Development 125, 3445-3459 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV2310

Determination of the identity of the derivatives of the cephalic neural crest: incompatibility between *Hox* gene expression and lower jaw development

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Accepted 23 June; published on WWW 6 August 1998

SUMMARY

In addition to pigment cells, and neural and endocrine derivatives, the neural crest is characterized by its ability to yield mesenchymal cells. In amniotes, this property is restricted to the cephalic region from the mid-diencephalon to the end of rhombomere 8 (level of somites 4/5). The cephalic neural crest is divided into two domains: an anterior region corresponding to the diencephalon, mesencephalon and metencephalon (r1, r2) in which expression of *Hox* genes is never observed, and a posterior domain in which neural crest cells exhibit (with a few exceptions) the same *Hox* code as the rhombomeres from which they originate. By altering the normal distribution of neural crest cells in the branchial arches through appropriate embryonic manipulations, we have investigated the relationships between *Hox* gene expression and the level of plasticity that neural crest cells display when they are led to migrate to an ectopic environment. We made the following observations. (i) *Hox* gene expression is not altered in neural crest cells by their transposition to ectopic sites. (ii) Expression of *Hox* genes by the BA ectoderm does not depend upon an induction by the neural crest. This second finding further supports the concept of segmentation of the cephalic ectoderm into ectomeres (Couly and Le Douarin, 1990). According to this concept, metameres can be defined in large bands of ectoderm including not only the CNS and the neural crest but also the corresponding superficial ectoderm fated to cover craniofacial primordia. (iii) The construction of a lower jaw requires the environment provided by the ectomesodermal components of BA1 or BA2 associated with the *Hox* gene non-expressing neural crest cells. *Hox* gene-expressing neural crest cells are unable to yield the lower jaw apparatus including the entoglossum and basihyal even in the BA1 environment. In contrast, the posterior part of the hyoid bone can be constructed by any region of the neural crest cells whether or not they are under the regulatory control of *Hox* genes. Such is also the case for the neural and connective tissues (including those comprising the cardiovascular system) of neural crest origin, upon which no segmental restriction is imposed. The latter finding confirms the plasticity observed 24 years ago (Le Douarin and Teillet, 1974) for the precursors of the PNS.

Key words: Regeneration, Neural crest, *Hox* code, Branchial arch, Aortic arch, Rhombomere, Plasticity, Facial skeleton, Hyoid bone, Neural derivative, Conotroncus, Heart, Glandular derivative, Pharynx, Quail, Chick.

INTRODUCTION

The neural crest is a highly pluripotent structure that plays a critical role in the construction of the vertebrate head. In addition to neurons and glia of the peripheral nervous system (PNS), and to melanocytes and endocrine cells, the neural crest also gives rise to mesenchymal cells capable of differentiating into bone, cartilage, connective and adipose tissues (see Le Douarin, 1982, for a review). Systematic investigations on the long-term fate of neural crest cells (NCC) exiting from the various levels of the neural axis have been carried out in the avian embryo using the quail-chick chimera system (see Le Douarin, 1982; Le Douarin et al., 1994 for reviews). This approach allowed the construction of a neural crest fate map, which revealed that the capacity to give rise to mesectodermal derivatives is restricted to the cephalic region of the neural axis down to the level of somite 5. The mesenchymal cells arising from the cephalic neural crest and forming the so-called mesectoderm yield most of the skull bones and all the facial and visceral skeleton (Couly et al., 1993). They also give rise to the dermis covering the neural-crest-derived skeletal structures and the connective cells associated with the head muscles (Couly et al., 1992; Köntges and Lumsden, 1996).

The recognition of the segmental structure of the rhombencephalon, which is divided from the early stages of neurogenesis into 8 rhombomeres (r), has been a landmark event in neural crest ontogeny research (see Lumsden and Keynes, 1989; Lumsden, 1990). The fact that rhombomeres are characterized by the expression of definitive sets of regulatory genes such as *Hox* genes, the vertebrate homologs of the

homeotic selector genes of *Drosophila* (see Krumlauf, 1994 for a review), raised a novel interest in the contribution of NCC to the complex processes of head morphogenesis. The fate of the midbrain and hindbrain neural crest was re-examined using more elaborate techniques in the avian embryo. The neural folds corresponding to each individual rhombomere were labelled with the fluorescent lipophilic dye DiI (Lumsden et al., 1991) or by using the quail-chick chimera technique (Köntges and Lumsden, 1996; Couly et al., 1996). In the latter approach, definite fragments of the chick neuroepithelium corresponding to the mesencephalon (anterior or posterior half) and to neuroepithelial segments corresponding to individual presumptive rhombomeres, were replaced in chick embryos by their quail counterparts. The contribution of the different transverse levels of the neural fold to the maxillary, mandibullary buds (i.e. the first branchial arch, BA1), the second (BA2), the third (BA3) and the posterior (BA4, BA5 and BA6, which are not clearly individualized and are designated hereafter as BA4-6) branchial arches were determined as indicated in Fig. 1A,B. It was also shown that (with some exceptions) *Hox* genes are expressed in the neural crest as they are in the rhombomere from which they originate (Fig. 1A). Thus, BA1 is colonized by NCC from the posterior mesencephalon and from r1 and r2 with a small contingent of cells from r3 that do not express any genes of the *Hox* clusters. They are therefore further designated as '*Hox*-negative BA1 presumptive NCC'. BA2, which is extensively colonized by NCC originating from r4 (with a minor contribution from r3 and r5), contains *Hoxa-2*-positive mesectodermal cells. BA3 is invaded by NCC expressing *Hox* genes of the second and third paralogous groups while, in the posterior BAs, the NCC express additional genes of the fourth paralogous groups, as summarized in Fig. 1A. Moreover, the long-term fate of the NCC exiting from each segmental level of the brain was established. The precise contribution to the skeleton is represented in Fig. 1C.

The role of certain *Hox* genes in controlling the morphogenetic program of NCC contributing to the hypobranchial structures was established in the mouse by targeted mutation. Thus, in mice lacking *Hoxa-2* function, the morphogenesis of BA1- and BA2-derived skeleton is profoundly altered with a duplication of the lower jaw at the expense of BA2-NCC (Rijli et al., 1993; Gendron-Maguire et al., 1993). Inactivation of other *Hox* genes did not produce homeotic transformations but malformations of neural-crest-derived structures. Thus null mutation of *Hoxa-1* resulted in the reduction of cranial nerve VIII (Lufkin et al., 1991; Chisaka et al., 1992; Mark et al., 1993) while ectopic expression of *Hoxa-1* in BA1 NCC of zebrafish resulted in the lack of BA1-derived structures and the fusion of ganglia V and VII (Alexandre et al., 1996). *Hoxa-3* inactivation profoundly affected the morphogenesis of structures derived from the mesectodermal derivatives of the posterior BAs reproducing the human DiGeorge's syndrome (Chisaka and Capecchi, 1991; Condie and Capecchi, 1994; Manley and Capecchi, 1995, 1998).

In the present study, we have addressed the problem of the specification of NCC before they reach the BAs. We have investigated the relationships between *Hox* gene expression by these cells and the level of plasticity that they display if they are led to migrate to ectopic sites. In the past, the determination of NCC was investigated through heterotopic transposition of fragments of the neural primordium along the neural axis in the avian embryo (Le Douarin and Teillet, 1974). These experiments have shown that the differentiation of the PNS precursors into the various types of sensory and autonomic ganglion cells depends upon environmental cues rather than upon their level of origin along the anteroposterior (AP) axis (see also Le Douarin, 1982; Le Douarin and Smith, 1988, for reviews). Such an absence of early specification, however, is not shared by all the NC derivatives and particularly does not hold for the mesectoderm. These transposition experiments have revealed that, when transplanted ectopically to the trunk level, the cephalic NCC retain the capacity to differentiate into cartilage wherever they were implanted. Thus, nodules of cartilage of graft origin were regularly found in the dermis and kidney of chick host embryos. Moreover, Noden (1983) demonstrated that transplantation of the mesencephalic and metencephalic (corresponding to r1/r2) neural epithelium to otic levels of the neural axis resulted in the duplication of the bones of the lower jaw. This was taken as a proof that the NCC that normally yield BA1 are endowed with cell autonomous patterning activity.

The experiments described here were designed to determine whether NCC retain their initial *Hox* code when led to migrate to ectopic sites along the AP axis. The relationships that exist between the BA ectoderm and the invading NCC as far as expression of *Hox* genes is concerned was also explored. By examining the long-term fate of the ectopically migrating NCC, we could establish that the capacity to construct a lower jaw is present in the whole NC domain located anteriorly to r3 that does not express *Hox* genes and that this capacity can be expressed in the tissular context of BA1 or BA2 as well. We show that this capacity of NCC is expressed only if they are transplanted together with the corresponding neural tube epithelium. We also show that any part of the cephalic neural crest can take part in the construction of the portion of the hyoid bone that is normally derived from BA2-6 but not to the part derived from BA1. Patterning activity for the posterior part of the hyoid bone is thus contained in the stable tissue components of the BAs but not in the NCC that join them. Endoderm, mesoderm and ectoderm constituting the BAs are potential effectors of this function.

MATERIALS AND METHODS

(A) Preparation of graft and host for microsurgery

Quail (*Coturnix coturnix japonica*) and chick (*Gallus gallus*) eggs from commercial sources were used throughout this study. Microsurgery was performed on embryos at the 5- to 6-somite stage corresponding to approximately 30 hours of incubation in a humidified atmosphere at 38°C. A window was cut in the shell and India ink diluted 1:4 in PBS was injected into the sub-blastodermic cavity in order to visualise the embryonic structures. The vitelline membrane was opened and the neural fold was surgically removed on a definite AP length. The limits of the grafted tissues and of the sites of implantation were defined by using an ocular micrometer according to the fate map previously established (Grapin-Botton et al., 1995) where the rostral and caudal borders of the presumptive rhombomeres were localized. For operations on the midbrain and forebrain, the pro-mesencephalic and mes-metencephalic constrictions were taken as landmarks. The donor neural fold was removed from quail embryos

