblasts (Goulding et al., 1994; Williams et al., 1994), suggesting that they are also myoblasts.

Expression of catenins in neural crest cells

Finally, we asked whether neural crest cells express αE- and β-catenins at their mutual contact sites. This question is important to assess the activity of cadherins in these cells, as the catenins are known to accumulate in cell-cell contacts only when cadherins are functioning (see Fig. 4). Sections of embryos at stage 10 were stained for these two catenins, and also for N-cadherin for comparison. The results showed that not only the premigratory neural crest (Fig. 8A,B) but also crest cells that had initiated migration, evenly expressed both catenins (Fig. 8D,E). Importantly, these catenins accumulated at cell-cell contacts. N-cadherin was detected neither in the premigratory neural crest nor in migrating crest cells (Fig. 8C,F), confirming the above in situ hybridization results.

We also examined catenin expression in neural tube explant cultures. Neuroepithelial cells of the neural tube strongly expressed both αE- and β-catenins (Fig. 9A,D). Migrating neural crest cells also strongly stained for β-catenin at fine process mediating cell-cell contacts (Fig. 9E). Likewise, we could detect αE-catenin on migrating crest cells (Fig. 9B), although its staining was quite faint compared with that on neuroepithelial cells. Cells located in the peripheral regions of neural crest outgrowth in the culture tended to show stronger staining for αE-catenin (Fig. 9C) as well as for β-catenin (Fig. 9F), suggesting that their expression increases during differentiation. Importantly, all neural crest cells in the culture appeared to be positive for the expression of both catenins, irrespective of their location.

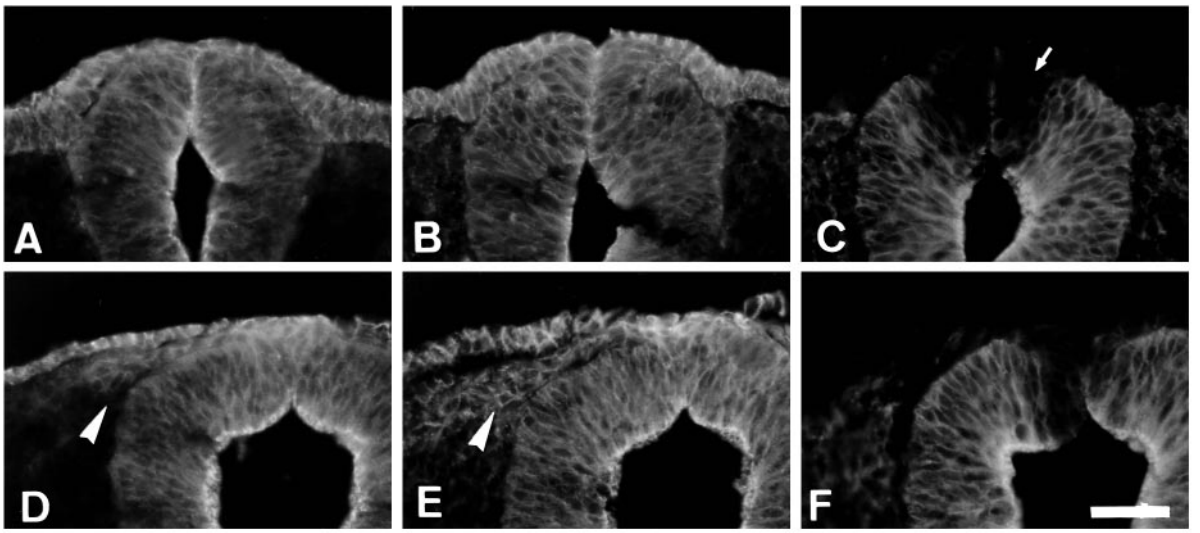


Fig. 8. Expression of αE- and β-catenin, and N-cadherin in the neural crest. Immunostaining for αE-catenin (A,D), β-catenin (B,E), and N-cadherin (C,F). A stage 10 embryo was sectioned at the hindbrain level (A-C), and at the midbrain level (D-F). Adjacent or nearby sections were stained in each level. In A-C, future neural crest cells are still localized in the neural tube at the dorsal N-cadherin-negative region (arrow); this region, however, expressed both α- and β-catenin. In D-F, neural crest cells are migrating out of the neural tube; they express both catenins at their cell-cell contact sites (arrowheads). N-cadherin signals observed in the mesenchymal region are presumably on mesodermal cells, but not neural crest cells, as assessed by histological observations. Bar, 50 μm.

