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The regeneration of the cephalic neural crest, a problem revisited: the regenerating cells originate from the contralateral or from the anterior and posterior neural fold

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SUMMARY

The mesencephalic and rhombencephalic levels of origin of the hypobranchial skeleton (lower jaw and hyoid bone) within the neural fold have been determined at the 5-somite stage with a resolution corresponding to each single rhombomere, by means of the quail-chick chimera technique. Expression of certain Hox genes (*Hoxa-2*, *Hoxa-3* and *Hoxb-4*) was recorded in the branchial arches of chick and quail embryos at embryonic days 3 (E3) and E4. This was a prerequisite for studying the regeneration capacities of the neural crest, after the dorsal neural tube was resected at the mesencephalic and rhombencephalic level. We found first that excisions at the 5-somite stage extending from the mid-mesencephalon down to r8 are followed by the regeneration of neural crest cells able to compensate for the deficiencies so produced. This confirmed the results of previous authors who made similar excisions at comparable (or older) developmental stages. When a bilateral excision was followed by the unilateral homotopic graft of the dorsal neural tube from a quail embryo, thus mimicking the situation created by a unilateral excision, we found that the migration of the grafted unilateral neural crest (quail-labelled) is bilateral and compensates massively for the missing crest derivatives. The capacity of the intermediate and ventral neural tube to yield neural crest cells was tested by removing the chick rhombencephalic neural tube and replacing it either uni- or bilaterally with a ventral tube coming from a stage-matched quail. No neural crest cells exited from the ventral neural tube but no deficiency in neural crest derivatives was recorded. Crest cells were found to regenerate from the ends of the operated region. This was demonstrated by grafting fragments of quail neural fold at the extremities of the excised territory. Quail neural crest cells were seen migrating longitudinally from both the rostral and caudal ends of the operated region and filling the branchial arches located inbetween. Comparison of the behaviour of neural crest cells in this experimental situation with that showed by their normal fate map revealed that crest cells increase their proliferation rate and change their migratory behaviour without modifying their Hox code.

Key words: rhombomeres, mesencephalon, Hox gene expression, Hox code, neural crest cell migration, lower jaw skeleton, hyoid bone

INTRODUCTION

The neural crest is at the origin of a number of tissues and structures among which the peripheral nervous system, the melanocytes, some endocrine and paraendocrine cells and also the so-called mesectoderm which, in higher vertebrates, is restricted to the cephalic region of the neural axis. The mesectoderm also called ectomesenchyme was discovered more than one hundred years ago by Kastschenko (1888), Goronowitsch (1892, 1893) and Platt (1893, 1897) and was shown to form most of the skeleton, dermis and connective tissue of the vertebrate head (see Le Douarin, 1982 for a review and Couly et al., 1993). Patterning of the facial and hypobranchial structures involves morphogenetic events of a high level of complexity, the underlying mechanisms of which are far from understood. A great deal of attention has recently been directed to the rhombencephalon from which most of the facial and hypobranchial structures were shown to be derived via the neural crest (see Le Douarin, 1982; Thorogood, 1993; Langille and Hall, 1993). Neural crest cells were even proposed to have a role in patterning branchial derivatives when Noden (1983) heterotopically grafted the neural crest, which normally yields the 1st branchial arch (BA1) ectomesenchyme, to the position of BA2 or BA3 and so induced the duplication of the mandible.

The rhombencephalon was further shown to exhibit a transient metamerisation into true segmental units designated as rhombomeres (Fraser et al., 1990) which are characterized by the expression of a defined combinatoire of developmental selector gene homologs to the HOM-C genes of *Drosophila* and designated as Hox genes in vertebrates (Wilkinson et al., 1989; see also Krumlauf, 1994). Hox genes of the four first paralog groups are expressed in the rhombencephalon in domains whose anterior limits respect the rhombomere boundaries with generally a two segment periodicity. This generates

a Hox code for each transverse double segment in the mouse (Wilkinson et al., 1989; Krumlauf, 1994) and in the avian embryos (Kuratani and Eichele, 1993; Prince and Lumsden, 1994; Grapin-Botton et al., 1995).

The possible involvement of Hox genes in patterning rhombencephalic neural crest derivatives was substantiated by two sets of observations. First the fact that the neural crest cells and superficial ectoderm of each BA were reported by Hunt et al. (1991) to express the same Hox code as the rhombencephalon at the same transverse level. Second the demonstration that altering the Hox code by targeted mutations or abnormal spatial expression of a given Hox gene results in abnormal development of branchial arch derivatives. Thus, mice lacking *Hoxa-2* function exhibit malformations which have been interpreted as anterior transformation of BA2 into BA1 (Gendron-Maguire et al., 1993; Rijli et al., 1993). Moreover, null mutations of *Hoxa-1* and *Hoxa-3* cause branchial arch defects which cannot be considered as homeotic transformations (Lufkin et al., 1991; Chisaka et al., 1992; Carpenter et al., 1993; Dollé et al., 1993; Mark et al., 1993 for *Hoxa-1*; Chisaka and Capecchi, 1991; Manley and Capecchi, 1995 for *Hoxa-3*).