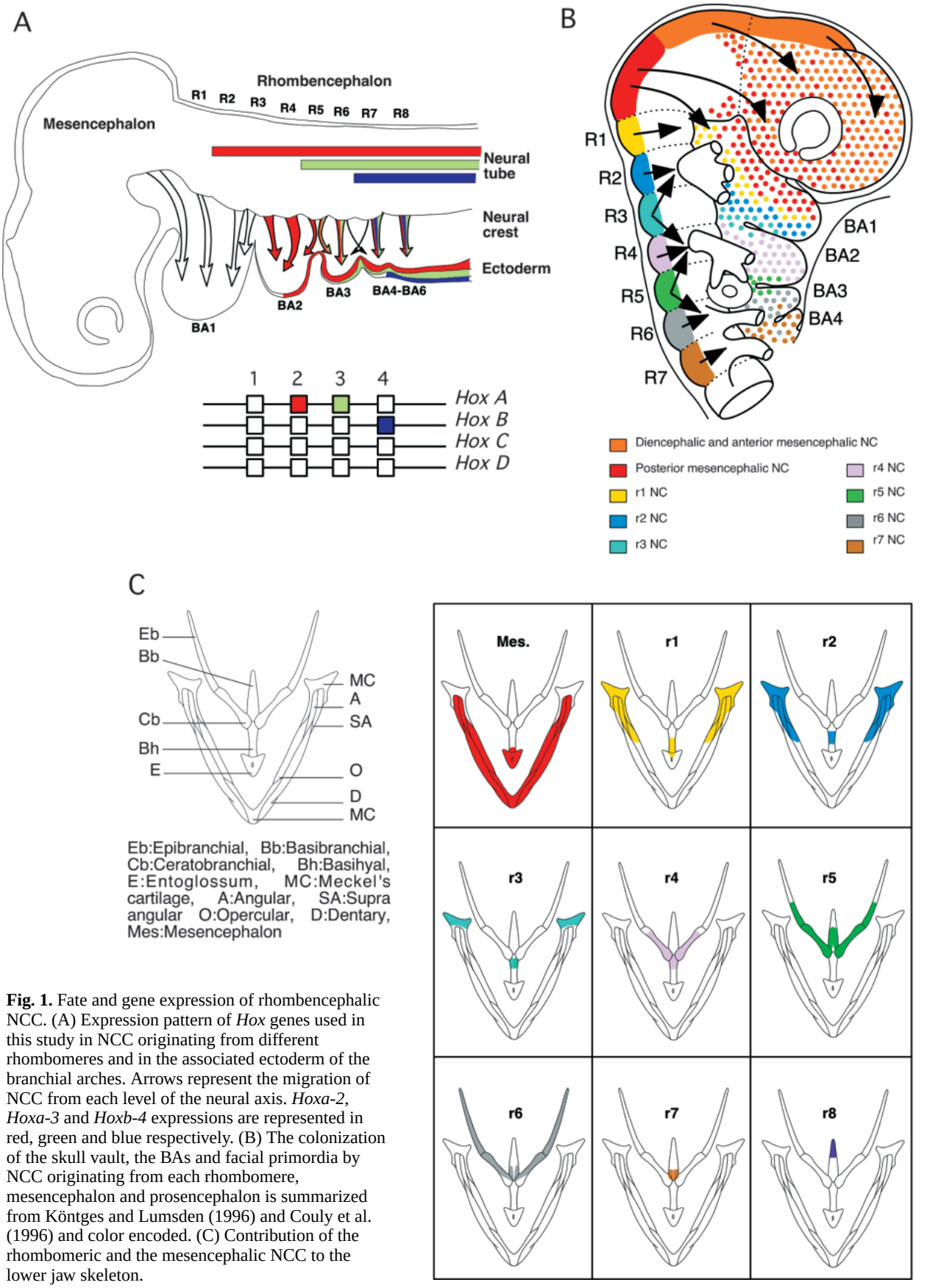

Fig. 1. Fate and gene expression of rhombencephalic NCC. (A) Expression pattern of *Hox* genes used in this study in NCC originating from different rhombomeres and in the associated ectoderm of the branchial arches. Arrows represent the migration of NCC from each level of the neural axis. *Hoxa-2*, *Hoxa-3* and *Hoxb-4* expressions are represented in red, green and blue respectively. (B) The colonization of the skull vault, the BAs and facial primordia by NCC originating from each rhombomere, mesencephalon and prosencephalon is summarized from Köntges and Lumsden (1996) and Couly et al. (1996) and color encoded. (C) Contribution of the rhombomeric and the mesencephalic NCC to the lower jaw skeleton.

and grafted isotopically or heterotopically to the recipient stage-matched chicks.

Two types of experiments were designated to reroute the NCC from their normal migration pathway. The first is based on the capacity of these cells to migrate along the AP axis (rostrocaudally or vice-versa) to compensate for excision of neural fold fragments in the mesencephalic and rhombencephalic domains. The second type of experiments concerned the heterotopic transpositions of the neural fold fragments.

The first series of experiments involved the bilateral resection of the neural fold from the pro-mesencephalic constriction down to the prospective limit between rhombomeres 6 and 7 (r6/r7). After ablation, fragments of quail neural fold were isotopically implanted bilaterally on the length corresponding to rhombomeres 4 to 6 (r4/r6; Figs 4A, 5A).

Some embryos were fixed in paraformaldehyde at embryonic day 3 and 4 (E3-E4) to study the colonization of the branchial arches (BAs) by quail cells after in toto treatment with the anti-quail monoclonal antibody (mAb) QCPN. Other embryos killed at E7 or later were fixed in Bouin's fluid, photographed, embedded in Paraplast and used to localize quail cells using QCPN mAb on sections. Some embryos killed at E7 or later were alternatively processed for whole-mount Alcian blue and alizarine red stainings to evidence the skeleton.

In the transposition experiments, the neural fold (i.e. less than the upper 1/4th of the lateral wall of the future neural tube) was removed at definite levels of the mesencephalon and rhombencephalon and replaced by the equivalent length of the quail neural fold originating from a different AP level, either more rostral (A→P transposition) or more caudal (P→A transposition). In certain experiments, the lateral wall of the neural tube was transplanted together with the neural fold. These grafts were refereed to as 'neural tube and neural fold' transplants.

The embryos subjected to these operations were examined either at E3-E4 or later from E7 to E14. At the early stages, the embryos were processed to evidence quail cells and expression of *Hox* genes on alternate serial sections. E7-E14 embryos were fixed in Bouin's or Carnoy's fluids and quail cells were localized on sections using QCPN mAb. In certain cases, the QCPN mAb staining was combined with Alcian blue to evidence cartilage. TTF1 mAb was sometimes used on E10 embryos on alternate sections to visualize the thyroid gland (Lazzaro et al., 1991). Alternate sections were also used to study *Hoxa-3* expression using radioactive in situ hybridization. Some embryos that had reached E10 or later were processed for whole-mount Alcian blue-alizarin red staining of the skeleton.

(B) Analysis of the chimeras

Immunocytochemistry

Identification of grafted cells was carried out by using the QCPN monoclonal antibody (DHSB), which recognizes an antigenic determinant common to all quail cells. Embryos were fixed in Bouin's or Carnoy's fixatives and embedded in Paraplast. 5 or 6 μm sections were incubated with QCPN mAb (undiluted supernatant) overnight in a humidified chamber at 4°C and, after washing in PBS, re-incubated for 90 minutes with horseradish-peroxidase-conjugated antibody directed against mouse IgG1 (Southern Biotechnology Associates). The reaction was developed in 250 ml PBS containing 20 mg diaminobenzidine tetrahydrochloride (DAB) and 36 μl H_2O_2 30%. Slides were then briefly rinsed in water and sections were stained with Gill's hematoxylin, ethanol-dehydrated and mounted with XAM (Gurr) or Entellan (Merck). Using the same procedure, TTF-1 mAb was occasionally used on adjacent sections to identify thyroid cells (Lazzaro et al., 1991). Secondary antibody was a goat anti-mouse IgG.

The QCPN mAb was also used in whole-mount preparations. Embryos were fixed in 4% paraformaldehyde, rinsed in PBS/0.1% Tween 20, progressively dehydrated in methanol and stored at –20°C. After rehydration, they were rinsed in PBS/0.2% gelatin/0.25% Triton/1 g/l sodium azide (PGTA), twice for 15 minutes. They were then incubated 48 hours in the same solution containing QCPN mAb diluted 1/20. They were washed for 1 day in PBS/2% gelatin/0.25% Triton (PGT), changing the solution about every hour. Embryos were then incubated overnight in the same solution containing biotin-conjugated antibody directed against mouse IgG1, 1/50. They were rinsed in PGT twice for 45 minutes, in PBS/0.25% Triton twice 45 minutes and in PBS 45 minutes and then transferred in avidin/biotin amplification solution (Vectastain HRPO, Vector) for 2 hours. They were washed in PBS, 3 times 15 minutes and then preincubated in DAB solution (10 mg/30 ml) 30 minutes. Reaction was started under microscope by adding H_2O_2 1/10,000. Staining developed within 15 seconds to 1 minute.

Identification of bones and cartilage

Embryos that were killed at E10-E14 were eviscerated and eyes were removed. They were fixed in a solution containing 80% alcohol 100°, 20% acetic acid 100% and 15 mg/100 ml Alcian blue 8GX. The fixation time varied between 16 and 30 hours depending on the stage between E8 and E14. Embryos were rinsed in 100° alcohol until the alcohol was clear. Embryos were then incubated in Alizarine Red 10 mg, KOH 0.5 g/100 ml in H_2O until the skeleton was visible.

In situ hybridization

The expression of the *Hoxa-2, a-3* and *b-4* genes was studied at stages HH20 (Hamburger and Hamilton, 1951) to E10. The position of quail cells and the expression of the three *Hox* genes were examined on adjacent sections. The *Hoxb-4* probe corresponds to the full-length mRNA (Sasaki and Kuroiwa, 1990). The *Hoxa-3* probe was generated from a 900 bp *Kpn*I-*Eco*RI fragment of the chick cDNA subcloned into pBluescript previously used by Grapin-Botton et al. (1995) and kindly provided by Robb Krumlauf. The *Hoxa-2* probe was generated from a 700 bp partial cDNA subcloned into pBluescript, including the whole coding sequence 3′ to the *Eco*RI site within the homeobox (Prince and Lumsden, 1994). RNA probes were labelled by incorporation of ^{35}S-UTP (Amersham, 1000 Ci/mmol) during synthesis (Promega, Riboprobe Gemini II). Radioactive in situ hybridizations were performed as previously described (Eichmann et al., 1993).

RESULTS

(1) Maintenance of the *Hox* code in neural crest cells following heterotopic transplantation of the neural folds

In order to investigate the effect of the environment on the fate of NCC, we carried out heterotopic transposition experiments of the neural fold, a few hours before the onset of migration, at 5- to 6-somite stage. In these experiments, the intermediate and ventral part of the neural tube was left in situ and the neural fold of the quail, originating either from a more rostral or a more caudal level of the neuraxis, was implanted at the site of the excision (Figs 2A,D,G, 3A,E).