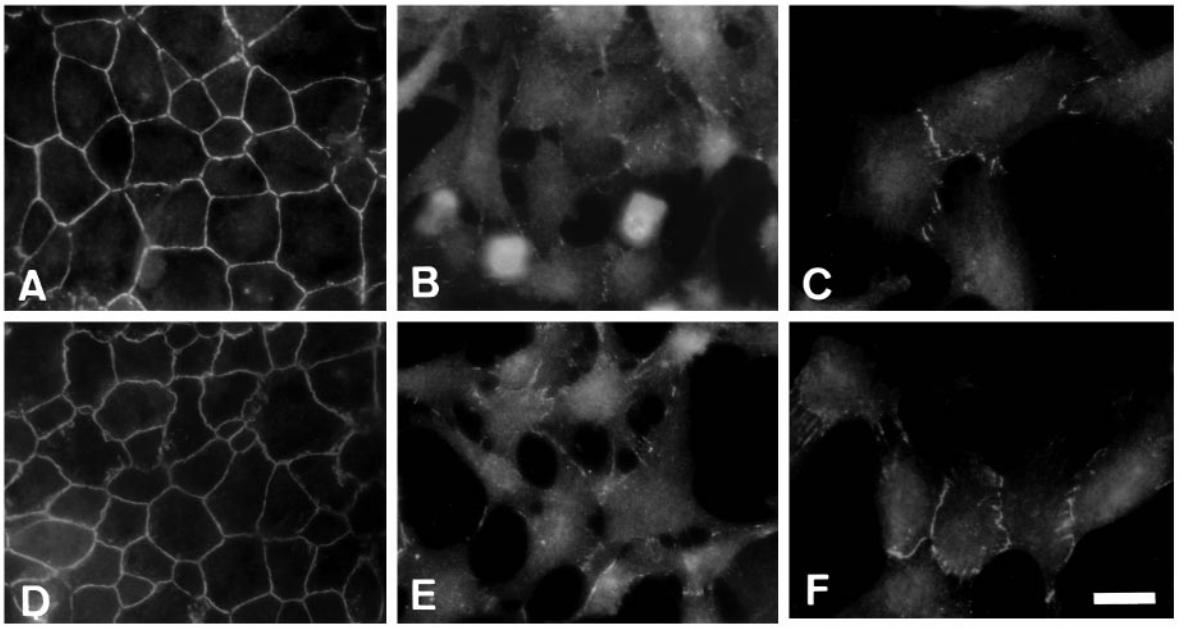


Fig. 9. Expression of αE- and β-catenin in neural crest cultures. Immunofluorescent staining for αE-catenin (A-C) and β-catenin (D-F) in a neural tube explant culture incubated for 24 hours. (A,D) Neuroepithelial cells composing the neural tube. (B,E) Neural crest cells that have just migrated out of the neural tube. (C,F) Neural crest cells located in the periphery of the explant culture, which are presumably at a more differentiated state than those in D and E. Bar, 20 μm.

DISCUSSION

In the present study, we identified two cadherins expressed by presumptive and migrating neural crest cells. The transfection of L cells with c-cad6B and c-cad7 cDNAs showed that both cadherins are functional. In these transfectants, α- and β-catenins were up-regulated at least at the protein level and accumulated at cell-cell boundaries. A similar profile of the up-regulation of the two catenins was observed when L cells had been transfected with E-cadherin (Nagafuchi et al., 1991, 1994). These observations suggest that c-cad6B and c-cad7 function through the interaction with catenins, as do other cadherins (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; Hirano et al., 1992; Watabe et al., 1994). The upregulation of

catenin levels presumably reflects their retarded turnover that might have resulted from the association with cadherins. Our preliminary immunoprecipitation experiments showed that each of these cadherins directly associated with the catenins (S. Nakagawa and M. Takeichi, unpublished data).

Concerning the adhesion specificity of c-cad6B and c-cad7, they could crossinteract with each other, although they preferred interacting with like molecules. A similar type of crossinteraction was observed between N-cadherin and R-cadherin (Inuzuka et al., 1991; Matsunami et al., 1993). The molecular basis of these crossinteractions, however, remains to be investigated. Neither c-cad6B nor c-cad7 interacted with N-cadherin, as has been found in many other combinations of cadherins (Nose et al., 1988; Miyatani et al., 1989; Friedlander et al., 1989; Kimura et al., 1995), suggesting that these novel cadherins can be involved in selective cell adhesion, as discussed before (Takeichi, 1987, 1988).

