Rhombomere transplantation repatterns the segmental organization of cranial nerves and reveals cell-autonomous expression of a homeodomain protein

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SUMMARY

The developing vertebrate hindbrain consists of segmental units known as rhombomeres. Hindbrain neuroectoderm expresses 3′ Hox 1 and 2 cluster genes in characteristic patterns whose anterior limit of expression coincides with rhombomere boundaries. One particular Hox gene, referred to as *Ghox 2.9*, is initially expressed throughout the hindbrain up to the anterior border of rhombomere 4 (r4). Later, *Ghox 2.9* is strongly upregulated in r4 and Ghox 2.9 protein is found in all neuroectodermal cells of r4 and in the hyoid crest cell population derived from this rhombomere. Using a polyclonal antibody, Ghox 2.9 was immunolocalized after transplanting r4 within the hindbrain. Wherever r4 was transplanted, Ghox 2.9 expression was cell-autonomous, both in the neuroectoderm of the graft and in the hyoid crest cell population originating from the graft.

In all vertebrates, rhombomeres and cranial nerves (nerves V, VII+VIII, IX, X) exhibit a stereotypic relationship: nerve V arises at the level of r2, nerve VII+VIII at r4 and nerves IX-X extend caudal to r6. To examine how rhombomere transplantation affects this pattern, operated embryos were stained with monoclonal antibodies E/C8 (for visualization of the PNS and of even-numbered rhombomeres) and HNK-1 (to detect crest cells and odd-numbered rhombomeres). Upon transplantation, rhombomeres did not change E/C8 or HNK-1 expression or their ability to produce crest cells. For example, transplanted r4 generated a lateral stream of crest cells irrespective of the site into which it was grafted. Moreover, later in development, ectopic r4 formed an additional cranial nerve root. In contrast, transplantation of r3 (lacks crest cells) into the region of r7 led to inhibition of nerve root formation in the host.

These findings emphasize that in contrast to spinal nerve segmentation, which entirely depends on the pattern of somites, cranial nerve patterning is brought about by factors intrinsic to rhombomeres and to the attached neural crest cell populations. The patterns of the neuroectoderm and of the PNS are specified early in hindbrain development and cannot be influenced by tissue transplantation. The observed cell-autonomous expression of *Ghox 2.9* (and possibly also of other Hox genes) provides further evidence for the view that Hox gene expression underlies, at least in part, the segmental specification within the hindbrain neuroectoderm.

Key words: neural crest, rhombomere, homeobox protein, hindbrain, head segmentation mechanisms, head development, chick embryo, tissue transplantation, quail-chick chimera

INTRODUCTION

The developing vertebrate hindbrain consists of eight distinct segments known as rhombomeres (r). Rhombomeres had been discovered and histologically characterized in the last century (e.g. von Baer, 1828; Remak, 1855; Orr, 1887). Only recently, however, have molecular and cell biological studies shed light on the role of rhombomeres in development. Silver impregnation of neurons and retrograde dye labeling in the chick have revealed that rhombomeres and branchial nerves (cranial nerves V, VII+VIII, IX and X) are organized in a pairwise fashion (Tello, 1923; Lumsden and Keynes, 1989). For example, motoneurons of nerve V derive from r2 and r3, those of nerve VII+VIII arise in r4 and r5. Cephalic neural crest populations, sensory ganglion primordia, and pharyngeal arches exhibit a similar periodicity (Remak, 1855; Beranek, 1884; Orr, 1887; His, 1887; Kuhlenbeck, 1935; Wen, 1928; Bartelmez and Dekaban, 1962; Kuratani and Tanaka, 1990). Grafting an even (odd) numbered rhombomere next to another even (odd) numbered rhombomere, results in a single enlarged rhombomere that lacks the interrhombomeric boundary (Guthrie and Lumsden, 1991). In contrast, boundaries will form if even and odd rhombomeres are juxtaposed. Dye labeling studies

by Fraser et al. (1990) showed that cells will not cross inter-rhombomeric boundaries once such boundaries are formed. Taken together, these data suggest that rhombomeres are compartments of distinct identity in which cells share a common developmental program. This is reminiscent of insect segments, an idea already suggested by Dohrn (1875).

This point of view is further supported by the discovery that a number of Hox genes are expressed in a pattern that reflects the segmental organization of the hindbrain (reviewed by Hunt et al., 1991). For example, chick Ghox 2.9 and its mouse homolog Hox 2.9 are both expressed at high levels in r4 (Frohman et al., 1990; Sundin and Eichele, 1990; Maden et al., 1991; Guthrie et al., 1992). Moreover, this gene is also transiently expressed in hyoid crest cells that derive from r4 and eventually migrate into the hyoid arch. Thus, cell fates and hence pattern formation in the branchial arches might partly depend on a program of gene expression primarily originating in hindbrain neuroectoderm (reviewed by Hunt et al., 1991; McGinnis and Krumlauf, 1992).

To study the role of rhombomeres in the establishment of the cranial pattern, we have transplanted rhombomeres from donor embryos into host embryos at different axial levels. These recombinant embryos were subsequently examined for structural changes in the CNS and PNS. To detect these changes, we have used an antibody against Ghox 2.9 protein, and antibodies HNK-1 and E/C8 which specifically label certain cranial neural crest cells and neurons.

MATERIALS AND METHODS

Construction of chimeric embryos

Fertilized eggs of White Leghorn chicken and Japanese quail were incubated in a humid chamber at 37°C. A window was cut through the shell and embryos were stained either with an agar film releasing neutral red or with India ink (Rapidograph 3080-F, Bloomsbury, NJ) diluted 1:3 in 0.9% NaCl/distilled water, injected into the subgerminal cavity. Chick and quail embryos used ranged from stage 9⁻ to 10⁺ (affix '−' means 6 somites, i.e., one somite less than stage 9, '+' means one somite more than stage 10; Hamburger and Hamilton, 1951). Stages of host and donor embryos were matched as closely as possible. Using a sharpened tungsten needle, the dorsal portion of the donor neuroectoderm was excised, immediately transferred with a pipet to the recipient and inserted into an appropriately sized gap prepared with a tungsten needle in the neuroectoderm of the host. At the stages employed mesodermal cells are not attached to the neuroectoderm and therefore, were not part of the graft.

Selection of graft tissues and of sites of implantation was as follows (see also Tables 1-3). Between stages 9 and 10, boundaries between r2 and r3 as well as between r3 and r4 become readily visible (see also Fig. 1). Such embryos also exhibit a boundary between mid- and hindbrain, a demarcation which specifies the anterior border of future r1. Therefore, neuroectoderm corresponding to r1, r2, r3 and r4 can be identified by that time. The position of r7 which has not appeared by stage 9⁺, was predicted using the first somite as a landmark, since somite 1 later resides lateral to r7 (Vaage, 1969).

Time of surgery and whole-mount immunostaining

For Ghox 2.9 studies, surgery was performed in embryos between stages 9⁻ and 10. Embryos were fixed between stages 11⁻ and 12⁻ (9-12 hours after the surgery, 12-15 somites) when hyoid crest cells express significant levels of Ghox 2.9 protein (Fig. 1; see also Sundin and Eichele, 1990). Whole-mount embryos were prepared as described (Sundin and Eichele, 1990) with the following modifications. After the TST wash (TST is Tris-HCl-buffered saline: 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100), samples were sequentially blocked with 50 mM ethanolamine in TST, with aqueous 1% periodic acid and with 5% dry non-fat milk in TST (TSTM).

In the case of E/C8 staining of rhombomeres and crest cells and for HNK-1 staining of crest cells following r4 ablation, surgery was performed between stages 9 and 10⁻ (see Table 2). Experimental embryos were fixed 22-26 hours after the surgery when embryos had reached stage 14 (22 somites). To analyze cranial nerve morphology by E/C8 immunolabeling, surgery was performed between stages 9⁻ and 10⁺ (Table 3). Embryos were incubated for additional 2-3 days prior to fixation.

Immunostaining methods with HNK-1 (Leu-7, Becton Dickinson, San Jose, CA) and E/C8 (Ciment et al., 1986) were basically the same as described for Ghox 2.9 above. Both antisera were diluted 1/20 in spin-clarified TSTM. Incubations with antisera were for either 48 hours or 72 hours (larger specimens). As secondary antibody, horseradish peroxidase-anti-mouse IgM (1/200 in TSTM, Southern Biotechnology Inc., Birmingham, AL) was used.