A→P transposition of the neural fold from the posterior mesencephalon or r1/2 levels to the r4-6, r5-7 or r8 levels

In the first series of experiments, the neural fold of rhombomeres 1/2 from quail donors was transposed unilaterally to the position of r4/r6 in chick recipients (Fig. 2A). The embryos were examined at E3 (n=8) and E4 (n=4). The NCC derived from r1/r2 are normally found in the posterior part of BA1 (see Fig. 1B). In these heterotopic positions, they mainly filled BA2 but in certain cases a few

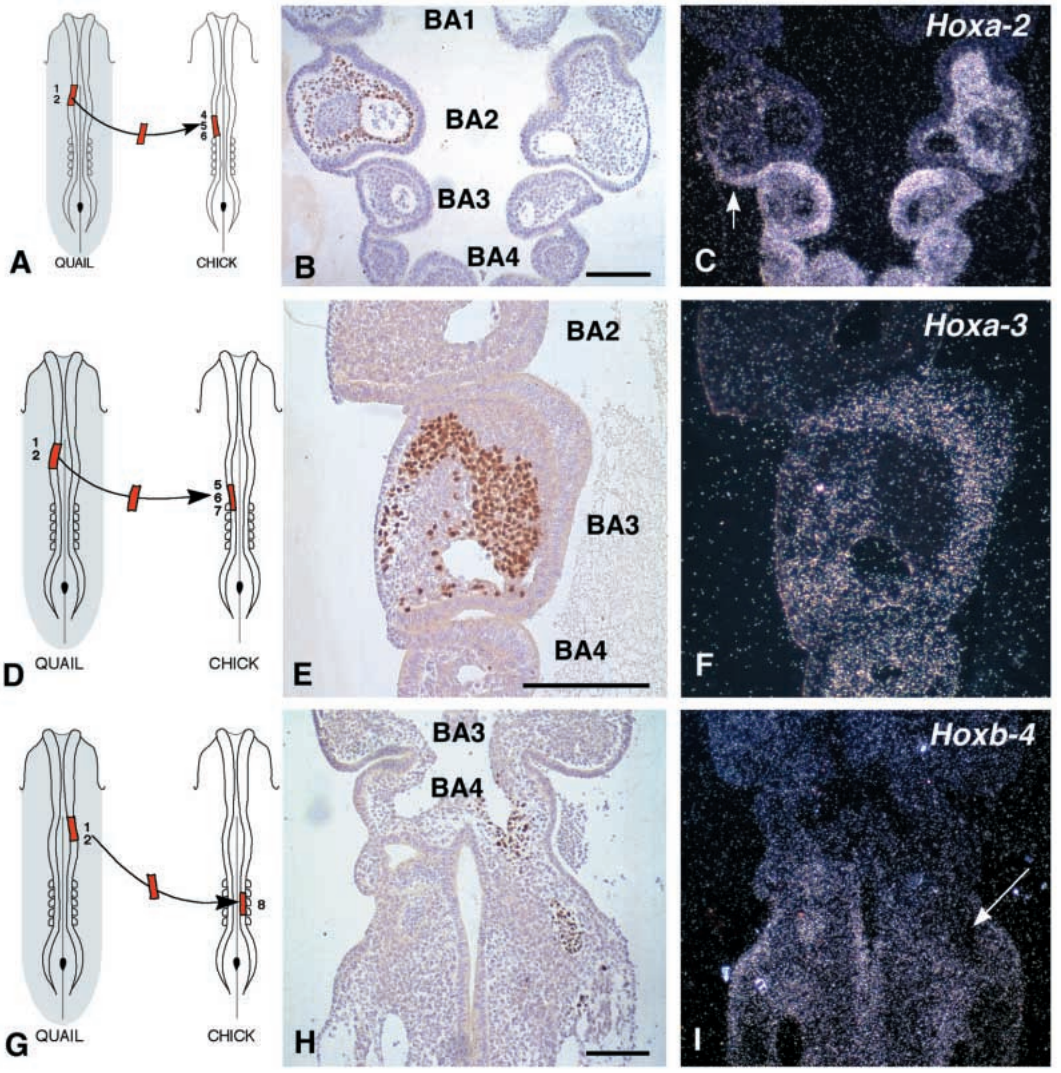

Fig. 2. *Hox* gene expression in posteriorly transposed r1/r2 NCC. (A) Unilateral transposition of r1/r2 quail neural fold to the position of r4/r6 of a chick host. The chimeras shown here are cut frontally at E3. (B) The localization of quail cells in BA2 using QCPN mAb. The center of the arch, made up mostly of mesoderm, is of host origin. (C) *Hoxa-2* expression is shown on an adjacent section. *Hoxa-2* is not expressed in NCCs of BA2 on the grafted side whereas it is in the opposite, control side. The arrow shows expression of *Hoxa-2* in the ectoderm of BA2. (D) Unilateral transposition of r1/r2 quail neural fold to the position of r5/r7 of a chick host. (E) The localization of quail cells in BA3 using QCPN mAb. NCCs in this arch are both of host and donor (brown) origin. (F) *Hoxa-3* expression is shown on an adjacent section. *Hoxa-3* is absent from cells of quail origin. (G) Unilateral transposition of r1/r2 quail neural fold to the position of r8 of a chick host. (H) The localization of quail cells in BA4-6 using QCPN mAb. (I) *Hoxb-4* expression is shown on an adjacent section. *Hoxb-4* is not expressed in quail cells of r1/2 origin (arrow). Bars, 150 μm.

cells were also found in BA1 (see Fig. 2B) and BA3 (not shown). BA2 was therefore of chimeric composition with endoderm, ectoderm and mesoderm derived from the host and NCC of graft origin (Fig. 2B). In normal embryos, *Hoxa-2* is expressed in none of the tissue components of BA1 but is expressed in the NCC and in the posterior half of the surface ectoderm of BA2. The paraxial mesoderm and the endoderm of BA2 (Fig. 2C, control side-right) are *Hoxa-2*-negative. The NCC arising from the transplant and invading BA2 did not acquire *Hoxa-2* expression, as evidenced 48 hours after grafting in Fig. 2C. When the posterior mesencephalic fragment of the neural fold was transposed, instead of r1/r2, similar results were obtained (n=2).

In the second series of experiments, the neural fold of r1/r2 was transposed more caudally in r5/r7 on one side of the embryo. The NCC of these levels normally express *Hoxa-2* and *Hoxa-3*. In normal embryos examined at E3, *Hoxa-3* was expressed in the NCC from the level of BA3 downward. When r1/r2 neural fold was grafted into r5/r7 (Fig. 2D), at E3 the quail cells were found in BA3 (Fig. 2E) and BA4 (n=6). *Hoxa-3* was not induced in these cells (Fig. 2F) nor in those that aggregated to form the proximal IX-X ganglion (n=6).

Lastly, r1/r2 neural fold was grafted unilaterally at r8 level, facing somites 3/4 (Fig. 2G). NCC derived from r8 normally migrate into BA4-6 and express *Hoxa-2*, *Hoxa-3* and *Hoxb-4*. The cells derived from the graft migrated normally and

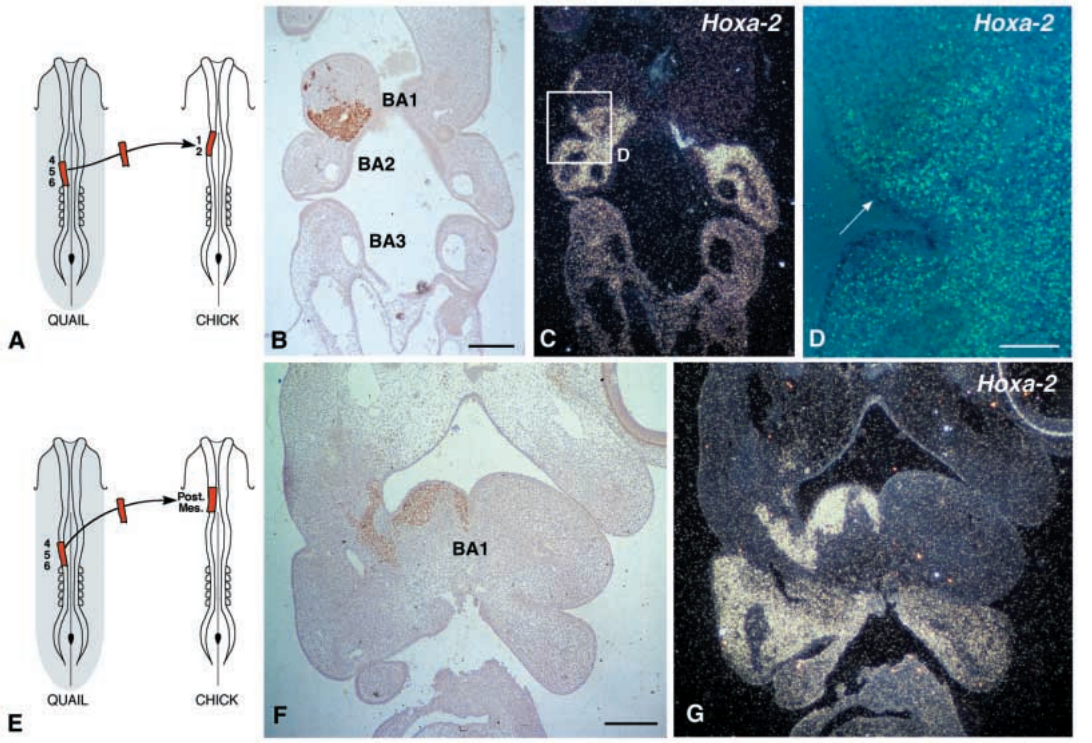

Fig. 3. *Hoxa-2* expression in anteriorly transposed r4/r6 NCC. (A) Unilateral transposition of r4/r6 quail neural fold to the position of r1/r2 of a chick host. The chimera shown in B-D is cut frontally at E4. (B) The localization of quail cells in the posterior part of BA1 using QCPN mAb. (C) *Hoxa-2* expression is shown on an adjacent section. *Hoxa-2* is maintained in NCCs of BA2 on the grafted side whereas it is not expressed in the opposite, control side. The boxed area is enlarged in D and photographed under epifluorescence in order to show both *Hoxa-2* expression and histologic landmarks. The arrow shows the absence of *Hoxa-2* in the ectoderm of BA1. (E) Unilateral transposition of r4/r6 quail neural fold to the position of the posterior mesencephalon of a chick host. The chimera shown in F and G is cut frontally at E4. The localization of quail cells in the rostral part of BA1 is shown in F using QCPN mAb. (G) *Hoxa-2* is maintained in cells of quail origin. Bars, 200 μm in B,C,F,G and 50 μm in D.

colonized BA4 to BA6 (Fig. 2H; n=3). In two embryos, it was evident the remaining chimera, the cells were too sparsely distributed for their expression of the gene to be distinguished using radioactive in situ hybridization.

Taken together, these results show that the expression of *Hox* genes is not induced in caudally transplanted NCC.

P→A transpositions

We next transplanted neural fold segments from posterior to anterior. Fragments of neural fold corresponding to r4/r6 were transplanted unilaterally at r1/r2 level (n=6 at E3 and n=2 at E4; Fig. 3A), or at the level of the posterior mesencephalon (n=1 at E2, n=8 at E3 and n=5 at E4; Fig. 3E) or at the prosencephalon (n=2 at E2).