Possible roles for c-cad6B

c-cad6B expression occurred in the neural fold, and persisted during its fusion process. The neural fold is composed of two cell populations: one gives rise to the neural crest and the other differentiates into components of the neural tube (Selleck et al., 1993). Since the neural fold homogeneously stained for c-cad6B transcripts, both populations likely express this cadherin.

The localization of c-cad6B suggests it has multiple roles. The ectoderm forming the neural plate originally expresses L-CAM or E-cadherin (Thiery et al., 1984; Nose and Takeichi, 1986). During the invagination of the neural plate, this cadherin is gradually replaced by N-cadherin. This N-cadherin expression, however, avoids the future neural crest (Akitaya and Bronner-Fraser, 1992; Bronner-Fraser et al., 1992) (Fig. 8C). Thus, at least three cadherins are differentially expressed in this area, that is, L-CAM/E-cadherin in the overlying ectoderm, c-cad6B in the neural fold, and N-cadherin in the remaining part of the forming neural tube. One role of c-cad6B is possibly the establishment of the future neural crest domain in the neural plate/tube. The inability of c-cad6B to interact with N-cadherin could serve to prevent the future neural crest cells from random mixing with adjacent neural plate cells. A similar principle may also work for the segregation of those cells from the future epidermis expressing L-CAM. The second possible role for c-cad6B is that it may be involved in the fusion of the neural fold, because the initial contacts between the closing neural folds occur through the cells expressing this cadherin. These possibilities can be tested when antibodies to block c-cad6B function become available. However, crest cells that have left the neural plate/tube no longer express c-cad6B. The cessation of c-cad6B expression could be a prerequisite for these cells to detach from the neural tube.

Possible roles for c-cad7 and other cadherins in migrating neural crest cells

The expression of c-cad7 in neural crest cells began when they were still present in the ‘crest’ region. This finding supports the idea that there exists a heterogeneity in the premigratory neural crest cells (reviewed by Marusich and Weston, 1991; Selleck et al., 1993). c-cad7-positive cells then migrated lateral to the neural tube, but not into the dermatome-ectoderm interface, indicating that their differentiation fate is restricted. The c-cad7-positive cells ceased migration at restricted regions such as the dorsal root, ventral root, and spinal nerve; the strongest expression of c-cad7 was observed at the proximal dorsal root and proximal ventral root. In the hindbrain, c-cad7 was most strongly expressed at the junctions between the neural tube and cranial ganglia or nerves; this localization is consistent with that in the trunk, as the afferent sensory fibers and motor axons use the same routes to enter into or exit from the neural tube in the cranial region. These localization patterns of c-cad7-positive crest cells suggest that their differentiation fate is restricted to a particular class of Schwann cells; their distribution is indeed reminiscent of that of Schwann cells identified by an early differentiation maker, Po (Bhattacharyya et al., 1991). The ring-shaped arrangement of c-cad7-positive cells surrounding the cranial nerves suggests that they may play a specific role in the organization of these nerves.

However, we do not know whether only a fixed crest population expresses c-cad7 throughout their migration process, or whether its expression is induced in other crest cells during their migration. It is also unknown if the observed changes in c-cad7 expression involve its turn-off in certain cell populations or reflect only relocalization of the originally positive cells. c-cad7 was also expressed in non neural crest-derived tissues. The expression of c-cad7 in motor neurons is intriguing, because their axons interact with Schwann cells and also with myoblasts, both expressing the same cadherin.

An important question is whether migrating neural crest cells are actively using their cadherins. An early study showed that neural crest cells display cadherin-like activity (Aoyama et al., 1985). In the present study, we demonstrated that both α- and β-catenins are concentrated at cell-cell contact sites in migrating crest cells. The catenins are known to be crucial for cadherin function (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; Hirano et al., 1992), and they localize at cell-cell contacts only when cadherins are active. It is therefore very likely that even migrating neural crest cells are linked together with cadherins. Another important finding is that all migrating neural crest cells were immunostained for the catenins, implying that c-cad7-negative crest cells express other types of cadherin, as yet unidentified. In fact, we recently showed that crest cells migrating into branchial arches express cadherin-11 in the mouse embryo (Kimura et al., 1995). Interestingly, crest cells with different destinations seem to express different cadherins, leading us to postulate that each subpopulation of migrating neural crest cells may express certain specific cadherins. If this is the case, neural crest cells can selectively interact with cells belonging to the same subpopulation by use of cadherins that have homophilic binding specificity, as found for c-cad7 and cadherin-11 (Kimura et al., 1995). Consistent with this idea, we observed that c-cad7-positive crest cells tended to form clusters segregated from the others.