Peroxidase substrates 3,3′-diaminobenzidine (DAB, 500 µg/ml) and hydrogen peroxide dissolved at 0.03% in TS, were added to the specimens and allowed to react for 7 minutes at 0°C. The reaction was stopped by rinsing embryos in 30% glycerol/PBS. The stained embryos were transferred through a graded series of glycerol/water mixtures into 80% glycerol containing a trace amount of thymol.
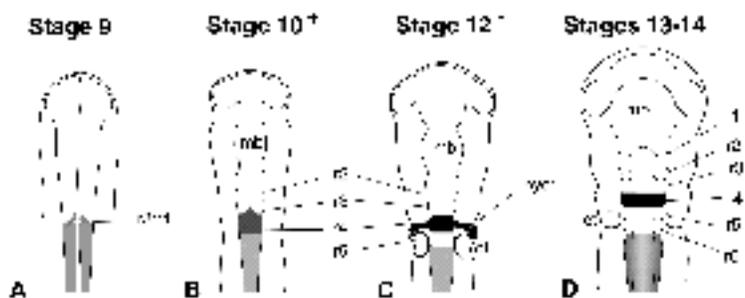

Fig. 1. Scheme of Ghox 2.9 protein expression in chick embryos (for details, see Sundin and Eichele, 1990). (A) Stage 9 embryo. Ghox 2.9 protein is expressed up to the level of the future boundary between r3 and r4 (r3/r4). (B) By stage 10⁺, the morphological boundary between r3 and r4 is clearly visible. Note, r3 contains a small number of Ghox 2.9 positive cells. (C) At stage 12⁻, Ghox 2.9 protein is strongly upregulated in r4 and downregulated in r5. In addition, hyoid crest cells express Ghox 2.9 protein. (D) After stage 13, Ghox 2.9 protein disappears in hyoid crest cells but persists at high levels in r4 and at moderate levels in neuroectoderm posterior to r6. hycr, hyoid crest cells; ot, otocyst.

Immunostaining of cryostat sections

Embryos were fixed and sectioned as previously described (Sundin and Eichele, 1990). HNK-1 antibody was applied after specimens had already been treated with Ghox 2.9 antibody. HNK-1 antibody was diluted 20- to 40-fold. To reveal HNK-1 antibody, alkaline phosphatase-conjugated anti-mouse IgM (Southern Biotech., Inc., diluted 1/400 in TSTM) was used. Sections were air-dried followed by additional drying in an oven at 40°C for 30 minutes and cover slips were mounted with Canada Balsam diluted 1:1 with methyl salicylate.

Histochemical staining and immunohistochemistry of chick-quail chimera

Chick-quail chimeric embryos were fixed with Carnoy's fixative (mixture of 10 ml acetic acid, 60 ml ethyl alcohol and 30 ml chloroform), embedded in paraffin and stained according to the Feulgen-Rossenbeck method (1924) to reveal the nuclear marker characteristic of interphase quail cells. A few embryos were subjected to both E/C8-immunostaining and the nuclear marking. In this case, specimens were first fixed in Carnoy, immunostained with E/C8 (see above) and then carried through the Feulgen-Rossenbeck procedure.

RESULTS

Peripheral nervous system and hindbrain segmentation revealed by E/C8 and HNK-1 immunostaining

E/C8 antibody recognizes an epitope on the neurofilament-associated protein NAPA-73 (Ciment et al., 1986). At stage 10$^+$, when E/C8 immunoreactivity is first detected, only the premigratory neural crest extending between presumptive r2 and r4 stain (Fig. 2A). Hyoid crest and the caudal portion of the trigeminal crest begin to express E/C8 around stage 12 (Fig. 2B). By stage 13, E/C8 continues to be present in hyoid crest (Fig. 2C), but has vanished in the trigeminal crest. Circumpharyngeal crest cells do not stain at these early stages (circumpharyngeal crest is defined as the dorsolateral subpopulation of the vagal crest, see Kuratani and Kirby, 1991). However, circumpharyngeal crest cells express E/C8 by stage 17, and expression persists until 6 days of incubation (Ciment and Weston, 1985; Kuratani and Kirby, 1992).

In addition to neural crest, the neuroectoderm of r2 and especially of r4 exhibits E/C8-immunoreactivity (Fig. 2B,C). This selectivity reflects the fact that neuronal differentiation initiates in even-numbered rhombomeres (Lumsden and Keynes, 1989; Kuratani, 1991). After stage 16, the antibody preferentially stains neuroblasts in even-numbered rhombomeres (now including r6) and in the cranial ganglion primordia (Fig. 3A). By stage 19, E/C8 stains even-numbered rhombomeres and cranial nerves V, VII+VIII, IX, and X (Fig. 3B) and thus accentuates the relationship between rhombomeres, cranial nerves and branchial arches.

The carbohydrate epitope of cell adhesion molecules, L1, J1, neural cell adhesion molecule (NCAM), ependymins, and myelin-associated glycoprotein (MAG) is recognized by HNK-1 antibody (Kruse et al., 1984, 1985; McGarry et al., 1983; Shashoua et al., 1986). In the chick embryo, all presumptive neural crest cells are stained with this antibody (Tucker et al., 1984). In contrast to E/C8 immunostaining,
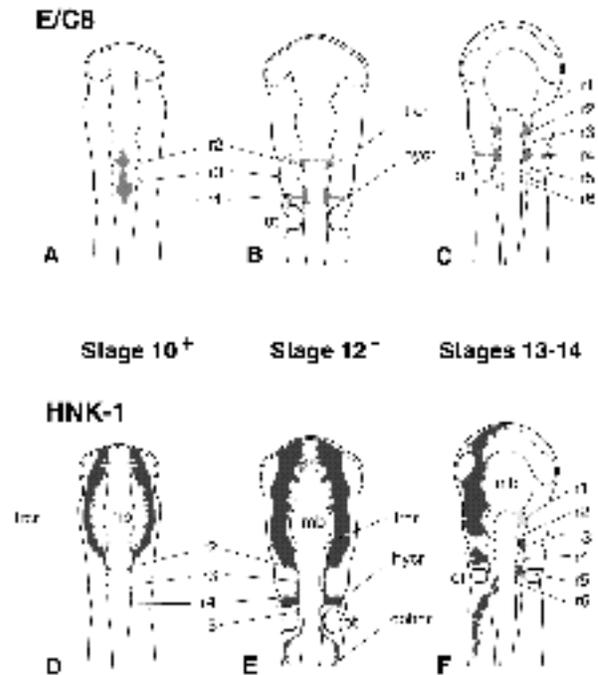

Fig. 2. Crest cell populations and rhombomeres in E/C8 (A-C) and HNK-1 (D-F) immunolabeled whole-mount chick embryos. Stage 10$^+$: E/C8 stains only premigratory crest between r2 and r4 (A). In contrast, HNK-1 staining is widespread encompassing the migrating crest cells in the trigeminal area (D). Stage 12$^-$: E/C8-immunoreactivity is restricted to the proximal portion of the trigeminal crest cell population and to the central portion of the hyoid crest cell population (B), while HNK-1 also labels the circumpharyngeal crest cell population (E). Note, E/C8 labels even-numbered rhombomeres r2 and r4 (B), while HNK-1 labels the ventricular surface of odd-numbered rhombomeres 3 and 5 (E, right side). Stages 13-14: Hyoid crest cell population is labelled with E/C8 (C). HNK-1 epitope is found throughout the cephalic crest cell populations (F, left side). Note, alternating staining pattern of rhombomeres for E/C8 (C) and HNK-1 (F), see also Fig. 7C. HNK-1 staining in r1 is less intense, as reflected by the weaker shading (F, right side). cphcr, circumpharyngeal crest cell population; hycr, hyoid crest cell population; ot, otocyst; trcr, trigeminal crest cell population; r1-6, rhombomeres.