In 6/8 cases examined at E3 (n=5) and E4 (n=3), the transplantations at r1/r2 level resulted in the colonisation of the posterior region of BA1 (Fig. 3B). In the two remaining embryos, the cells were only found in the anlage of the trigeminal ganglion and not in the branchial arch. Transplantation of r4/r6 at the level of the posterior mesencephalon yielded cells localized mainly in the trigeminal ganglion and in the trochlear nerve (n=14/14) but not in the mesenchyme of BA1 except for some dispersed cells found in mandibullary and maxillary buds (n=11/14). In fact, BA1 was filled with large numbers of chick host cells. In three cases only, analyzed at E4, graft-derived cells were numerous in the anterior part of BA1 (Fig. 3F). Cells derived from the quail graft were sometimes found surrounding the eye (n=5/14). Transplantation to the diencephalic level resulted in the migration of grafted cells around the eye and in the nasofrontal bud (n=2 observed at E3).

In all cases, quail cells retained their capacity to express *Hoxa-2* in this ectopic position (Fig. 3C,G). These results show that, when they begin to migrate, the AP identity of NCC revealed by their *Hox* gene expression is already fixed and cannot be altered by environmental signals.

(2) Expression of *Hox* genes by the surface ectoderm does not result from an induction by NCC

The experiments described above provided an experimental paradigm to test the hypothesis put forward by Hunt et al. (1991) according to which the NCC might be responsible for the induction of *Hox* genes in the surface ectoderm of the BAs. When the neural fold of r1/r2 is transplanted to the r4/r6 level, the NCC migrate into BA2 instead of BA1 (see above). Although they remain *Hoxa-2* negative, the surface ectoderm of BA2 expresses *Hoxa-2* normally, in its posterior part (Fig. 2B,C). Thus, expression of this *Hox* gene by the ectoderm does not depend on an induction by NCC.

In the reverse experiment (graft of r4/r6 neural fold to the level of the r1/r2), where NCC expressing *Hoxa-2* migrate into BA1, they fail to induce expression of this gene in the surface ectoderm (Fig. 3A-D).

Hox gene expression in the surface ectoderm is thus independent of NCC.

(3) R4/r6 NCC are unable to differentiate into a lower jaw in the context of BA1

The experiments aimed at leading r4/r6 NCC to migrate to BA1 were carried out according to two different paradigms.

Ectopic migration of r4/r6 neural crest after bilateral ablation of mesencephalic to r3 fold

In our previous work (Couly et al., 1996), we have demonstrated that the bilateral ablation of definite segments of the neural fold at the rhombencephalic level is followed by the longitudinal migration along the AP axis of NCC exiting from the neural folds adjacent to the excised area. This regulation is able to restore the colonization of the BAs corresponding to the transverse level of the excision. Moreover, NCC migrating either rostrocaudally or caudorostrally were shown to retain their original *Hox* code. The present experiment was devised to determine whether the prosencephalic neural crest can migrate in a rostral-to-caudal direction and to participate in the regulation of BA1 structures.

The neural fold was excised bilaterally from the pro-mesencephalic constriction down to the level of the presumptive limit between r6 and r7 in a chick embryo at the 5-somite stage. Then the r4/r6 neural folds from a stage-matched quail were orthotopically implanted on both sides (Fig. 4A). It was found that NCC exiting from the diencephalon do not significantly change their migration pathway. They migrate normally around the optic vesicles and rostrally to form the frontal bud. In contrast to those of the mesencephalon and rhombencephalon, the prosencephalic NCC do not migrate rostrocaudally and do not participate in the regulation of the deficiencies resulting from excision of the mesencephalic neural crest.

In contrast, the quail NCC exiting from the transplanted r4/r6 neural folds invaded BA2 and BA3, thus following their normal fate, but also progressed in a caudorostral direction to reach BA1 (Fig. 4B,C). This pattern of migration was observed in 2 out of 4 cases at E3 and in 2 cases at E4. In this experiment, *Hoxa-2*-expressing NCC migrated to BA1 (data not shown).

In view of the results obtained by genetic manipulations in the mouse embryos by Rijli et al. (1993) and Gendron-Maguire et al. (1993) where the authors showed that *Hoxa-2* null

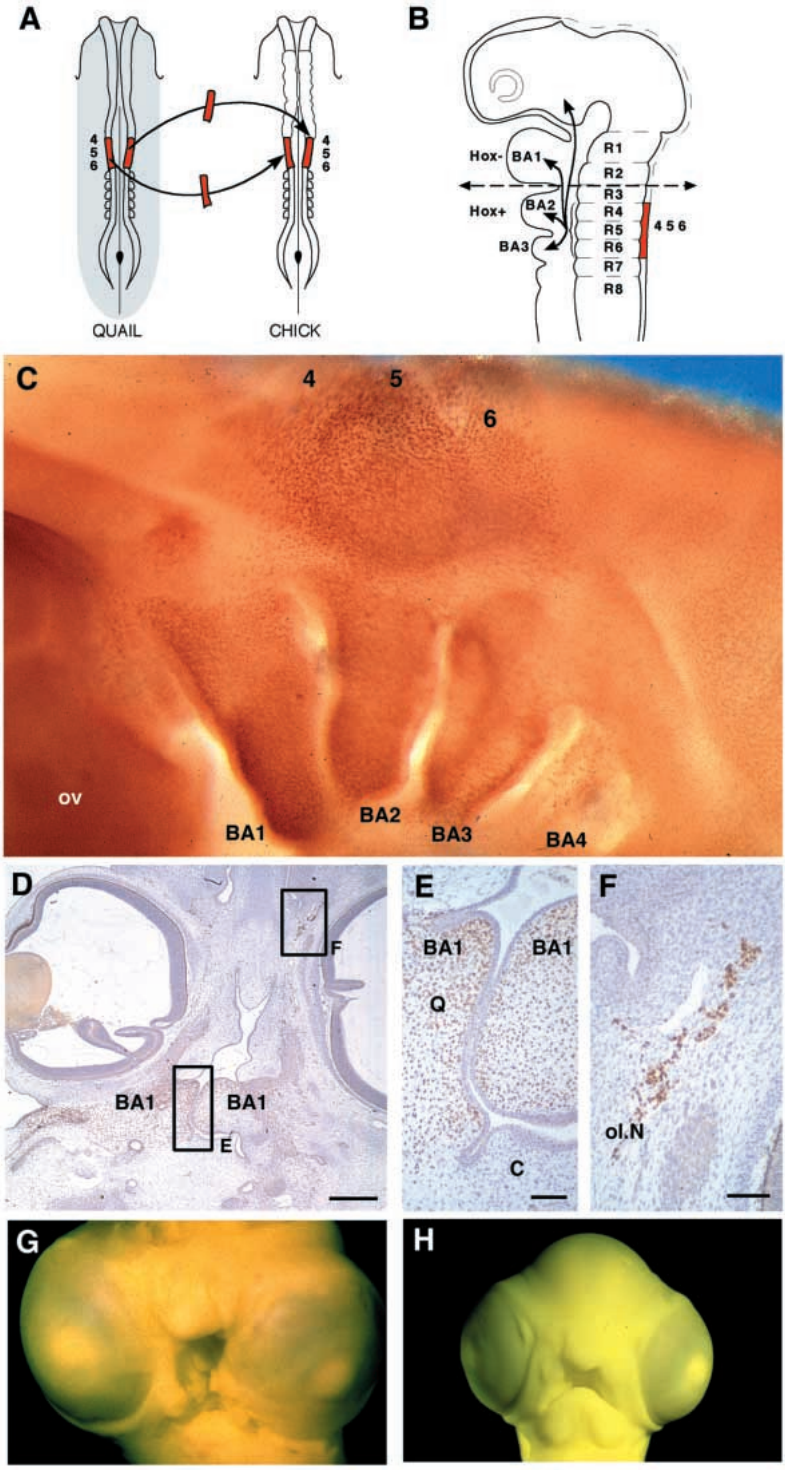

Fig. 4. Ectopic migration of r4/r6 neural crest after bilateral ablation of the neural fold from the diencephalic-mesencephalic constriction down to r6 inclusively followed by the isotopic graft of r4/r6 quail neural folds. (A) The quail r4/r6 neural folds were bilaterally and orthotopically grafted in r4/r6 position after bilateral excision from the diencephalic to r6 fold inclusively. (B) Arrows represent NCC migration from the grafted r4/r6 neural fold. The limit between *Hox*-expressing (*Hox*+) and *Hox*-non-expressing (*Hox*–) is indicated. (C) Whole-mount QCPN-Mab immunocytochemistry showing r4/r6 grafted NCC (brown spots) at E4 close to the optic vesicle (ov) and in BA1 to BA4. (D-F) Frontal sections of a chimeric E7 embryo where the quail (Q) NCC have migrated into BA1 without undergoing any chondrocytic differentiation into Meckel's cartilage. Boxed areas are magnified in E and F. (F) The Schwann cells of olfactory nerve (ol.N) are of quail origin. (G) Face of an E7 embryo showing the complete absence of a lower jaw. A normal embryo is shown for comparison in H. Bars = 300 μm in D and 50 μm in E,F.

mutations resulted in the duplication of the lower jaw skeleton at the expense of BA2 NCC, it seemed interesting to explore the effect of a 'gain-of-function' experiment in which BA1 NCC would be led to express *Hoxa-2* by embryonic manipulation in the avian embryo. We thus decided to allow the embryos subjected to this operation reach E7 and E9.

In the E7 and E9 embryos, it was found that the lower jaw did not develop (n=5; Fig. 4G,H). At E9, the Meckel's cartilage, the primordia of membrane bones and the tongue skeleton were absent (n=3; Fig. 4D,E). Histological observation of the embryos at E7 also revealed the presence of quail cells in the olfactory nerves (Fig. 4D,F), the maxillary and mandibulary branches of nerve V, the trigeminal ganglion, the connective tissue associated with muscles and the dermis. The parts of the hyoid bone that are normally derived from r4-r6 were of quail origin (e.g. rostral basibranchial, ceratobranchial and epibranchial) but the entoglossum and basihyal, which are derived from the neural crest corresponding to the level of the mesencephalon to r3 (see Fig. 1C), were absent. Thus, the NCC of r4/r6 origin migrated into BA1 but did not form the corresponding skeletal structures. They participated in the formation of those parts of the hyoid bone that normally descend from *Hox* gene-expressing NCC (see the fate map of neural crest, Fig. 1B).

In this experiment, the only NCC left in situ rostrally corresponded to the posterior diencephalon. These cells migrated to their normal sites: around the eyes where they formed the sclerotic, the choroid membrane and the nasofrontal bud. Cells of posterior diencephalic origin did not contribute to mandibulary skeletal structures of the face.