The selective interaction between migrating neural crest cells should have certain importance for their developmental process. For example, by forming subpopulation-specific clusters, cells of the clusters can share intercellular signals specific for this particular subgroup, which may be used for coordination of their migration and differentiation. It should be noted that gastrulating mesodermal cells lost their directional movement when their cadherin-mediated contacts were blocked (Winklbauer et al., 1992), suggesting that these cells exchange signals via cadherin-mediated contacts for directing

themselves to particular destinations. It could even be possible that, the majority of migrating cells may merely follow a small number of 'guide' cells, and therefore their specific intercellular connections are necessary, hence the activity of cadherins. The cadherin-mediated specific interaction may also be used for sorting of heterogeneous crest cells during their final aggregation at their destinations. This idea is based upon the observation that, while cells accumulating in dorsal and ventral roots express c-cad7, cells forming dorsal root ganglia express N-cadherin and also R-cadherin (Hatta et al., 1987; Inuzuka et al., 1991), locating side by side. In addition, we found that catenin expression levels increase during crest cell differentiation, as shown by in vitro culture experiments. The increase in the catenin levels may allow tighter cell-cell associations, and could be a crucial step for neural crest cells to settle down to the immobile state and to form solid tissues. Meanwhile, there is a report that migratory neural crest cells are negative for α-catenin staining in *Xenopus* embryos (Schneider et al., 1993), but it is possible that the level of α-catenin expression was simply too low to be detected in this material, even though it was present. In any case, this report does not seem to contradict our observation that the level of α-catenin expression in migrating crest cells is not so high.

The adhesive interaction between migrating cells was also observed in primordial germ cells (Gomperts et al., 1994). We also showed that migrating myoblasts expressed c-cad7. These observations indicate that it may be a general rule for migrating cells to form networks between themselves. These findings provide a novel insight into the mechanisms controlling migration and homing of cells.

We thank Kenji Shimamura and Yoshiko Takahashi for advice on experiments and data analysis; Akira Nagafuchi and Sayumi Shibamoto for antibodies; Akinao Nose, Harukazu Nakamura, and Nobue Itasaki for technical advice and for providing laboratory space. We also thank Kenneth Yamada and Jean Paul Thiery for thoughtful discussions. This work was supported by Grant-in-Aid for Creative Fundamental Research from the Ministry of Education, Science, and Culture of Japan, and by a grant from the Human Frontier Science Program.

REFERENCES

Akitaya, T. and Bronner-Fraser, M. (1992). Expression of cell adhesion molecules during initiation and cessation of neural crest cell migration. *Dev. Dynamics* **194**, 12-20.

Aoyama, H., Delouvee, A. and Thiery, J. P. (1985). Cell adhesion mechanisms in gangliogenesis studied in avian embryo and in a model system. *Cell Differ.* **17**, 247-260.

Bhattacharyya, A., Frank, E., Ranter, N. and Brackenbury, R. (1991). Po is an early marker of the Schwann cell lineage in chickens. *Neuron* **7**, 831-844.

Bronner-Fraser, M. (1985). Alterations in neural crest migration by a monoclonal antibody that affects cell adhesion. *J. Cell Biol.* **101**, 610-617.

Bronner-Fraser, M. (1986). Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. *Dev. Biol.* **115**, 44-55.

Bronner-Fraser, M., Wolf, J. J. and Murray, B. A. (1992). Effects of antibodies against N-cadherin and N-CAM on the cranial neural crest and neural tube. *Dev. Biol.* **153**, 291-301.

Delannet, M., Martin, F., Bossy, B., Cheresh, D. A., Reichardt, L. F. and Duband, J. L. (1994) Specific roles of the αVβ1, αVβ3 and αVβ5 integrins in avian neural crest cell adhesion and migration on vitronectin. *Development* **120**, 2687-2702.

Duband, J. L., Volberg, T., Sabanay, I., Thiery, J. P. and Geiger, B. (1988). Spatial and temporal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis. *Development* **103**, 325-344.