HNK-1 reactivity in early embryos is more widespread (compare Fig. 2A-C with Fig. 2D-F). At stage 10$^+$, HNK-1 antibody reveals the trigeminal crest cell population (Fig. 2D). Thereafter, immunoreactivity in hyoid- and circumpharyngeal crest cells appears (Fig. 2E), and by stage 13, all three crest populations are distinctly stained (Fig. 2F, left part). In the neuroectoderm of stage 12 and older embryos, HNK-1 immunoreactivity is found at the ventricular side of r3 and r5 (Fig. 2E,F, see also Fig. 7C and Kuratani, 1991). Even prior to the morphological separation of r1 and r2, anterior and posterior halves of the pre-rhombomere show a difference in HNK-1 immunoreactivity, forecasting the subsequent segregation. By stage 13, the alternate rhombomere expression pattern of HNK-1 epitope becomes very distinct throughout the hindbrain (Figs 2F, 7C). The rule that emerges is that HNK-1 immunoreactivity is found in crest-free, odd-numbered rhombomeres,
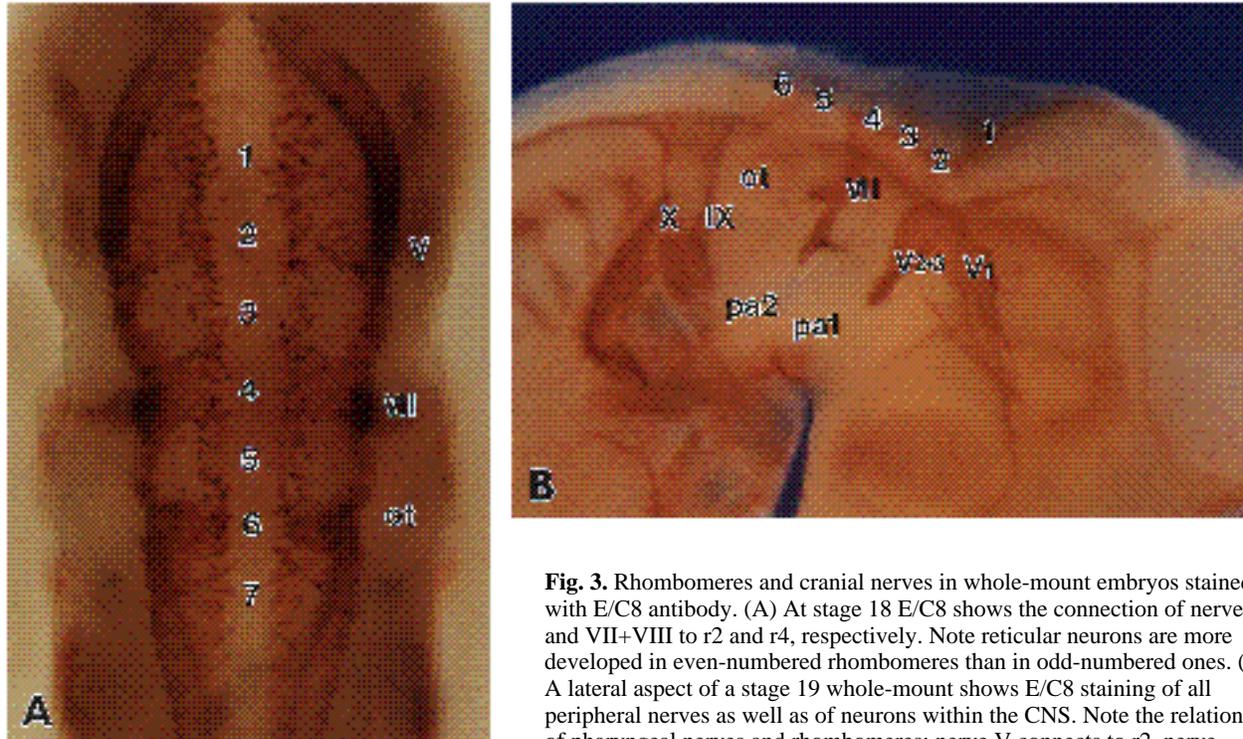
Fig. 3. Rhombomeres and cranial nerves in whole-mount embryos stained with E/C8 antibody. (A) At stage 18 E/C8 shows the connection of nerves V and VII+VIII to r2 and r4, respectively. Note reticular neurons are more developed in even-numbered rhombomeres than in odd-numbered ones. (B) A lateral aspect of a stage 19 whole-mount shows E/C8 staining of all peripheral nerves as well as of neurons within the CNS. Note the relationship of pharyngeal nerves and rhombomeres; nerve V connects to r2, nerve VII+VIII to r4, and the root of nerve IX and X extends caudal to r6. Each of these cranial nerves is associated with one particular pharyngeal slit. ot, otocyst; pa, pharyngeal arches; 1-7, rhombomeres; IX, glossopharyngeal nerve; V1, ophthalmic portion of the trigeminal nerve; V_{2+3}, maxillomandibular portion of the trigeminal nerve; VII, acustico-facial nerve complex (nerves VII+VIII); X, vagus nerve.

whereas E/C8 immunoreactivity resides in even-numbered rhombomeres with crest attached.

Ghox 2.9 protein is initially expressed in the spinal cord and hindbrain neuroectoderm up to the anterior border of r4 (Fig. 1B; for details see Sundin and Eichele, 1990). Around stage 10, Ghox 2.9 is downregulated in r5, and simultaneously upregulated in r4 (Fig. 1C). Caudal rhombomeres 7 and 8 also express Ghox 2.9, but always at a substantially lower level than r4 (Fig. 1D). Immunoreactivity in hyoid crest cells is restricted to the early migration period (around stage 11). In contrast, Ghox 2.9 expression in neuroectoderm continues at least up to stage 18 (B. Lutz, S.C. Kuratani and G. Eichele, unpublished data). We conclude that HNK-1 and E/C8 and Ghox 2.9 epitopes reveal the metameric organization of hindbrain neuroectoderm and of cranial neural crest cells and thus provide markers to study structural changes that result from experimental manipulations in the hindbrain.

Cell-autonomous expression of *Ghox 2.9* gene in rhombomere 4 and hyoid crest

Rhombomere 4 was transplanted to various axial levels. Donor and host chick embryos used ranged from stage 9⁻ to 10, i.e., before crest cells begin to migrate. In all cases, irrespective of axial position of the graft site, r4 transplants exhibited Ghox 2.9 immunoreactivity (Fig. 4A-C). This suggests that the expression of this homeodomain protein is cell-autonomous, i.e., not influenced by surrounding tissue. It should be noticed that Ghox 2.9 levels in r4 are

upregulated around stage 11 (for details, see Sundin and Eichele, 1990). Fig. 4A-D illustrates that the intensity of antibody staining in graft and host r4 are very similar, demonstrating that upregulation, i.e. synthesis of new protein occurs in the graft. Hence the signal seen in the graft is not caused by Ghox 2.9 protein already present in the neuroectoderm at the time of transplantation.

To test whether mesoderm underneath the r4 region is capable of inducing Ghox 2.9 in overlying neuroectoderm, a piece of Ghox 2.9-negative neuroectoderm of r2, r3, or r5 was placed into an appropriate gap within r4 (see Table 1). Fig. 4D shows that an r2 r4 graft does not express Ghox 2.9 protein. Likewise, r3 or r5 placed into a gap at the level of r4, will not begin to express Ghox 2.9. This finding indicates that expression of Ghox 2.9 is regulated within the neuroectoderm and at this time in development is not influenced by mesodermal signals.

In normal development, hyoid crest cells derived from r4 transiently express Ghox 2.9. Rhombomere 4 grafts also produce crest cells (Fig. 4A,C) and these express Ghox 2.9. Because of the transient nature of expression, Ghox 2.9 immunoreactivity in ectopic crest cells is often difficult to visualize in whole-mounts (Fig. 4A) or even in sections. Moreover, for unknown reasons, crest cells were least frequently observed associated with the graft in specimens in which r4 was inserted into the region of r2 (Fig. 4B, Table 1). Taken together, these results show that the expression of Ghox 2.9 is cell-autonomous, both in r4 neuroectoderm and in the hyoid crest cells.

Fig. 4. Cell-autonomous expression of Ghox 2.9 protein after unilateral heterotopic transplantation of rhombomeres. (A) r4   r1 transplantation. Arrows indicate slightly immunoreactive crest cells released from the graft, and from r4 of the host. (B) r4   r2 transplantation. Graft-derived Ghox 2.9-positive crest cells are not seen in this particular specimen. (C) r4   r3 transplantation. Note a band of Ghox 2.9-positive crest cells coming from the graft (arrow). This cell population fuses with the hyoid crest cells of the host. Note that in all heterotopic transplants, the r4 graft is stained with Ghox 2.9 antibody. (D) r2   r4 transplantation. In grafted r3 (arrow) Ghox 2.9 protein is not induced. A small fragment of host r4 neuroectoderm is seen anterior to the graft. Immunoreactive crest cells are seen at the control side of the embryo (arrowhead). In contrast, very few crest cells are found lateral to the graft. mb, midbrain; 1-5, rhombomeres.