We next decided to test the capacity of the quail mesencephalic NCC to migrate to BA1 and to generate lower jaw structures if they were grafted in r4/r6 and then led to colonize BA2 in the same experimental paradigm. After bilateral removal of the neural fold from the pro-mesencephalic constriction down to the r6/r7 presumptive limit, the r4/r6 neural folds were replaced bilaterally by the neural fold from the posterior or anterior half of the mesencephalon or by the neural fold of r1/r2 of a quail embryo (Fig. 5A). The embryos were examined at E7 (n=4) and at E9 (n=2) in the case of the mesencephalic graft and at E7 (n=4) for r1/r2 grafts. In all cases, a normal lower jaw and tongue were generated (Fig. 5F,G). In these embryos, Meckel's cartilage, entoglossum and basihyal were entirely made up of quail cells (Fig. 5C,E). Ceratobranchial, epibranchial and basibranchial were mostly of chick origin although quail cells forming chondrocytic clusters of various sizes regularly contributed to the ceratobranchial (Fig. 5C,D). In addition, ectopic nodules of cartilage were found in BA2. There was no duplication of the lower jaw in BA2.

The conclusion from this series of experiments is that the facial skeleton (maxillary, mandibulary, anterior part of the hyoid bone) can be generated equally well by NCC from either the posterior or the anterior mesencephalon as well as from the neural fold of r1 and r2. In contrast NCC from a more posterior origin, i.e. r4-6, turned out to be unable to yield skeletal structures in the context of BA1.

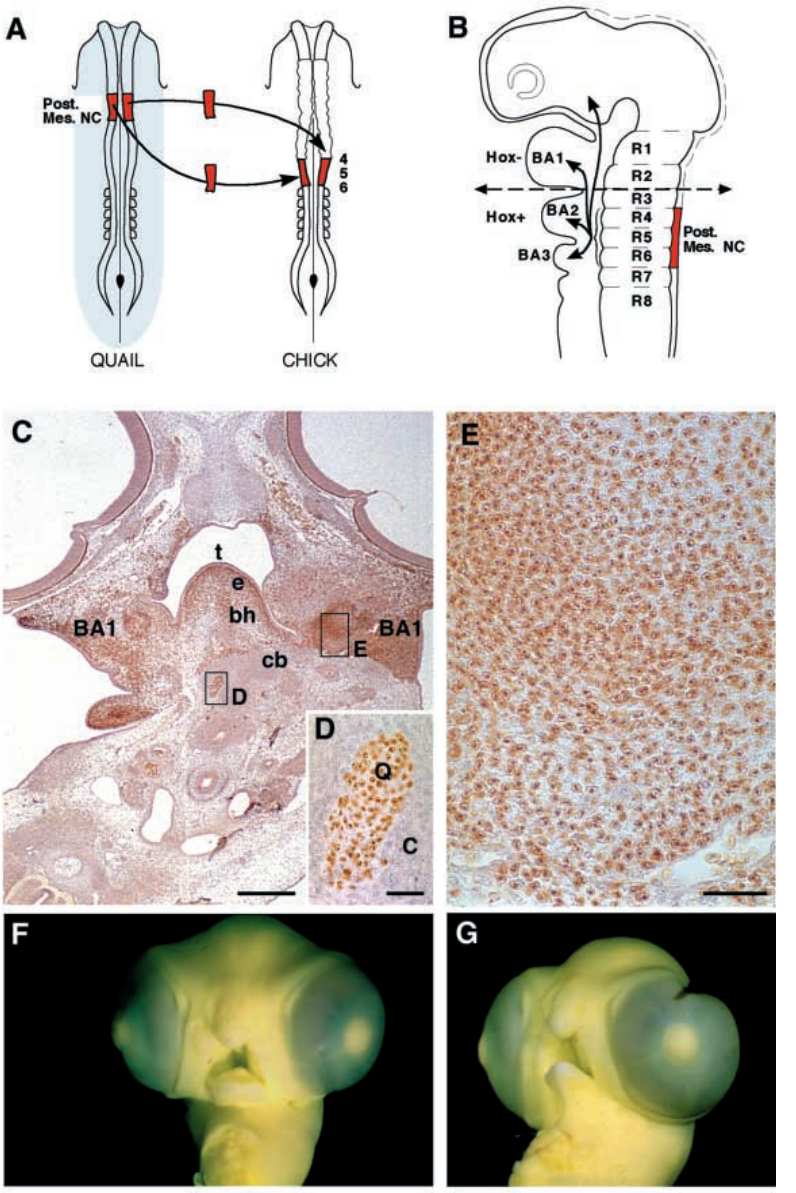

Fig. 5. Rostral migration and differentiation of mesencephalic NCC grafted into r4/r6 after bilateral ablation of diencephalic to r6 endogenous neural fold. (A,B) The posterior mesencephalic neural fold (Post. Mes. NC) was heterotopically and bilaterally grafted in r4/r6 position after bilateral excision from the limit between diencephalon and mesencephalon down to r6. (B) The Post. Mes. NCC invade BA3, BA2, BA1 and the periocular region as did r4/r6-derived NCC in the experiment represented on Fig 4. (C) Frontal section of an E7 chimeric embryo, showing quail cells (brown) forming the mesenchyme of the tongue (t), of BA1 and the entoglossum (e) and basihyal (bh) as well as the Meckel's cartilage (enlarged in E). (D) A chondrocytic cluster of quail cells origin (Q) is located in the ceratobranchial cartilage (cb). (F,G) The facial gross anatomy of two E7 chimeric embryos is normal and the lower jaw has developed. Compare to the control in Fig. 4H and to Fig. 4G devoid of lower jaw. Bars, 200 μm in C and 25 μm in D,E.

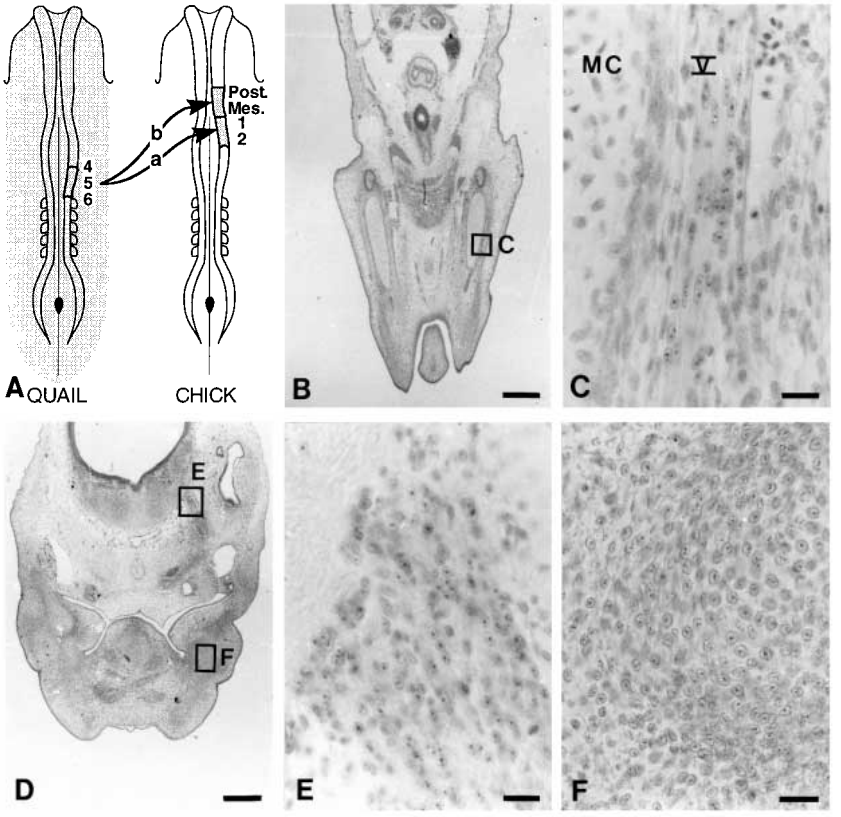

Fig. 6. Transposition of r4/r6 neural fold to r1/2, and to the mesencephalic level. (A) The neural fold was transplanted from r4/r6 to the r1/2 level (a), or to the posterior mesencephalon (b). (B,C) After transplantation into r1/2, a transverse section at E9 shows that the glial cells of the mandibular nerve (V) are of quail origin whereas chondrocytes of the Meckel's cartilage (MC) are of host origin. (D-F) After transplantation of r4/r6 to the posterior mesencephalic level, a transverse section at E7 shows that the root of the trigeminal ganglion is of the quail type (magnified in E) and that the Meckel's cartilage derived from host cells (magnified in F). Bar, 2.5 mm in B and D, 20 μm in C,E,F.

One characteristic that distinguishes these two regions of the neural crest is that the former does not express *Hox* genes whereas the latter does.

Transposition of r4/r6 to r1/r2 and to the mesencephalic levels

To further explore the capacity of r4/r6 NCC to regulate the deficiencies resulting from the ablation of the cells that normally colonize BA1, we transposed r4/r6 neural fold cranially by implanting it to either the posterior mesencephalic or the r1/r2 levels as already described above but, in this case, the embryos were allowed to reach E7 or E9 and their facial skeleton was examined (Fig. 6A). According to the above described results, we expected that the embryos subjected to this experiment would have deficiencies in their lower jaw apparatus. In fact, these embryos, examined at E7 (n=4 for grafts at the mesencephalic level) and E9 (n=4 for grafts at the r1/r2 level; Fig. 6B,D), had apparently normal lower jaws. Sections through these chimeras revealed that the chondrocytes of the skeleton were of host origin (Fig. 6B-D,F). Quail cells were abundant in the proximal region of the trigeminal ganglion and nerves (Fig. 6C,E) and sparse in the ciliary ganglion. Some dermal cells were also of graft origin in BA1. This was not a result of migration since quail cells were found in BA1 at E3 and E4 as already mentioned (Fig. 3B,F,G). This was not due either to an inability of these r4/r6 NCC to give rise to chondrocytes since these crest cells contribute to the posterior part of the hyoid bone in normal development.

We conclude that r4/r6 NCC grafted either at the level of r1/r2 or at the level of the caudal mesencephalon do not produce skeletal structures in the environment of BA1. Moreover, in this experimental design where the mesencephalic neural fold is still (partly or completely) present, mesencephalic NCC compensate for the inability of their r4/r6 counterparts to differentiate into bone and cartilage. It is noticeable that NCC originating from posterior rhombencephalic levels are able to contribute to the neural and connective tissue derivatives of the neural crest of BA1.

(4) Patterning capacities of the BA1 NCC revisited

It has been demonstrated by Noden (1983) that transplantations of the neural crest 'rostral to the midmetencephalic' boundary, which normally migrate to BA1, to the otic levels of the neural axis resulted in the duplication of the lower jaw skeleton and to the formation of a supernumerary beak and auditory pit. These experiments were performed using quail-to-chick combinations at stages ranging from 9^- to 9HH for donors and 9^+HH for recipients (about 6- to 8-somite stage).

In our exploration of the regulatory capacities of the cephalic neural crest, we decided to investigate the precise developmental fate of the neural fold that normally colonizes BA1 after posterior transplantation.