Duband, J. L., Dofour, S., Yamada, S. S., Yamada, K. M. and Thiery, J. P. (1991). Neural crest cell locomotion induced by antibodies to β1 integrins: a tool for studying the roles of substratum molecular avidity and density in migration. *J. Cell Sci.* **98**, 517-532.

Erickson, C. A. and Perris, R. (1993). The role of cell-cell and cell-matrix interactions in the morphogenesis of the neural crest. *Dev. Biol.* **159**, 60-74.

Friedlander, D. R., Mége, R., Cunningham, B. A. and Edelman, G. M. (1989). Cell-sorting out is modulated by both the specificity and amount of different cell adhesion molecules (CAMs) expressed on cell surfaces. *Proc. Natl. Acad. Sci. USA* **86**, 7043-7047.

Fujimori, T., Miyatani, S. and Takeichi, M. (1990). Ectopic expression of N-cadherin perturbs histogenesis in *Xenopus* embryos. *Development* **110**, 97-104.

Gomperts, M., Garcia-Castro, M., Wylie, C. and Heasman, J. (1994). Interactions between primordial germ cells play a role in their migration in mouse embryos. *Development* **120**, 135-141.

Goulding, M., Lumsden, A. and Paquette, A. J. (1994). Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. *Development* **120**, 957-971.

Hamburger, V. and Hamilton, H. (1951). A series of normal stages in the development of the chicken embryo. *J. Morphol.* **88**, 49-92.

Hatta, K. and Takeichi, M. (1986). Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chicken embryos. *Nature* **320**, 447-449.

Hatta, K., Takagi, S., Fujisawa, H. and Takeichi, M. (1987). Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. *Dev. Biol.* **120**, 215-227.

Herrencknecht, K., Ozawa, M., Eckerskorn, C., Lottspeich, F., Lenter, M. and Kemler, R. (1991). The uvomorulin-anchorage protein α-catenin is a vinculin homologue. *Proc. Natl. Acad. Sci. USA* **88**, 9156-9160.

Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S. and Takeichi, M. (1992). Identification of a neural α-catenin as a key regulator of cadherin function and multicellular organization. *Cell* **70**, 1-20.

Inuzuka, H., Miyatani, S. and Takeichi, M. (1991). R-cadherin: A novel Ca^{2+}-dependent cell-cell adhesion molecule expressed in the retina. *Neuron* **7**, 69-79.

Kato, K., Takahashi, Y., Hayashi, S. and Kondoh, H. (1988). Improved mammalian vectors for high expression of G418 resistance. *Cell. Struct. Funct.* **12**, 575-580.

Kemler, R. (1993). From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* **9**, 317-321.

Kimura, Y., Matsunami, H., Inoue, T., Shimamura, K., Ueno, K., Miyazaki, T. and Takeichi, M. (1995). Cadherin-11 expressed in association with mesenchymal morphogenesis in the head, somite, and limb bud of early mouse embryos. *Dev. Biol.* in press.

Lallier, T. E. and Bronner-Fraser, M. (1990). The role of extracellular matrix in neural crest cell migration. *Sem. Dev. Biol.* **1**, 35-44.

Lallier, T. E. and Bronner-Fraser, M. (1993). Inhibition of neural crest cell attachment by integrin antisense oligonucleotides. *Science* **259**, 692-695.

LeDouarin, N. (1982). *The Neural Crest.* Cambridge: Cambridge University press.

Marusich, M. and Weston, J. (1991). Development of the neural crest. *Curr. Opin. Gen. Dev.* **1**, 221-229.

Matsunaga, M., Hatta, K. and Takeichi, M. (1988). Role of N-cadherin cell adhesion molecules in the histogenesis of neural retina. *Neuron* **1**, 289-295.

Matsunami, H., Miyatani, S., Inoue, T., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Takeichi, M. (1993). Cell binding specificity of mouse R-cadherin and chromosomal mapping of the gene. *J. Cell Sci.* **106**, 401-409.

McCrea, P. D., Turck, C. W. and Gumbiner, B. (1991). A homolog of the armadillo protein in *Drosophila* (plakoglobin) associated with E-cadherin. *Science* **254**, 1359-1361.

Miyatani, S., Shimamura, K., Hatta, M., Nagafuchi, A., Nose, A., Matsunaga, M., Hatta, K. and Takeichi, M. (1989). Neural cadherin: Role in selective cell-cell adhesion. *Science* **245**, 631-635.