Morphological changes in the distribution of crest cells 24 hours after grafting

During the 24 hours of incubation after transplantation surgery, embryos progress to stage 14. At this time in development, Ghox 2.9, HNK-1 and E/C8 are still good markers for neuroectoderm and HNK-1 and E/C8 are also markers for the neural crest cells (Fig. 2C,F). In a first series of experiments E/C8 antibody was used as a marker to examine neuroectoderm and hyoid crest cell behavior in embryos with an ectopic r4. As has been shown by dye labeling (Lumsden et al., 1991), hyoid crest cells derive only from r4. In agreement we find that upon bilateral removal of this rhombomere, HNK-1 positive crest cells in the hyoid arch are absent (Fig. 5A,B). This observation is consistent with the absence of Ghox 2.9-positive crest cells in the same type of experiment (Table 1).

When r4 was placed either into r1 or r3, the grafted neuroectoderm appeared as E/C8-positive area (Fig. 6A-C, summarized in Fig. 9, center). For example, when r4 was grafted into the region of r3, graft and host r2 and r4 formed a single continuous block of E/C8 staining (Fig. 6C). In addition to the grafted neuroectoderm, crest cells derived from the graft were also immunolabeled (Fig. 6B,C, Table 2). In the case of r4 r1 transplantations, these crest cells were usually difficult to visualize (Fig. 6A,B). In contrast, in r4 r3 transplantations, graft-derived crest cells were well visible filling the gap between the trigeminal and hyoid crest populations of the host (Fig. 6C). In the case of r4   r2 transplantations (not shown), the resulting combined crest looked almost normal, since E/C8 immunoreactivity and crest cell distribution are similar in r2 and r4 (Fig. 2B,C, Table 2). Only in one out of three cases, the quantity of E/C8 positive crest cells increased on the experimental side of the embryo (data not shown).

Table 1. *Expression of Ghox 2.9 protein 9 to 12 hours after transplantation*

| Type of transplantation at st. 9⁻ to 10	Number of embryos	Number of embryos examined	Number of embryos with ectopic expression of Ghox 2.9 in	
			Neuroectoderm	Ectopic crest cells
r4 r1 unilateral	19	17	17	9 (Fig. 4A)
r4 r2 unilateral	32	27	27	8 (Fig. 4B)
r4 r3 unilateral	27	18	18	8 (Fig. 4C)
r4 r3 bilateral	1	0		
r2 r4 unilateral	2	2	none (Fig. 4D)	
r3 r4 unilateral	1	1	none	
r3 r4 bilateral	1	1	none	
r5 r4 unilateral	1	1	none	
r4 bilateral ablation	5	4		no crest cells found
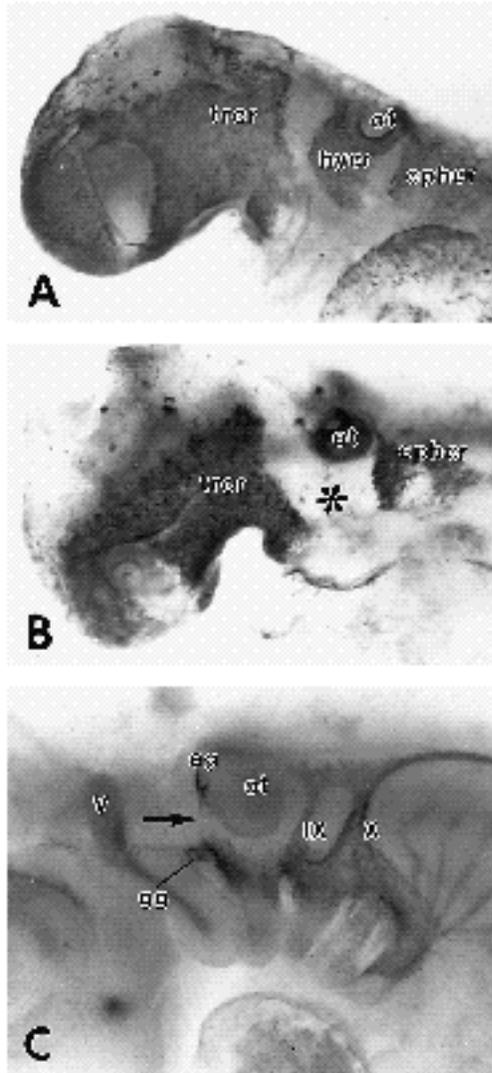
Fig. 5. Distribution of crest cell populations and cranial nerve development after bilateral extirpation of r4. (A) Control embryo at stage 14. HNK-1 immunostaining shows the three distinct crest cell populations. (B) Experimental embryo labeled with HNK-1 at a comparable stage. Rhombomere 4 has been removed bilaterally at stage 9 (before the onset of hyoid crest migration). Only two crest cell populations appear and the hyoid arch (asterisk) is not occupied by HNK-1-positive crest cells. (C) E/C8 staining, two days later, reveals that the facial nerve root is reduced or sometimes lost (arrow points at location where nerve VII would have formed). A geniculate ganglion develops in isolation and generates a few sensory branches. ag, acoustic ganglion; cphcr, circumpharyngeal crest cell population; gg, geniculate ganglion; hycr, hyoid crest cell population; IX, nerve IX; ot, otocyst; trcr, trigeminal crest cell population; V, nerve V; X, nerve X.

Table 2. *Appearance of graft-derived E/C8 positive crest cells 1 day after surgery*

Type of transplantation at st. 9⁻ to 10	Number of embryos examined	Embryos with ectopic E/C8 positive crest cells
r4 → r1 unilateral	4	3
r4 → r1 bilateral	1	1, bilateral (Fig. 6AB)
r4 → r2 unilateral	3	1*
r4 → r3 unilateral	3	2 (Fig. 6C)
r4 → r3 bilateral	1	1, bilateral

  *Host r2 is occasionally associated with E/C8 positive crest cells. Only one transplant showed a significantly increased amount of E/C8 positive crest cells on the operated side.

sectioned transversely and revealed that crest cells in r2 were derived from the transplanted r4 of quail (Fig. 6D).

In another series of grafting experiments we examined whether cell-autonomous behavior of Ghox 2.9 and HNK-1 epitope persists to stage 14. Fig. 7C-D shows the left side control (C) and the operated side (D) of a double-stained specimen. In this embryo, host r3 and r4 were replaced by donor r4 and r5. The r4 portion of the graft showed Ghox 2.9 immunoreactivity (Fig. 7D) and it formed a distinct boundary with the non-immunoreactive r5 area of the graft. Within the r5 region, HNK-1 antigen was detected in more ventral sections (not shown). In none of the embryos was HNK-1 epitope expression induced on the ventricular side of grafted r4, nor was there any induction of the Ghox 2.9 protein in neuroectoderm when a fragment of an odd-numbered rhombomere was placed above mesodermal tissue at the level of r4. This is consistent with our finding in earlier embryos that gene expression in rhombomeres is not influenced by mesoderm.

The morphology of the crest cell mass is considerably changed in stage 14 embryos. When the r4 area of the graft is translocated into the r3 level of the host, separation of the hyoid and trigeminal crest cell populations is lost, resulting in a single huge ganglion primordium which adheres to host r2 and grafted r4 (Fig. 7D). The r5 area of the graft, now located at the host r4 level, did not exhibit lateral crest cells. Thus, the crest cell distribution and its segmental connection to the hindbrain are dependent on the segmental origin of the grafted rhombomere and not on the mesenchymal environment of the host.