After the unilateral transposition of the posterior mesencephalic or r1/r2 neural folds to the r4/r6 level (Fig. 7A), the embryos were examined at E7 (n=7 for the mesencephalic transplants and n=8 for the r1/r2 transplants) and at E9 (n=2 for the mesencephalic grafts). Examination of embryos on transverse serial sections stained with the QCPN-mAb revealed that quail cells participated in all cases in the formation of the entoglossum and the basihyal, in accordance with the observation reported above that some of these cells migrate more rostrally than r4/r6 NCC, i.e. into BA1. They also

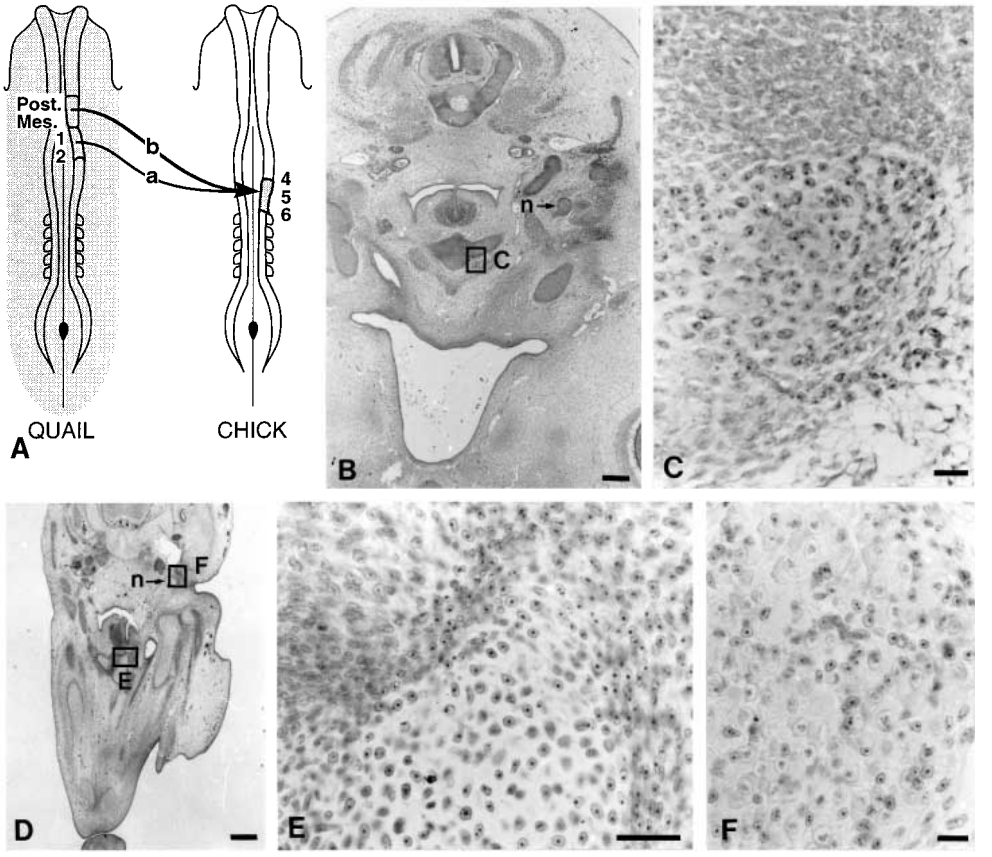

Fig. 7. Transposition of r1/r2 or the posterior mesencephalic neural fold to r4/r6 level. (A) Posterior transplantation of r1/r2 neural fold into r4/r6 (a). (B) Transverse section at E7 of a chimera subjected to r1/r2 transplantation shows clusters of quail chondrocytes in the ceratobranchial cartilage (magnified in C) and ectopic nodules of cartilage of quail origin (n). (D) Caudal transplantation of posterior mesencephalic neural fold into r4/r6 (A,b): transverse section at E9 shows quail cells in the ceratobranchial cartilage (magnified in E) and in the jugular (IX) ganglion (magnified in F). Nodules of cartilage neighbouring the jugular vein are indicated (n). Bars, 100 μm in B, 10 μm in C and F, 200 μm in D and 20 μm in E.

contributed in certain cases (n=5) to the formation of the ceratobranchial (Fig. 7B-E) in which the quail cells were clustered. Schwann cells were found in the acousticofacial and glossopharyngeal ganglia and nerves (Fig. 7F). In addition, ectopic pieces of quail cartilage were regularly observed in BA2 neighbouring the normally formed ceratobranchial (Fig. 7B,D). Quail cells were present in the dermis and in the normally patterned muscles of the second branchial arch. Thus, in these experiments, transplantation of the neural fold alone did not result in the duplication of the lower jaw as described by Noden (1983).

A contribution to the hyoid bone was also observed when r1/r2 neural fold was grafted at the level of r5/r7 rather than r4/r6 (Fig. 9G). Dispersed or clustered quail cells were found in the ceratobranchial (n=6/7), the epibranchial (n=3/7), the basibranchial (n=5/7) and the basihyal (n=2/7). The shape of this bone was altered only in three cases. As in the previously described experimental series, ectopic pieces of superficially located cartilage were found but with a lower frequency: in about half of the embryos examined (3 cases out of 7). In normal embryos, *Hoxa-3* is still expressed at E10 in the cartilage pieces derived from the posterior BAs. *Hoxa-3* transcripts were abundant in the perichondrium and also, but to a lower level, in the differentiated cartilage, which is derived from the neural fold of r5/r8. Accordingly the ceratobranchial, epibranchial and basibranchial, which are derived from r5/r8 neural fold, expressed *Hoxa-3*. The entoglossum, basihyal and Meckel's cartilage, which originate from NCC exiting from the mid-brain and r1/r2 levels, are *Hoxa-3*-negative. When r1/2 neural fold was grafted to the r5/r7 level, quail cells participated in the ceratobranchial and epibranchial cartilages without acquiring *Hoxa-3* expression (Fig. 9G,H). Thus the capacity to form the posterior part of the hyoid bone does not require *Hoxa-3* expression by NCC.

The next step consisted of similar transplantations of the neural fold unilaterally but, in this case, it was associated together with the lateral wall of the neural tube (Fig. 8B). The embryos were collected and examined at E7 (n=4 for transplantation of r1/r2 to r4/r6), at E9 (n=2 for transplantation of posterior mesencephalon to r4/r6 and n=2 for transplantation of r1/r2 to r4/r6) and at E14 (n=1 for transplantation of r1/r2 to r4/r6). In all these cases, the embryos exhibited a typical duplication of the cartilages of BA1, i.e. Meckel, articular and quadrate cartilages (Fig. 8A,C-F) as previously described by Noden (1983) in the avian embryo. In addition to the duplicated bones, the grafted quail cells also participated in the formation of those parts of the hyoid bones normally derived from r4/r6. Therefore, mesencephalic and r1/r2 NCC can differentiate into a lower jaw in the context of BA2 if they are transplanted posteriorly together with the neural tube but not if they are transplanted alone and therefore associated with a *Hox*-expressing neuroepithelium.

(5) Capacity of r1/r2 NCC to contribute to the cardiovascular system and to the glands of the neck

The mesencephalic and r1/r2 neural folds were implanted unilaterally at the r5/r7 levels corresponding to the origin of the cells colonizing BA3 and BA4-6 (Fig. 9A). In this case, in addition to the contribution to the hyoid bone described above, quail NCC participated in neural structures (Fig. 9C; the

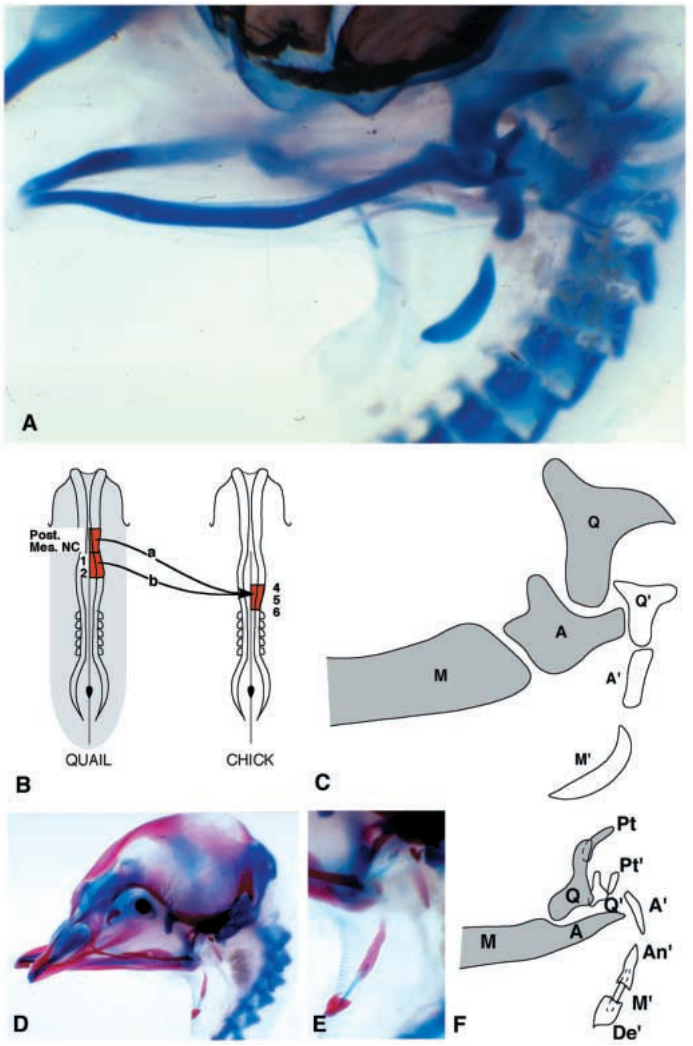

Fig. 8. Transposition of r1/r2 or of the posterior mesencephalic neural fold + tube to r4/r6 level. (A) Side view of an Alcian blue-alizarin red stained E9 chimera, in which the posterior mesencephalic quail neural tube and neural crest where grafted into r4/r6 of a five somite stage host according to the protocol schematized in B (experiment a). The lower jaw is schematized in C. (A,C) At E9, the skeleton of the first branchial arch (M, Meckel's cartilage; A, articular; Q, quadrate) is duplicated in the second branchial arch (M′, A′, Q′). (D,E) Side view of an Alcian blue-alizarin red stained E14 chimera, in which the r1/r2 quail neural tube and neural crest were grafted into r4/r6 of a five somite stage host (experiment b). The lower jaw is schematized in F. The first branchial arch skeleton (M, A, Q, Pt: pterygoid) is duplicated in BA2 (M′, A′, Q′, Pt′, De′: dentary, An′: angular).

nodose ganglion and Schwann cells of nerve X), and gave rise to the musculoconnective component of the aortic arches (common carotid artery, pulmonary artery, aortic trunk including the sigmoid valves; Fig. 9B,E). They also contributed to the glandular and lymphoid structures of the neck (the thymus, parathyroid glands, carotid body and ultimobranchial body; Fig. 9C,D) and migrated to the gut (see Le Douarin, 1982; Fig. 9C).

This therefore indicates that, when transplanted to this posterior level of the hindbrain, the anterior NCC are able to respond to the environmental patterning cues of the posterior BAs leading them to develop into normal posterior components of the cardiovascular structures.