Nagafuchi, A., Shirayoshi, Y., Okazaki, K., Yasuda, K. and Takeichi, M. (1987). Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature* **329**, 341-343.

Nagafuchi, A. and Takeichi, M. (1989). Transmembrane control of cadherin-mediated cell adhesion: A 94kd protein functionally associated with a specific region of the cytoplasmic domain of E-cadherin. *Cell Regul.* **1**, 37-44.

Nagafuchi, A., Takeichi, M. and Tsukita, S. (1991). The 102kd cadherin-associated protein: similarity to vinculin and posttranscriptional regulation of expression. *Cell* **65**, 849-857.

Nagafuchi, A., Ishihara, S. and Tsukita, S. (1994). The role of catenins in the cadherin-mediated cell adhesion: functional analysis of E-cadherin-α catenin fusion molecules. *J. Cell Biol.* **127**, 235-246.

Nose, A. and Takeichi, M. (1986). A novel cadherin adhesion molecule: Its expression patterns associated with implantation and organogenesis of mouse embryos. *J. Cell Biol.* **103**, 2649-2658.

Nose, A., Nagafuchi, A. and Takeichi, M. (1988). Expressed recombinant cadherins mediate cell sorting in model system. *Cell* **54**, 993-1001.

Ozawa, M., Baribault, H. and Kemler, R. (1989). The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J.* **8**, 1711-1717.

Sano, K., Tanihara, H., Heimark, R. L., Obata, S., Davidson, M., John, T. S., Taketani, S. and Suzuki, S. (1993). Protocadherins: a large family of cadherin-related molecules in central nervous system. *EMBO J.* **12**, 2249-2256.

Schneider, S., Herrenknecht, K., Butz, S., Kemler, R., Hausen, P. (1993). Catenins in *Xenopus* embryogenesis and their relation to the cadherin-mediated cell-cell adhesion system. *Development* **118,** 629-640.

Selleck, M. A. J., Scherson, T. Y. and Bronner-Fraser, M. (1993). Origins of neural crest cell diversity. *Dev. Biol.* **159**, 1-11.

Shimamura, K., Takahashi, T. and Takeichi, M. (1992). E-cadherin expression in a particular subset of sensory neurons. *Dev. Biol.* **152**, 242-254.

Shimamura, K., S., H., McMahon, A. P. and Takeichi, M. (1994). Wnt-1-dependent regulation of local E-cadherin and αN-catenin expression in the embryonic mouse brain. *Development* **120**, 2225-2234.

Stemple, D. L. and Anderson, D. J. (1992). Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell* **71**, 973-985.

Suzuki, S., Sano, K. and Tanihara, H. (1991). Diversity of the cadherin family: Evidence for eight new cadherins in nervous tissue. *Cell Regul.* **2**, 261-270.

Takeichi, M. (1977). Functional correlation between cell adhesive properties and some cell surface proteins. *J. Cell Biol.* **75**, 464-474.

Takeichi, M. (1987). Cadherins: a molecular family essential for selective cell-cell adhesion and animal morphogenesis. *Trends Genet.* **3**, 213-217.

Takeichi, M. (1988). The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development* **102**, 639-655.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251**, 1451-1455.

Thiery, J. P., Duband, J. L., Rutishauser, U. and Edelman, G. M. (1982). Cell adhesion molecules in early chicken embryogenesis. *Proc. Natl. Acad. Sci. USA* **79**, 6737-6741.

Thiery, J. P., Delouvee, A., Gallin, W. J., Cunningham, B. A. and Edelman, G. M. (1984). Ontogenetic expression of cell adhesion molecules: L-CAM is found in epithelia derived from the three primary germ layers. *Dev. Biol.* **102**, 61-78.

Vincent, M., Duband, J.-L. and Thiery, J.-P. (1983). A cell surface determinant expressed early on migrating neural crest cells. *Dev. Brain Res.* **9**, 235-238.

Watabe, M., Nagafuchi, A., Tsukita, S. and Takeichi, M. (1994). Induction of polarized cell-cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a dispersed carcinoma line. *J. Cell. Biol.* **127**, 247-256.

Winklbauer, R., Selchow, A., Nagel, M. and Angres, B. (1992). Cell interaction and its role in mesoderm cell migration during Xenopus gastrulation. *Dev. Dynamics* **195**, 290-302.

Williams, B. A. and Ordahl, C. P. (1994). Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* **120**, 785-796.

(Accepted 9 February 1995)