Changes of the cranial nerve morphology resulting from rhombomere transplantations

The morphology of peripheral cranial nerves was examined 2-3 days after the surgery and results are summarized in Table 3 and in the right hand column of Fig. 9. Rhombomere 4 was chosen for most of these experiments, since this rhombomere gives rise to single crest cell population, the hyoid crest cells (Fig. 5A). In the case of r4 → r1 transplantations, a new connection was formed between the trigeminal nerve and a nerve emerging from the graft (Fig. 8A,B). When r4 was grafted into r2, no significant changes were observed in the morphology of the trigeminal nerve, although an ectopic small otocyst was often found, demonstrating that the graft was incorporated (data not shown). In the case of r4 → r3 grafts, two types of results were

To demonstrate that in these experiments neuroectoderm and crest cell both derive from the donor, we used chick-quail chimeras. A section (Fig. 7B) shows that the grafted r4 neuroectoderm that was inserted in a gap made at the level of host r3 consists of quail cells. Another chick embryo that had undergone a r4 → r2 transplantation was
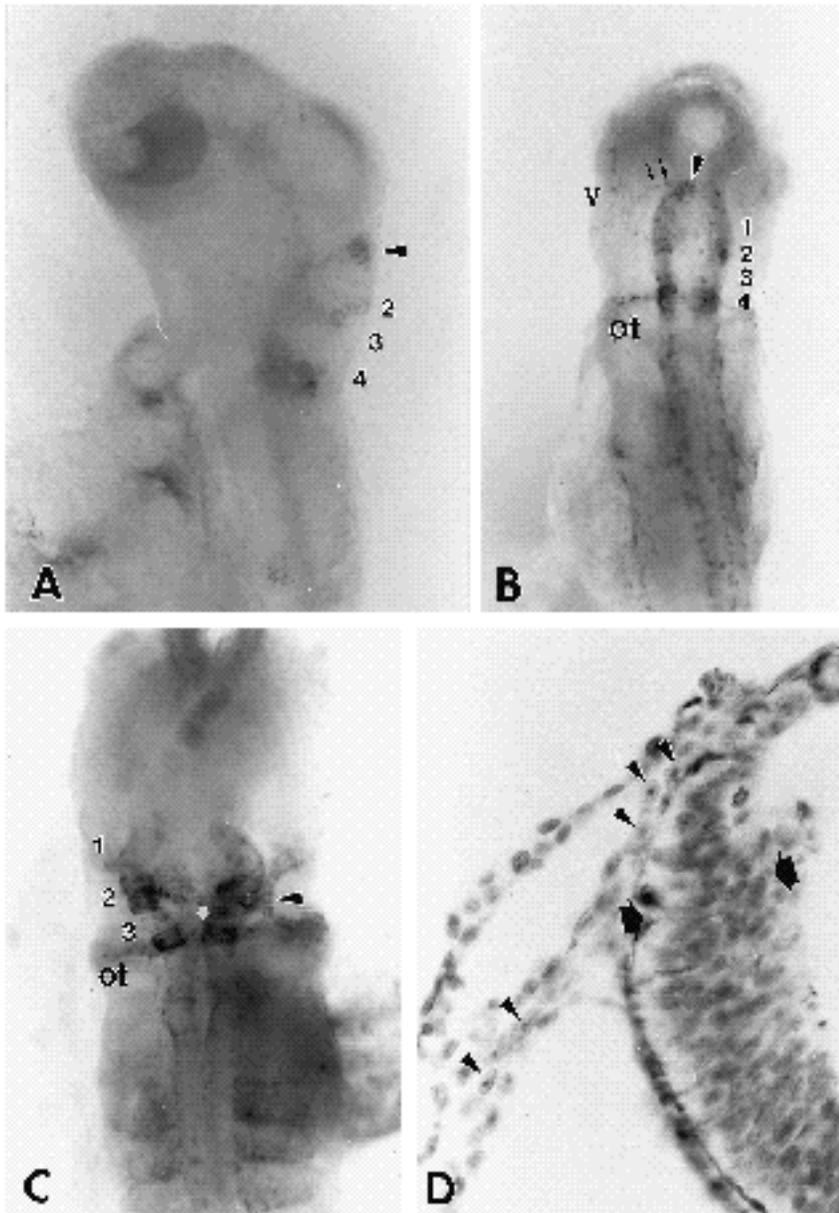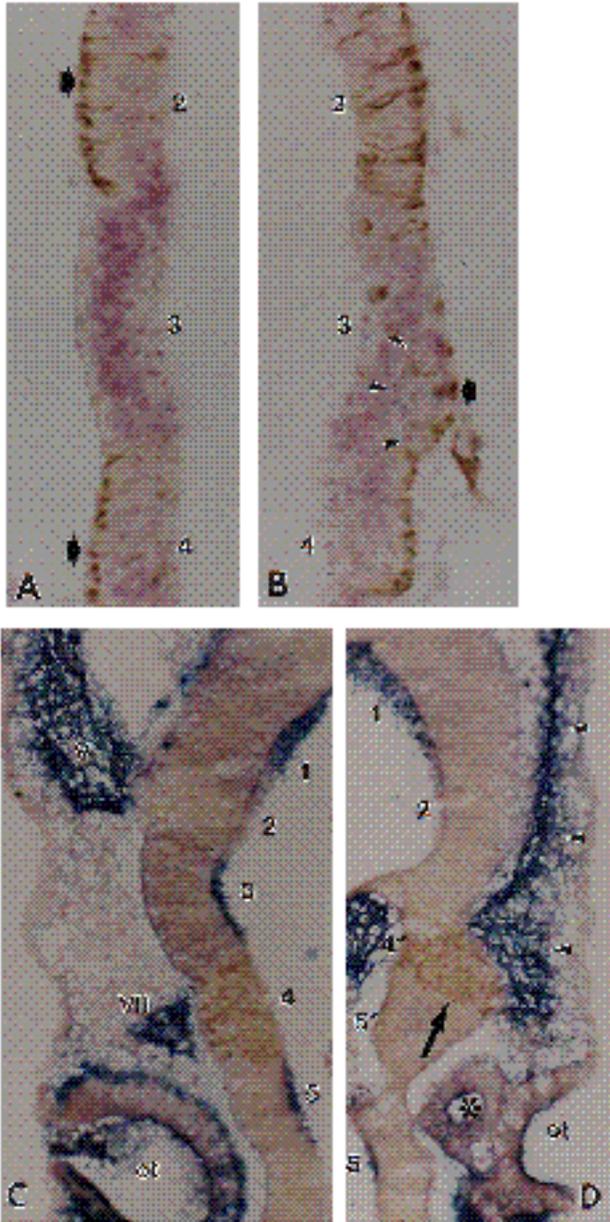
Fig. 6. Appearance of ectopic crest cells one day after heterotopic transplantation of r4. (A-C) E/C8 stained embryo. (A,B) r4 r1 bilateral transplantation. Lateral (A) and dorsal (B) aspects of the same embryo. The graft is the immunoreactive tissue in the anterior region of the r1 area (arrowheads). Immunoreactive cells migrate from the graft (arrows in B) and fuse with the trigeminal sensory ganglion primordium (B). (C) r4 r3 unilateral transplantation. The graft fills the gap of immunoreactivity between r2 and r4 (asterisk) and the graft-derived immunoreactive crest cells fill the gap between trigeminal and facial ganglion primordia (arrowhead). (D) Feulgen-stained chimeric embryo between chick and quail in which r4 of quail has been inserted into a space made at the r2 level. The dorsal portion of the neuroectoderm is replaced with quail tissue (thick arrows indicate the ventral chick-quail boundary). Crest cells emerging from the graft are revealed by their quail nuclear marker (arrowheads). ot, otocyst; V in (B), trigeminal nerve primordium; r1-4, rhombomeres.