Recent work from our laboratory (Grapin-Botton et al., 1995) showed that heterotopic transplantations of rhombomeres from a rostral to a caudal position within the rhombencephalon result in the posteriorisation of the Hox code in the graft, followed by a complete homeotic transformation of the corresponding central nervous system (CNS) structures.

This showed therefore that the rhombomere-specific expression of a particular set of Hox genes is not autonomous but depends on positional cues unevenly distributed along the anteroposterior (AP) axis and therefore specific for a given transverse level. This also means that the neural epithelium possesses a considerable degree of plasticity even up to a developmental stage where the rhombomeres are well individualized (our unpublished observations).

These results led us to consider that the assumption that the neural crest has a role in patterning the hypobranchial structures and that this role might be mediated through the expression of the Hox selector genes should be further investigated. This was particularly timely since the problem of the capacity of neural crest cells to regenerate after ablation was recently raised in a series of articles (Scherson et al., 1993; Hunt et al., 1995; Sechrist et al., 1995). One of the conclusions reached in these works was that the lateral wall of the neural tube at the mes- and rhombencephalic level (even its most "ventral midline region"; Scherson et al., 1993, p.1056-1057) was able not only to regenerate normal hindbrain structures but also to produce neural crest cells which, on the basis of DiI labelling, "followed migratory patterns identical to neural crest cells in unoperated controls" (Scherson et al., 1993, p.1057). In view of such an impressive capacity of the neural epithelium to regulate large deficiencies occurring at a relatively late stage of the neurulation process, we decided to further examine this question and to use the cell marker provided by the quail-chick chimera system, to assess the extent and the source of the regeneration.

Although the migration routes taken by neural crest cells at the head level have already been determined (see Le Douarin, 1982 for a review of early work; Couly and Le Douarin, 1990; Lumsden et al., 1991; Couly et al, 1993) and their fate known in general terms, the derivatives of each single rhombomere has not been established in long term experiments allowing their contribution to neural, skeletal and connective tissues to be precisely demonstrated. We have thus mapped the derivatives of the posterior rhombencephalic and mesencephalic NC by the quail-chick substitution technique at 5-somite stage and looked at the presence of quail cells in the hypobranchial skeleton of the chimeras at E5-7. This gave us the necessary reference to interprete the outcome of ablation experiments in which more or less extended regions of the neural fold and neural tube were removed at the mid- and hindbrain presumptive level of 5-somite stage embryos. Ablation experiments of embryonic territories are well known to produce more or less extended regeneration processes from the remaining adjacent areas. This has been well illustrated in the past for the neural crest, the ablation of which has given rise to variable results going from no effect to more or less extended deficiencies (e.g. Stone, 1929; Hammond and Yntema, 1947, 1964; Le Lièvre, 1974; McKee and Ferguson, 1984; Langille and Hall, 1988a,b). Considering the transient existence of the neural crest and the highly dynamic character of this structure during a relatively short developmental period, these discrepancies are understandable since they may depend upon the AP length and the precise stage of the ablation. In recent experiments (Scherson et al, 1993; Hunt et al, 1995; Selleck and Bronner-Fraser, 1995) attempts have been made to take these parameters into careful consideration, and the demonstration that regeneration of neural crest derivatives takes place after its ablation at the early somitic stages is convincing. The problem remains as to what is (are) the source(s) of the regenerating cells.

The capacities of regenerating a neural crest from the lateral (or intermediate) and ventral neural tube were tested in appropriate quail-chick chimeras at 5-somite stage. These experiments failed to confirm the previous claims that the lateral and ventral neural tube can regenerate a neural crest. The considerable power of regeneration of the neural crest was however confirmed and shown to result from the extensive growth and invasive properties of the neural fold cells limiting rostrally and caudally the excised territories. Hox gene expression of the regenerating cells was shown at E3 and E4 to match their level of origin whatever their final position may be in the hypobranchial area.

MATERIALS AND METHODS

Experimental series

All the experiments were done in 5-somite stage quail and chick embryos. The fate map established in our previous work (Grapin-Botton et al., 1995) served as a basis to design all the operations described below, involving transplantations of defined regions of the cephalic neural primordium between quail and chick embryos. In this map (Fig. 2A) the boundaries of the presumptive territory corresponding to each rhombomere (r) were defined by using DiI particles as described by Grapin-Botton et al. (1995) with a precision evaluated at ±20 μm. All the excision and grafting experiments were thus performed using a dissecting microscope equipped with an ocular micrometer.