We then decided to explore the regulatory capacities of the *Hox*-negative and *Hox*-positive NCC when they are transposed within these respective domains.

(6) Transposition of neural fold segments within the *Hox*-negative domain of the neural primordium

The limit between the *Hox*-negative and *Hox*-positive domains of the NCC is located at the level of the r3/r4 boundary. Transpositions of neural fold fragments were then systematically carried out within these two domains, respectively.

Fragments of the neural folds corresponding to the r1/r2 and diencephalic levels were exchanged. When r1/r2 was transposed unilaterally to the diencephalic region (n=2 at E6.5; n=3 at E8.5), the NCC of quail origin migrated in the nasofrontal bud, around the eye and yielded normal sclerotical and choroidal structures. They also differentiated into Schwann cells of olfactory nerves and of the optic chiasma and optic nerves. They formed normally leptomeninges and pachymeninges in the prosencephalon and chondrocytes in the nasal cartilage. When the diencephalic neural fold was transposed unilaterally to the r1/r2 level (n=3 at E6.5; n=4 at E8.5) the quail cells formed the proximal part of the Meckel's cartilage as well as the quadrate and articular cartilages, that is, similar to the normal fate of r1/r2-derived cells.

Transposition of neural fold segments within the *Hox*-positive domain of the neural primordium

Segments of the neural folds were exchanged as follows : the r4/r5 neural folds were transposed unilaterally to the r6/r7 level. The embryos were observed at E6 (n=5) and at E8 (n=2). Quail cells were found in neural derivatives of the neural crest and in the ceratobranchial, in the epibranchial and in the rostral part of basibranchial cartilages. No abnormalities were observed in neural and connective tissue derivatives.

When the neural folds of the r6/r7 were grafted unilaterally to the r4/r5 level (n=4), the embryos were examined at E6 and at E8.5 (n=2). The graft contributed cells to the ceratobranchial, the rostral part of the epibranchial cartilages but not to the basibranchial.

Taken together these results confirm that the NCC are interchangeable inside *Hox*-negative or *Hox*-positive domains.

DISCUSSION

In the experiments described here, we have challenged the migratory behaviour, the capacity to express *Hox* genes and the fate of NCC when they are derouted by various microsurgical procedures applied to chick embryos at 5- to 7-somite stages in vivo. Fragments of quail neural folds were grafted into chick embryos either heterotopically or homotopically after removal of large fragments of the rhombencephalic and mesencephalic chick neural fold. In the latter circumstances, NCC migrate not only transversally to reach their normal sites of arrest but they also compensate for the deficiencies due to the ablation by migrating longitudinally along the AP axis to colonize adjacent BAs (Couly et al., 1996).

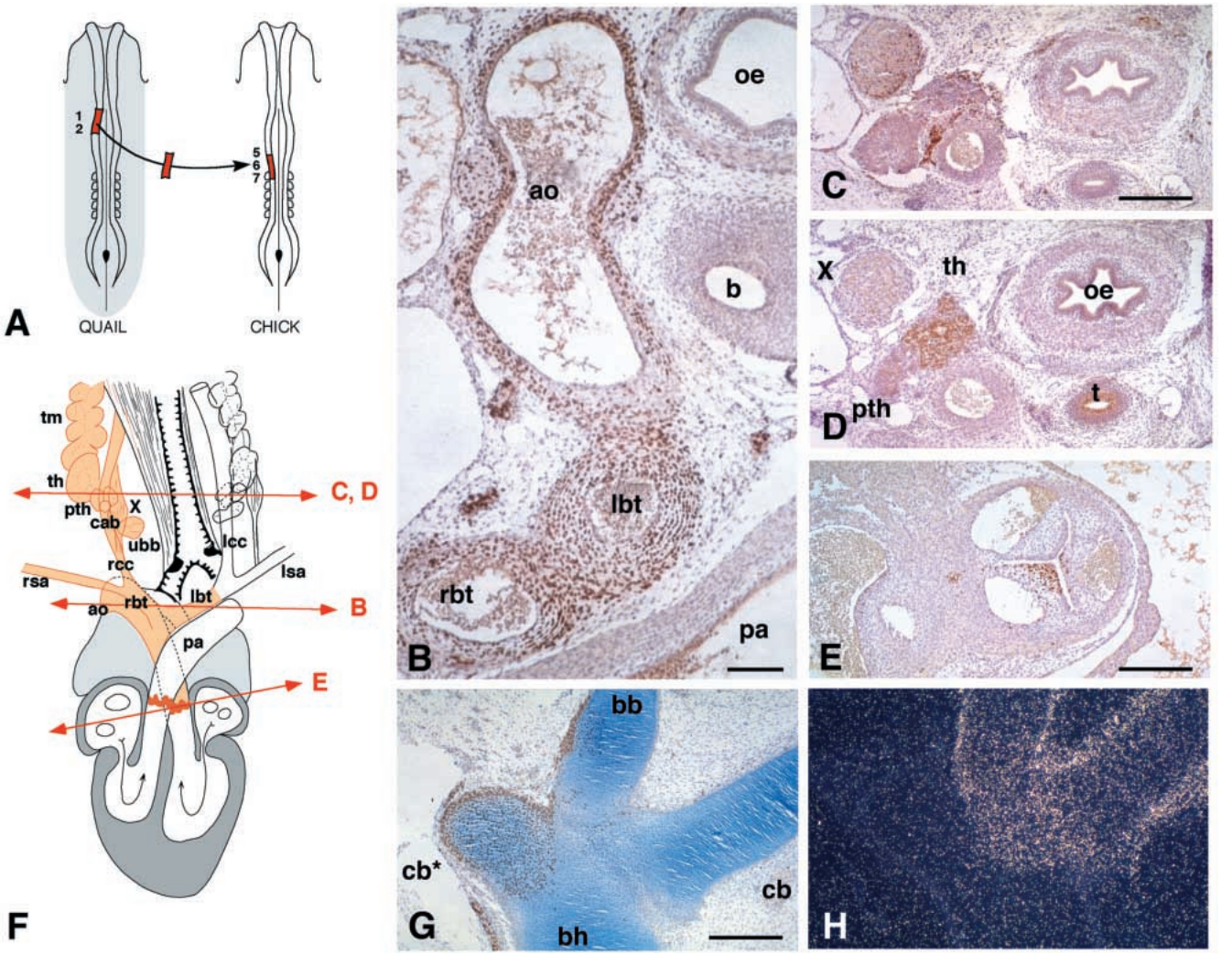

Fig. 9. Contribution of NCCs of r1/2 origin to r5/7-derived structures. (A) Unilateral transposition of r1/r2 quail neural fold to the position of r5/r7 of a chick host. The chimeras shown are cut transversally at E10. (B,C,E,G) The localization of quail cells using QCPN mAb. They contribute to the muscular layers of the blood vessels of the conotruncus on the side of the graft (B), to glial cells of the nodose ganglion (X), connective cells of the thyroid (th) and parathyroid (pth) to nerve plexus of the oesophagus (oe) (C). (D) The localization of the thyroid is shown in a section adjacent to C using TTF-1 antibody. This antibody also labels the epithelium of the trachea (t). (E) Quail cells of the graft also contribute to the sigmoid valves. The contribution to the glands of the neck, the blood vessels and ganglia is summarized in brown in F, as well as the planes of section. (G) Section at the level of the hyoid cartilage. The cartilage is shown using Alcian blue and quail cells in brown using QCPN mAb. Quail cells contribute to the ceratobranchial cartilage on one side (cb*) and to a lower extent to the basibranchial (bb). (H) Adjacent section to G hybridized with *Hoxa-3* probe. *Hoxa-3* is normally expressed in the ceratobranchial and basibranchial but not the basihyal. Quail cells of r1/r2 origin form a ceratobranchial without acquiring *Hoxa-3* expression. ao, aorta; b, bronchus; bb, basibranchial; bh, basihyal; cab, carotid body; cb, ceratobranchial; lcc, left common carotid; pa, pulmonary artery; lsa, left subclavian artery; rcc, right common carotid; rsa, right subclavian artery; oe, oesophagus; pth, parathyroid; tm, thymus; th, thyroid; ubb, ultimobranchial body; X, nodose ganglion. rbt, right branchial trunk, lbt, left branchial trunk. Bars, 200 μm.

Migratory behaviour of ectopically transplanted NCC

Our experiments first show that ectopically transplanted neural fold gives rise to NCC which, with a few exceptions, migrate like the cells normally exiting from the level to which they were implanted. This observation is not obvious since NCC of different AP levels express different sets of genes whose products are involved in pathfinding. It was recently shown that EphA4 and EphB1 are expressed in the third and third plus fourth arches, respectively (Smith et al., 1997). One of their ligands, ephrin-B2 is expressed in the mesoderm of the second BA. When EphA4 and EphB1 are dominantly inactivated in Xenopus, third arch NCC migrate ectopically into BA2. It was interpreted that these receptors would prevent the mixing of NCC of different arches by responding to repulsive cues. In our experiments, such a mechanism could be responsible for the fact that, when r1/r2 NCC are grafted into r4/r6, although they populate BA2 according to their new position, they, in several cases, extend their migration up to BA1 where they participate in the formation of the basihyal and entoglossum. This may be due to the fact that, in this kind of graft, the onset of the boundary that normally exists between BA1 and BA2 NCC precursors is not established. Our experiments show, in

addition, that the mesoderm lateral to r4/r6 is permissive to the migration of r1/r2 NCC and vice-versa. Therefore, in birds, ephrins define preferential but not absolute migration pathways. Differences in ephrins, according to the level of the neural axis considered, also account for the formation of clusters of cells within the host tissues rather than a random mingling of host and donor cells. The observation that, after ablating neural folds from the diencephalon down to r4, r4/r6 NCC undergo an anterior migration, suggests that the Ephrin/Eph system is mediating repulsion between the NCC themselves rather than between NCC and mesoderm.

As a whole, the experiments described here in which a large excision of the neural fold in the recipient embryo (from r4 up to the diencephalic-mesencephalic constriction) was performed, showed that the tendency to regulate the deficiencies consecutive to the excision largely overcomes the cues that normally restrict NCC to migrate in transversally distributed streams of cells.

Hox gene expression in NCC seems to be irreversibly determined prior to migration

We show here that, when NCC are led to migrate to an ectopic site, they maintain the *Hox* code that they express in their original position. This was observed at early stages, E3 and E4, and later at E10 (see Fig. 9G,H). Accordingly, posteriorly transposed *Hox*-negative cells do not become positive and vice-versa. Hence, normally patterned NC derivatives may, in these experimental embryos, exhibit *Hox* gene expression patterns that are abnormal.