Table 3. *Cranial nerve morphology 2 to 3 days after surgery*

Type of transplantation at st. 9⁻ to 10⁺	Number of embryos examined	Resulting cranial nerve morphology
r4 r1 bilateral	3	2 with ectopic roots on n. V on both sides (Fig. 8A,B); 1 normal
r4 r2 bilateral	2	normal
r4 r3 unilateral	1	fusion between n. V and VII (Fig. 8D)
r4 r3 bilateral	3	2 with unilateral fusion between n. V and VII (Fig. 8C); 1 normal
r4 bilateral ablation	12	10 with thin n. VII root; 2 with complete loss of n. VII root (Fig. 5C)
r3 r7 unilateral	2	1 with ipsilateral notch (Fig. 8E); 1 with deformed n. IX root
r3 r7 bilateral	1	unilateral notch only

obtained. Nerves V and VII were proximally fused at the level halfway between r2 and r4 (Fig. 8C). Alternatively, nerve roots were still separate but a fused ganglion appeared (Fig. 8D). Moreover, an ectopic otocyst often formed which was innervated by an ectopic acoustic nerve (Fig. 8C). The morphological changes observed in these stage 18/19 embryos are the result of fusion of sensory ganglion primordia that were seen in the younger stage 14 specimens

Fig. 7. (A,B) Quail r4 was unilaterally inserted into chick r3 area and the embryo was analyzed one day later. The section was stained with E/C8 antibody (brown) before the Feulgen-Rossenbeck staining (purple). (A) Control side. Even-numbered rhombomeres 2 and 4 show E/C8-positive reticular neurons accumulating on the meningeal side of the epithelium (arrows). Rhombomere 3 does not contain such neurons. (B) Experimental side. The E/C8-negative region of r3 is now filled with the r4 graft tissue. The graft consists of quail cells (arrowheads) and contains E/C8-positive neurons. (C,D) Ghox 2.9 (brown) / HNK-1 (blue)-double stained sections of an embryo, in which a graft containing r4′ and r5′ was inserted into a gap at the level of host r3 and r4 (grafted rhombomeres were labeled by a prime). Operated between stages 9+ and 10, fixed 24 hours later. (C) Control side. Rhombomere 4 expresses Ghox 2.9 protein. The ventricular surface of odd-numbered rhombomeres stains strongly with HNK-1. In contrast, the neuroepithelial portion is stained lightly. Even-numbered rhombomeres are associated with the sensory ganglion primordia for nerves V and VII. The otocyst is found between r5 and r6. (D) Experimental side. The graft (4′, 5′) was inserted into a gap at level of r3 and r4. Only one large sensory ganglion primordium develops lateral to the inserted r4′ (arrowheads). Within the neuroectoderm of the graft there is a sharp boundary (arrow) between Ghox 2.9-positive r4′ (brown) and r5′. Note, HNK-1 immunoreactivity in r5′ appears in more ventral sections. Asterisk indicates an ectopic otocyst associated with the graft. ot, otocyst; V, sensory ganglion primordium of nerve V; VII, sensory ganglion primordium of nerve VII+VIII; 1-5, rhombomeres; 4′, 5′, grafted rhombomeres.

ence over a short-range. This is reminiscent of grafts of r4 that affected cranial nerves proximally but not distally (Fig. 8A-D). Moreover, the r3 → r7 experiments emphasizes that a piece of neuroectoderm that does not form nerve roots will maintain this property even when placed into a region where nerves would normally form.

DISCUSSION

Cell-autonomous expression of a homeodomain protein during hindbrain development

Hox genes encode DNA binding proteins which are thought to be involved in the specification of segmental structures along the anteroposterior body axis (Holland and Hogan, 1988; Graham et al., 1989; Kessel and Gruss, 1990; McGinnis and Krumlauf, 1992). One particularly striking example of segmental expression is provided by Ghox 2.9 (Hox 2.9 in mouse) that is expressed at high levels in rhombomere 4 (r4) of the hindbrain. By transplanting r4 to different axial positions within the hindbrain followed by immunolabeling of Ghox 2.9 protein, we demonstrate that this gene is expressed in a cell-autonomous fashion. This finding is consistent with similar experiments by Guthrie et al. (1992) who showed that Ghox 2.9 mRNA persists in transplanted r4 neuroectoderm. Substituting r2, r3 or r5 for r4 did not upregulate Ghox 2.9, indicating that the underlying mesodermal tissue is not able to affect Ghox 2.9 expression. This agrees with the observation of Guthrie et al. (1992) who showed that grafting r2 to the level of r4 does not induce Ghox 2.9 mRNA expression in the graft. Cell-autonomous behavior of a protein possibly involved in defining cell identity is consistent with our finding that a
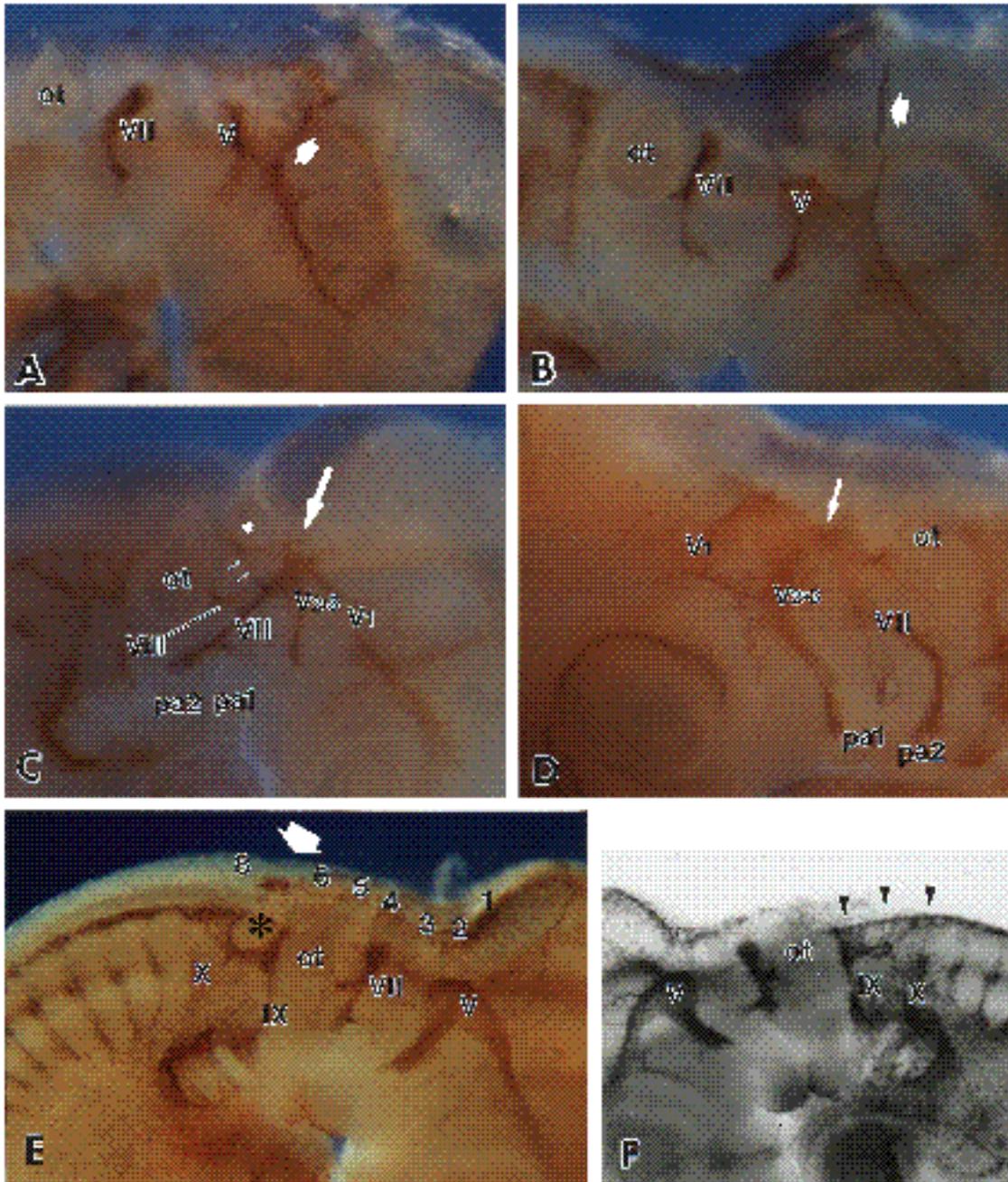
(compare Figs 6C and 8B and Fig. 9 center and right column).