1. Fate map of the neural fold

Experiment I: This was aimed at defining the skeletal tissues yielded by the mesencephalic and rhombencephalic neural fold from the level

of the mid-mesencephalic area down to r8 inclusively. Isotopic and isochronic grafts of fragments of 70-150 μm of quail neural fold, according to the level considered, were implanted unilaterally into chick embryos, hosts and donors being at the 5-somite stage. The limits and location of the grafts were defined according to the fate map of Grapin-Botton et al. (1995) and corresponded successively to the posterior half of the mesencephalon and to every single rhombomere. The recipient embryos were fixed at E5-E7 and their hypobranchial neural crest derivatives were studied on histological sections treated to be able to distinguish host from donor cells (see histological techniques below). Some embryos were killed 24 hours after the operation (stage 15 of Hamburger and Hamilton (1951) (HH), i.e. 24-28 somite-stage) in order to control the location of the graft with respect to the rhombomeric level (data not shown).

2. Contralateral regeneration

Experiment II (see Fig. 1): Following the excision of the neural fold in the rhombencephalic area, the capacity of the contralateral neural fold to produce a neural crest cell outflow bilaterally was tested. For this purpose, the neural folds of the chick were excised bilaterally at the level corresponding to r1, r2 and r3. Then a quail neural fold was isotopically and isochronically grafted on one side (Fig. 1, exp. II). The embryos were examined at three stages: 8- to 11-somite stage, 20- to 27-somite stage and at embryonic day 7 (E7). The results of this experiment were compared to those obtained when the chick neural fold was excised on one side only and replaced by its quail counterpart.

3. Regeneration from the intermediate/ventral neural tube?

Experiment III (A and B) (see Fig. 1): This experiment was aimed at testing the capacity of the intermediate/ventral neural tube to produce neural crest cells. The right or left half of the chick neural tube was surgically removed at the level of r1 to r3 and replaced by the right or the left ventral half of the neural tube of the quail taken at the same transverse level. Two experimental series were performed. In experimental series IIIA, the fragment of neuroepithelium was surgically isolated without enzymatic treatment. In series IIIB, to allow the internal part of the neuroepithelium to be completely free of contaminating mesodermal cells, the quail neuroepithelium was isolated from the donor then digested (for 1 minute) with pancreatin (GIBCO) diluted 1/3 in PBS.

Experiment IV (A and B) (see Fig. 1): This experimental design is similar to that described in experiment III except that the ablation and graft were bilateral and thus involved the whole ventral half of the neural tube. Therefore, the dorsal half of the neural tube including the neural folds were missing in the operated embryo. Series A and B correspond to the use (B) or not (A) of pancreatin to isolate the grafted neuroepithelium.

In experiments III and IV the superficial ectoderm was put back onto the surface of the neural tube excision at the end of the operation thus establishing an immediate contact between the two tissues (Fig. 5D).

4. Anterior and posterior regeneration of the neural fold?

Experiment V (see Fig. 1): This consisted of the bilateral resection of the neural fold from either the mid-mesencephalon or from the pro-mesencephalic constriction down to the limit between r4/r5, r7/r8 or to the level of somite 3 (i.e. corresponding to the anterior part of r8: r8a).

After ablation, isotopic fragments of quail neural fold were implanted bilaterally on the length corresponding to the caudal half of the mesencephalon (series VA) or the last rhombomere(s) included in the excision (series VB1 and VB2 included r7 and r7/8a respectively).

In certain experiments the quail grafts were implanted in the same embryo at both the rostral and caudal levels of the excised territory (series VC).

Experiments VD involved the removal of the bilateral neural folds from the pro-mesencephalic constriction down to that between r4/r5, followed by the bilateral graft of neural fold fragments corresponding to r3/r4.

Analysis of the chimeras

The embryos were observed from the 8-somite stage to E8 and the chimeras in which the graft had not been properly incorporated into the host's neural tube were discarded. The others were analysed histologically as follows.