Experiments in which the initial position of rhombomeres along the AP axis was modified were previously carried out by Prince and Lumsden (1994) and Saldivar et al. (1996). These transpositions, however, were performed over very short distances. Thus, Prince and Lumsden (1994) transposed r2 into r4 and observed that *Hoxa-2* expression was not gained in r2-derived NCC. Similarly, Saldivar et al. (1996) reverted r4/r5 and r4/r6 along the AP axis and showed that r5 and r6 NCC did not loose *Hoxa-3* expression and r4 derivatives did not gain it. These two sets of experiments can thus be interpreted, like those described here, as an absence of plasticity of NCC at least during their early migration. In addition to the experiments of Prince and Lumsden (1994) and Saldivar et al. (1996), which consisted of transplantation of the neural fold associated to the neural tube, we show here that, when the neural fold is transplanted alone, the subjacent neural tube does not modify *Hox* gene expression in NCC. Moreover, some discrepancy has to be mentioned between the present work and the experiments of Saldivar et al. (1996). The latter authors observed that, at the final steps of their migration, *Hoxa-3* expression by NCC was lost by r5 but not r6 NCC on their way to BA2. In our experiments, we never observed any change of *Hox* gene expression during migration. In particular, we did not notice a down-regulation of *Hoxa-2* in r4/r6 grafted either to r1/r2 or to the caudal mesencephalon. Therefore, the hypothesis put forward by Saldivar et al. (1996), according to which plasticity in *Hox* gene expression might exist only for the anteriomost rhombomere where a given *Hox* gene is expressed, seems likely. This would be the case of r3 for *Hoxa-2* and r5 for *Hoxa-3*. A later publication of Saldivar et al. (1997) suggests a second interpretation. These authors observed that, after ablating r5/r6, r4 and r7 cells were rerouted and filled up BA3. Rerouted r4 cells appeared to turn on *Hoxa-3*. This suggests that even rhombomere NCC can also change their *Hox* code. However, the regenerated BA3 mesectoderm was made up of 9/10th of r7 cells that normally express *Hoxa-3* and 1/10th of r4 cells. There could be a community effect resulting in the acquisition of *Hoxa-3* by the few r4-derived cells. This would explain why, in their rotation experiments (Saldivar et al., 1996), *Hoxa-3* is never turned on in r4 NCC. We designed our experiments in such a way to prevent this mixing of populations and massive invasions.

The absence of plasticity of the AP identity of NCC as recorded by *Hox* gene expression conflicts with our previous observation that another marker of AP identity in the hindbrain, the transcription factor *MafB/Kr*, can be upregulated in r4 NCC after grafting somites lateral to this rhombomere (Grapin-Botton et al., 1998). One explanation could be that segmentally expressed genes of different families do not follow the same regulatory rules. Alternatively, hindbrain NCC might have a plastic AP identity when they are still epithelial. In contrast, when they begin to migrate, either they lose this plasticity or they get out of range of the transforming signals. In the present paper, NCC begin to migrate a few hours after grafting, a time lapse that might not be sufficient for the signals acting in the neural fold to exert their effect considering the time necessary for healing of graft-to-host tissues.

Expression of *Hox* genes in branchial arch ectoderm does not depend on an induction by NCC

In their pioneering work describing the segmental expression of *Hox* genes in the various cell layers constituting a branchial arch, Hunt et al. (1991) suggested that, since *Hox* gene expression in the ectoderm takes place after the emigration of NCC, the latter might be responsible for inducing *Hox* gene expression in the ectoderm. In fact, our experiments show that, when *Hoxa-2*-expressing NCC are led to migrate into BA1, they do not induce *Hoxa-2* in the ectoderm. Conversely, *Hoxa-2* is normally expressed in BA2 ectoderm even if it is colonized by *Hox*-negative BA1 NCC. We conclude that the positional value in ectoderm, be it neural or epidermal in nature, is coordinated earlier and independently from the NCC. This supports the view put forward by Couly and Le Douarin (1990) according to which the ectoderm covering each branchial arch is part of a larger ‘segment’ designated as ‘ectomere’ which includes segments of the brain anlage (prosomeres or rhombomeres) and the corresponding neural fold yielding the NCC.

Expression of *Hox* genes and development of the lower jaw skeleton

Longitudinal migration from r4/r6 towards BA1 and transplantation of r4/r6 to r1/r2 or to mesencephalic levels show that r4/r6 NCC, while colonizing BA1, do not further develop into a lower jaw skeleton. Although they migrate at the appropriate level and are found filling up BA1 at E3-E4, these ectopic cells do not differentiate into BA1 cartilage and bones while they yield the neural derivatives corresponding to BA1. Thus, posterior *Hox*-positive NCC do not receive, in BA1, the appropriate cues to differentiate into skeletal structures in this environment.

Their inability to form a lower jaw is likely to be correlated with their expression of *Hox* genes. Recent experimental

evidence supports this view. Thus, ectopic expression of *Hoxa-1* following injection of *Hoxa-1* mRNA in ovo in zebrafish precludes the formation of the cartilaginous derivatives of BA1, i.e. Meckel's cartilage and palatoquadrate (Alexandre et al., 1996). Surprisingly, transgenic mice with widespread expression of *Hoxa-1* driven by the β-actin promoter were not described as having defects in jaw formation (Zhang et al., 1994). However, this work does not seem to have involved an analysis of the mice after E9.5, when skeletal pieces can be distinguished.

On the contrary, Noden (1983) showed that the mesencephalic and metencephalic (r1/r2) neural fold transplanted to a posterior (preotic) position yields NCC that migrate to BA2 and develop into an ectopic supranumerary lower jaw in this site. These results were confirmed in the present work. However, the NCC exerted such a patterning activity only if they were transplanted together with the subjacent neural tube. When the anterior neural fold was transplanted alone, there was no duplication of the jaw but merely ectopic nodules of cartilage in random locations. These results show that the BA1 NCC keep their original morphogenetic program provided that they remain under the influence of the corresponding level of the neural tube. Although not precisely indicated in Noden's article, one can assume that the graft of neural fold also included some neuroepithelium of the neural tube. The reason why the neural tube potentiates the patterning activity of NCC is unclear. The inability of the neural fold transplanted alone to yield an ectopic lower jaw cannot be explained by the induction of posterior *Hox* genes mediated by the subjacent neural tube: a *Hox*-negative neural fold above a *Hox*-positive neural tube remains negative and so do the NCC that it produces. Moreover, in the transposition experiments that yielded a duplication of the lower jaw the NCC did not express *Hox* genes and exited from a segment of neural tube which did not either. Therefore, r1/r2 and mesencephalic NCC colonizing BA2 were in the same situation, as far as *Hoxa-2* expression is concerned, as mouse embryos in which the *Hoxa-2* gene had been disrupted (Rijli et al., 1993; Gendron-Maguire et al., 1993). Similar to these findings in mice, the operated chicken showed a duplication of the lower jaw. Therefore it seems that, as proposed by these authors, *Hoxa-2* acts as an inhibitor of the intrinsic capacity of the mesencephalic and metencephalic neural crest to pattern a lower jaw. The gain-of-function experiments described above, in which *Hox*-positive NCC are led to colonize BA1 can be interpreted as if the inhibitory effect exerted by *Hox* genes prevents these NCC from proliferating and surviving if they are in the environment of BA1.

In contrast to this inadequacy of *Hox*-positive NCC in a *Hox*-negative environment, we found that transposition of the neural fold does not preclude the normal development of neural-crest-derived skeletal structures provided that it takes place within one or the other of these two cephalic neural crest domains. Thus, the r1/r2 neural fold transposed to the diencephalon contributes to normal structures characteristic of the site of the graft in the recipient and vice-versa. Moreover, exchanges between r4/r5 and r6/r7 domains do not result in malformations of the hyoid bone. Taken together these observations suggest that *Hox* gene expression in the hindbrain and associated NCC does not play a direct role in patterning the hypobranchial skeleton. It seems rather that expression of *Hox* genes by NCC is not compatible with the development of BA1 skeletal structures (whether they belong to the lower jaw proper or to the tongue skeleton : basihyal and entoglossum).

In the reverse situation where r1/r2 or mesencephalic NCC are transposed to r4/r6 or r5/r7 levels, we found that graft-derived cells contribute to the posterior part of the hyoid bone. This had already been mentioned by Noden (1983). We show here that this plasticity occurs without the acquisition of *Hoxa-3* expression by the effector NCC even during late development. It is interesting to note that, in *Hoxa-3*-null mice, morphogenesis of the posterior parts of the hyoid bone is deficient (Chisaka and Capecchi, 1991). This discrepancy might result from the fact that, in the chicken experiments, the tissue components other than NCC in the posterior BAs normally express *Hox* genes.

In conclusion, these experiments demonstrate that the cephalic neural crest exhibits a certain level of plasticity while also being endowed with patterning capacities. Along the cephalic neural axis, two domains can be distinguished in this respect: an anterior region corresponding to the posterior diencephalon, the mesencephalon and the metencephalon (i.e. r1, r2) in which no genes of the *Hox* clusters are expressed by NCC, and a posterior region corresponding to r3 to r8 inclusively in which *Hox* genes of the four first paralogue groups are expressed. When the NCC are transposed within these two respective domains, no abnormalities in facial and hypobranchial skeleton result. In contrast, *Hox* gene-expressing NCC cannot substitute for *Hox* gene non-expressing cells in construction of a lower jaw.

The epithelial and mesodermal environment of BA2, which is at the limit between the two domains and in which NCC expressing the most 'anterior' genes of the *Hox* clusters, is permissive for lower jaw skeleton formation if it is colonized by cells from the *Hox* gene non-expressing domain. Moreover, if *Hox* gene-expressing NCC are led, by embryonic manipulations, to colonize BA1, they fail to differentiate into cartilage and membrane bones thus leading to the absence of a lower jaw. The restrictions of the differentiating capacities of NCC in these two domains concern only the skeletal derivatives while a large level of plasticity is observed for the neural and connective cell types originating from the neural crest.

Our experiments support the contention put forward by previous authors (Rijli et al., 1993; Gendron-Maguire et al., 1993; Lohnes et al., 1995) who interpreted the duplication of the lower jaw skeletal apparatus resulting from the inactivation of *Hoxa-2* gene in the mouse as an indication that *Hox* genes, largely placed under the regulatory control of the morphogen retinoic acid (Lohnes et al., 1995; Mark et al., 1995), have played a major role in the diversification of the branchial arches during vertebrate evolution. According to this view, the BA1 phenotypic pattern would conform the most to an ancestral, basic type upon which modifications have been secondarily imposed in the course of vertebrate evolution.

We thank Françoise Viala, Francis Beaujean and Sophie Gournet for their help with the illustration, Chrystèle Guilloteau and Evelyne Bourson for typing the manuscript, and Marie-Ange Bonnin for technical assistance. We are indebted to Robb Krumlauf for *Hoxa-3* and *Hoxb-3* probes, Andrew Lumsden for *Hoxa-2* probe and Roberto

Di Lauro for TTF-1 antibody. The QCPN hybridoma was obtained from the Developmental Studies Hybridoma Bank (DHSB contract N01-HD-6-2915). This work was supported by the CNRS, Collège de France, and ARC.

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