An ectopic odd-numbered rhombomere inhibits the development of the cranial nerve root

Caudal to the anterior border of r6, an E/C8 positive nerve root for IX-X complex broadly extends lateral to the posterior hindbrain (Fig. 3B). Crest cells originate from along the entire length of the postotic neuroectoderm (Miyagawa-Tomita et al., 1991). A piece of r3 was grafted into presumptive r7 area before the onset of circumpharyngeal crest cell migration (at stage 9 presumptive r7 is situated medially to the first somite). We found that an ectopic r3 created a nerve-free gap within the IX-X nerve root, dividing it into two parts (Fig. 8E). More distally, nerve roots fused again resulting in a morphology very similar to that of a normal embryo (Fig. 8F). Hence, the graft exerts its influ-

Fig. 8. Morphological changes of the cranial nerves two days after r4 transplantations. Whole-mount embryos were stained with E/C8. (A,B) Two examples of r4 r1 bilateral transplantation. In A, an additional massive connection (arrow) is formed between the graft level and nerve V. In B, the connection (arrow) is much thinner and longer than in A, thus giving an appearance of a branch. In neither specimen is the distal portion of nerve V affected (compare with Fig. 3B). (C,D) r4 r3 transplantation (C, bilateral; D, unilateral). Two days (C) and 3 days (D) after the surgery. In C, nerves V and VII+VIII share a single nerve root that spans between r2 and r4 (large arrow). The anatomy of nerve branches and nerve distribution in pharyngeal arches shows that this compound nerve contains, in the distal portion, normal components of facial and trigeminal nerves. The compound nerve root possesses two acoustic nerves, one leading to the host otocyst (VIII and ot), and the other to the ectopic otocysts (asterisk) which is, in addition, innervated by a presumptive ectopic acoustic nerve (small arrows). (D) Fusion of ganglia between nerves V and VII+VIII is seen (arrow). Distal to the fused ganglion, the nerve branching pattern is normal (compare with Fig. 3B). (E) r3 r7 transplantation. The common nerve root for nerves IX and X normally extends evenly caudal to r6 (see Figs 7F, 3B). This root is now subdivided (asterisk) at the level of inserted r3 (arrow). However, the nerves fuse again distally. (F) The control side of the same specimen shown in E. The root for nerves IX and X extends continuously caudal to r6 (arrowheads). Note, in the specimen shown, the distal portion of the vagus nerve is not yet formed. ot, otocyst; pa1 and 2, first (mandibular) and second (hyoid) pharyngeal arch; V_1, ophthalmic portion of nerve V; V_{2+3}, maxillomandibular portion of nerve V; VII, nerve VII+VIII; VIII, nerve VIII portion of nerve VII+VIII; X, nerve X.
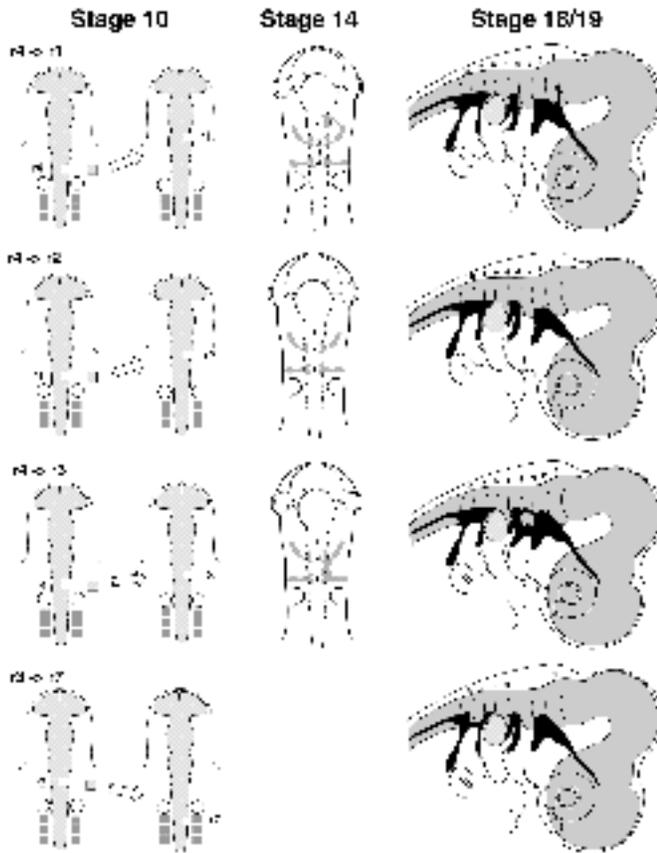
Fig. 9. Summary of the transplantation experiments analyzed by E/C8 staining. Experimental manipulations at stages 9⁻ to 10⁺ are shown on the left; E/C8 staining by stage 14 (center) is indicated by shading. By stage 18/19, E/C8 antibody reveals the peripheral morphology of cranial nerves following surgery. The distribution of the crest cells at stage 14 is consistent with the resulting morphological changes in cranial nerve root development seen by stage 18/19. No changes are seen in the distal portion of cranial nerves. This is also the case when the non-crest forming r3 is positioned into r7 area (bottom row).

transplanted r4 maintains its ability to form a cranial nerve root at an ectopic site.

The early vertebrate neural tube shows serially arranged bulges representing forebrain, midbrain, rhombomeres, and myelomeres. Although these bulges look similar, the mechanisms by which they are formed and specified is probably different. In the spinal cord, the segmental units, the myelomeres, are not clonal cell populations, but restricted domains of neuroepithelial cells that arise secondarily as the result of cues derived from adjacent somites (Stern et al., 1991; Lim et al., 1991).

Our transplantation studies suggest that segmental identity underlying the morphological uniqueness of each rhombomere is already acquired around stage 9 and apparently not changeable. Establishment of segmental identity requires cell lineage restriction (Slack, 1991). Dye labeling studies have indeed revealed that cells within a rhombomere do not intermingle once a boundary is formed (Fraser et al., 1990). Moreover, Guthrie and Lumsden (1991) have demonstrated that formation of rhombomere boundaries is

an intrinsic property of neuroectoderm and not imposed by mesoderm (Guthrie and Lumsden, 1991). These recent observations are consistent with pioneering work of Källen (1956) who showed that, in contrast to spinal cord and anterior CNS, rhombomeres develop in the absence of mesoderm and notochord. It thus appears that the delineation of rhombomeres and establishment of rhombomere identity are controlled by mechanisms different from those operating in the spinal cord. The molecular basis of this difference may be reflected in the fact that only in hindbrain are Hox genes (3 genes) expressed in domains limited by sharp boundaries which coincide with the morphological borders of rhombomeres. In contrast, more 5 Hox genes, which are expressed in the spinal cord, display anterior boundaries of expression that are less distinct (Graham et al., 1989). Still one cannot completely rule out the possibility that paraxial mesoderm, especially the somitomeres, may have a role in patterning of the hindbrain ectoderm similar to that proposed for somites in the spinal cord (Stern et al., 1991; Lim et al., 1991).

It is believed that the establishment of pattern in the midbrain is based on interactions within the neuroectoderm possibly involving *en* protein that forms an anteroposterior gradient in the neurepithelium (Alvarado-Mallart et al., 1990; Martinez et al., 1991; Itasaki et al., 1991). It should be mentioned that the gradient of *en* protein expression across the midbrain has a posterior high point. Upon experimental inversion of midbrain at stage 10, this high point of *en* protein expression becomes transiently located anteriorly. However, during subsequent development the original polarity is reestablished (Martinez and Alvarado-Mallart, 1990). This regulation of expression of *en* contrasts with the persistence of Ghox 2.9 protein in ectopic sites.

Cranial nerve segmentation

An ectopic graft of r4 gives rise to a population of ectopic crest cells that develops either into an extra cranial nerve root or merges with neighboring crest cells, producing fused cranial ganglia (Fig. 8A-D). Conversely, when r3, which does not generate crest cells, is inserted into r7, the graft prevents formation of a root for nerve IX-X which would normally extend caudal to r6 (Fig. 8E). The gap in this nerve root could simply be due to the ablation of crest in the vagal area as a consequence of removal of host r7. However, earlier studies have shown that removal of vagal crest will result in poor development of the IX-X nerve root (Kuratani et al., 1991) but not in the type of gap seen in Fig. 8E.

These findings seem to imply mechanisms of nerve patterning that are unique to the hindbrain. In the trunk, segmental organization of spinal nerves is entirely dependent on the presence of somites (Detwiler, 1934; Stern and Keynes, 1987; Tosney, 1988). Trunk crest cells originate uniformly along the neuraxis (Kalcheim and Le Douarin, 1986; Kalcheim and Teillet, 1989) and migrate only through the anterior half of the sclerotome, a restriction that eventually results in the segmental pattern of dorsal root ganglia (Lehmann, 1927; Detwiler, 1934, 1937; reviewed by LeDouarin et al., 1984; Bronner-Fraser and Stern, 1991). A similar segmentation mechanism is found in the formation of the ventral spinal root of the chick embryo; ventral horn
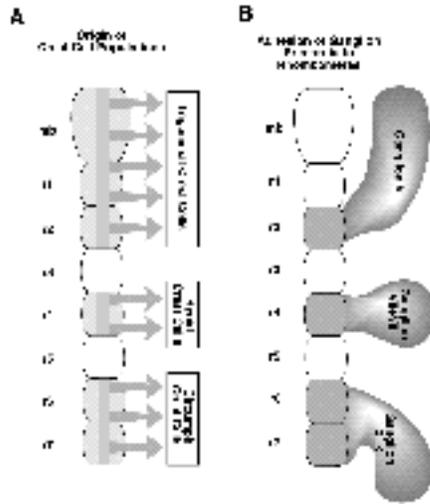
Fig. 10. A diagram to illustrate a hypothetical mechanism of cranial nerve patterning. (A) The origin of cephalic crest is segregated into three portions by r3 and r5 that do not give rise to crest cells. (B) The three crest cell populations form three sensory ganglion primordia which attach to r2, r4, and neuroectoderm posterior to the boundary of r5/r6 (gray rhombomeres). Presence of crest cells is required for formation of pharyngeal nerve roots (Moody and Heaton, 1983a,b,c). The gray rhombomeres thus become 'cranial nerve ports.' Unlike spinal nerve patterning, the rhombomere-dependent distribution of pharyngeal nerve roots can be altered by the heterotopic transplantation of rhombomeres. This reflects the precommitted cell-autonomous nature of the rhombomeres.

neurons can only grow at the level of the anterior half of the somite (Keynes and Stern, 1984). Thus, in the trunk, the patterning of the PNS is controlled by mesoderm and accordingly anteroposterior reversal of spinal cord has no effect on the PNS pattern (Keynes and Stern, 1984).

In the hindbrain, however, transplantation of neuroectoderm results in repatterning of cranial nerve roots. Fig. 10 emphasizes two features that we believe to be important in patterning of cranial nerves. Unlike in the trunk, crest cells appear in three distinct domains (Fig. 10A). These subpopulations result from the finding that r3 and r5 do not give rise to crest cells (Lumsden et al., 1991; see also Fig. 2F). The second factor worth considering is that ganglion primordia attach (we use 'attachment' in the sense of a morphological association, not necessarily to imply a direct cell-cell interaction between neuroectoderm and ganglion, see also below) to selected regions of the hindbrain (Fig. 10B). Fig. 10B illustrates that sensory ganglion primordia are in proximity only to restricted areas of the hindbrain (r2, r4, and region caudal to r6) and only these areas have the capacity to form nerve roots (Kuratani, 1991; also see Fig. 2). Note, the ability to attach crest cells is more restricted than the capacity to generate crest cells (compare Figs 10A and B). For example, r1 and midbrain both produce crest cells (Tan and Morriss-Kay, 1985; Lumsden et al., 1991), but neither of these regions permit attachment and thus are incapable of developing (pharyngeal) nerve roots (Fig. 3B). At present, it is not clear how such selective association of ganglion primordia with neuroectoderm

takes place. One possible scenario could involve localized expression of cell adhesion molecules in an alternating pattern on the surface of the hindbrain. There is some evidence that Hox genes regulate, for example, the expression of cell adhesion molecules, such as NCAM (Jones et al., 1992).

Attachment of sensory ganglia does not only specify the pattern of cranial nerves but is also an important factor in the development of cranial nerve roots. For example, Moody and Heaton (1983a,b,c) found that proximity of the cranial ganglion primordium and neuroectoderm is required for proper development of the motor nucleus, and for sprouting of motor axons from the hindbrain. Removal of crest or of the sensory ganglion primordium results in abnormal sprouting of motor axons (Moody and Heaton, 1983c; Chang et al., 1992) or in poor development of cranial nerve roots (Yntema, 1944; Kuratani et al., 1991; see also Fig. 5C). In conclusion, cranial nerve ports and hence cranial nerve roots arise only in those rhombomeres that are able to form crest cells and permit crest cell attachment. This property does not alter after grafting rhombomeres to a new site. Whereas in the trunk, spinal nerves are patterned by somites (Keynes and Stern, 1984), in the head, cranial nerve patterning has no obvious relationship to structures of paraxial mesoderm (Noden, 1988).

In the trunk and in the head crest cells are capable of forming enlarged ganglia. If a crest-producing rhombomere is substituted for either r3 or r5, the crest cell gap is filled and ganglia will fuse into a large single ganglion (Fig. 7C,D). Similarly, when somites in the trunk are eliminated crest cells will no longer segregate and coalesce to form a large dorsal root ganglion (Detwiler, 1934, 1937; Tosney, 1988). Thus, given an opportunity, both cranial and trunk crest can aggregate in a similar way as a result of removing a gap in which crest cells are normally absent.

A fusion of the trigeminal and acustico-facial ganglion primordia is also observed in *Xenopus*, mouse and chick embryos treated with retinoic acid (Morriss-Kay et al., 1991; Papalopulu et al., 1991; Sundin and Eichele, 1992). This finding can be explained by assuming that retinoic acid results, among other effects, in loss of identity of r3. Therefore, the gap between trigeminal and acustico-facial ganglion primordia would be abolished. This interpretation is supported by the observation that in retinoic acid-treated embryos Krox-20 gene expression typical for r3 (Wilkinson et al., 1989) is absent (Morriss-Kay et al., 1991; Papalopulu et al., 1991). It is also of interest that mouse embryos lacking Hox 1.6 (a paralog of Hox 2.9) exhibit fusion of trigeminal and acustico-facial nerves (Chisaka et al., 1992). These investigators also noticed that the boundaries between rhombomeres were apparently lost. In terms of our model (Fig. 10) this would result in an altered distribution of crest cells and in changes of the sites of crest cell adhesion and ultimately in a changed pattern of cranial nerves.

We found that the alteration of branchial nerve morphology in recombinant embryos was restricted to its proximal portion (Figs 8, 9); distally nerves were unaffected. A number of factors could determine distal nerve branching patterns. Axons could interact with pharyngeal ectoderm and endoderm, and in addition, with extracellular matrix known to influence axonal growth (Moody et al., 1989;

Riggot and Moody, 1987). It has also been shown that epibranchial placode-derived neurons are necessary for normal development of the trigeminal sensory branches in the face (Hamburger, 1961). More recent studies suggest that crest-derived neurons follow the axonal pathway first established by placode-derived neurons (Noden, 1980a,b). This would imply that not the neural crest, but the placode determines the specific distal branching pattern of cranial nerves. Thus, it seems likely that the placode influences distal nerve patterning, yet it remains possible that neurons coming from the graft retain function and phenotypic characteristics reflecting their site of origin. To further examine this issue it will be necessary to elucidate the morphology of central and peripheral projections and determine the type of neurotransmitters used in the graft-derived neurons.

Two-rhombomere repeat pattern of hindbrain development

Motoneurons derived from two rhombomeres form one cranial motor root (Tello, 1923; Lumsden and Keynes, 1989). Thus, patterning of cranial nerve roots and motoneuron organization exhibit a two-rhombomere periodicity. There are several proteins and genes whose expression pattern show two-rhombomere periodicity (e.g. CRABP, Maden et al., 1991; neurofilament protein, Lumsden and Keynes, 1989; HNK-1 antigen and NAPA-73 in the present study and in Kuratani, 1991). It is possible that the expression patterns of these proteins are related to the morphological segmentation of the hindbrain. Some of these proteins may be directly involved in aspects of cranial nerve specification and rhombomere boundary formation. For example, HNK-1 epitope is found on various cell adhesion molecules (NCAM, MAG, L1) and these may be directly involved in establishing the connections between sensory ganglion primordium and neuroectoderm and/or participate in the formation of rhombomere boundaries. Guthrie and Lumsden (1991) showed that rhombomere boundaries form only between an even- and an odd-numbered rhombomere. This observation predicts the existence of cell interaction molecules that are differently expressed in even- and odd-numbered rhombomeres such as those revealed by the HNK-1 antibody.

The combinatorial pattern of expression of the 3 genes of Hox-2 cluster also exhibits a two-rhombomere 'rhythm'. Specifically, the anterior border of the domain of Hox 2.8 expression coincides with the anterior sulcus of r3, that of Hox 2.7 with the anterior boundary of r5, and the expression domain of Hox 2.6 starts at the anterior boundary of r7 (see Hunt et al., 1991 for a review). It remains to be determined how Hox proteins regulate molecules that are responsible for producing the actual cellular patterns. One possibility is that Hox genes directly regulate genes encoding proteins involved in cell interactions. Evidence for this comes from recent studies demonstrating that Hox proteins may act as regulators of the NCAM expression (Jones et al., 1992).

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