1. Determination of the contribution of the grafted quail cells to the various derivatives of the mid- and hindbrain neural fold

Two staining methods were used, either the Feulgen-Rossenbeck's staining procedure for DNA (Le Douarin, 1969, 1973) after fixation in Zenker's fluid or the QCPN monoclonal antibody (mAb) (DHSB) which recognizes an antigenic determinant common to all quail cells. After fixation in Bouin's fluid, selected embryos were ethanol dehydrated and embedded in Paraplast Plus. 5 or 6 μm frontal, sagittal or transverse sections were incubated with QCPN mAb (supernatant not diluted) overnight in a humidified chamber at 4°C and, after washing in PBS, reincubated for 90 minutes with horseradish peroxydase-conjugated antibody against mouse IgG1 (Southern Biotechnology Associates) and the reaction was developed in 250 ml PBS containing 20 mg DAB and 36 μl 30% H_2O_2 . Slides were then briefly rinsed in water and sections were stained with Gill's hematoxylin, ethanol, dehydrated and mounted with XAM (Gurr). Using the same methodology, two other mAbs were occasionally used on adjacent sections: HNK1 to identify neural crest cells and anti-MB1 which recognizes an antigenic determinant common to endothelial and white blood cells of the quail, but to the exclusion of any cell type of the chick (Péault et al., 1983).

The QCPN mAb was also used in some cases in whole-mount preparations according to the method of Chédotal et al. (1995). The dilution of the culture supernatant was 1/20.

2. The expression of the Hox genes

Expression of *Hoxa-2*, *a-3* and *b-4* in normal and experimental embryos from stage 20 HH to E7 was examined. The position of quail cells and the expression of the 3 Hox genes were examined on adjacent sections. The *Hoxb-4* probe corresponds to the full length RNA (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). The *Hoxa-2* probe was generated from a 700 bp partial cDNA subcloned into Bluescript, including all the coding sequence 3′ of 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

I. Migration of neural crest cells from the mid-mesencephalon to r8 into the branchial arches and their contribution to the skeletal structures (Experiment I)

As a prerequisite for our experiments on the possible regeneration of the mesencephalic and rhombencephalic neural crest following neural fold resection at the early somitic stages, we have carried out the orthotopic replacement of the chick neural fold by its quail counterpart by doing small sized grafts corresponding to the length of a single rhombomere. Although the early steps of migration of neural crest cells from the rhombomeres have been carefully studied through the application of vital dyes followed by the direct observation of cell movement

(Lumsden et al., 1991; Sechrist et al., 1993), the fate of the crest cells originating from each single hindbrain segment and their participation in easily distinguishable skeletal tissues in the hypobranchial region had not yet been assessed. Moreover, *Hox* genes were used as anteroposterior markers of the neural crest cells. Their expression pattern was thus analyzed in the various tissue layers which form the BAs.

1. Skeletal derivatives of the rhombencephalic and mesencephalic NC

A total of 17 embryos were observed between E6 and E7. The results of the detailed analysis carried out in this work are summarized in Fig. 2. They show that the neural crest cells which arise from the posterior half of the mesencephalon and migrate, together with crest cells from r1, r2 and r3 (partly) into BA1 (Lumsden et al., 1991), are at the origin of the Meckel's cartilage and membrane bones of the lower jaw.

The hyoid bone was found to derive from all the rhombomeres and the posterior mesencephalon. Moreover, the contribution of the mesencephalic crest and of cells arising from every hindbrain segment to a particular region of this structure was assessed: the entoglossum receives the exclusive contribution of the mesencephalic crest. The basihyal derives from r1 to r4. The ceratobranchial is formed by r4 to r6 NC cells. The epibranchial derives from r5 and r6 and the basibranchial from r5 to r8.

2. Normal expression of Hox genes in the BAs

Hox gene expression has already been described in the mouse and chick branchial arches (Hunt et al., 1991; Prince and Lumsden, 1994; Hunt et al., 1995). We used three probes at stages HH20 (E3) to 22 (E4): *Hoxa-2*, *Hoxa-3* and *Hoxb-4*. Their expression in the BAs together with the level of origin of the cells which colonize them is summarized in Fig. 3A. At E3-E4, BA1 does not have any Hox gene expression. *Hoxa-2* is expressed in BAs 2, 3, 4 and 5 (Fig. 3A,D). In the surface ectoderm the expression begins approximately in the middle of BA2 downward. The neural crest derivatives strongly express *Hoxa-2* in all BAs except BA1. In BA2 the mesodermal core (Fig. 3B,D) and endoderm are *Hoxa-2* negative. *Hoxa-2* is expressed in the mesodermal derivatives and endoderm only from BA3 downward. *Hoxa-3* is expressed in the surface ectoderm in the caudal part of BA3 and in the neural crest cells present in BA3 at that stage (Fig. 3A,C). It is also present in all tissue layers of more caudal arches although at a lower level. *Hoxb-4* is expressed in an AP increasing gradient in the neural crest cells and surface ectoderm in BA4 and B5 and more caudally. Its expression is absent in the rostral part of BA4 and higher in BA5. The pharyngeal endoderm is highly labelled from BA4 downward. The paraxial mesoderm is labelled by *Hoxb-4* probe only from the level of somite 5.

In summary (Fig. 3A), the neural crest cells of BA1, derived from the mesencephalon and r1, 2 and 3 do not express any

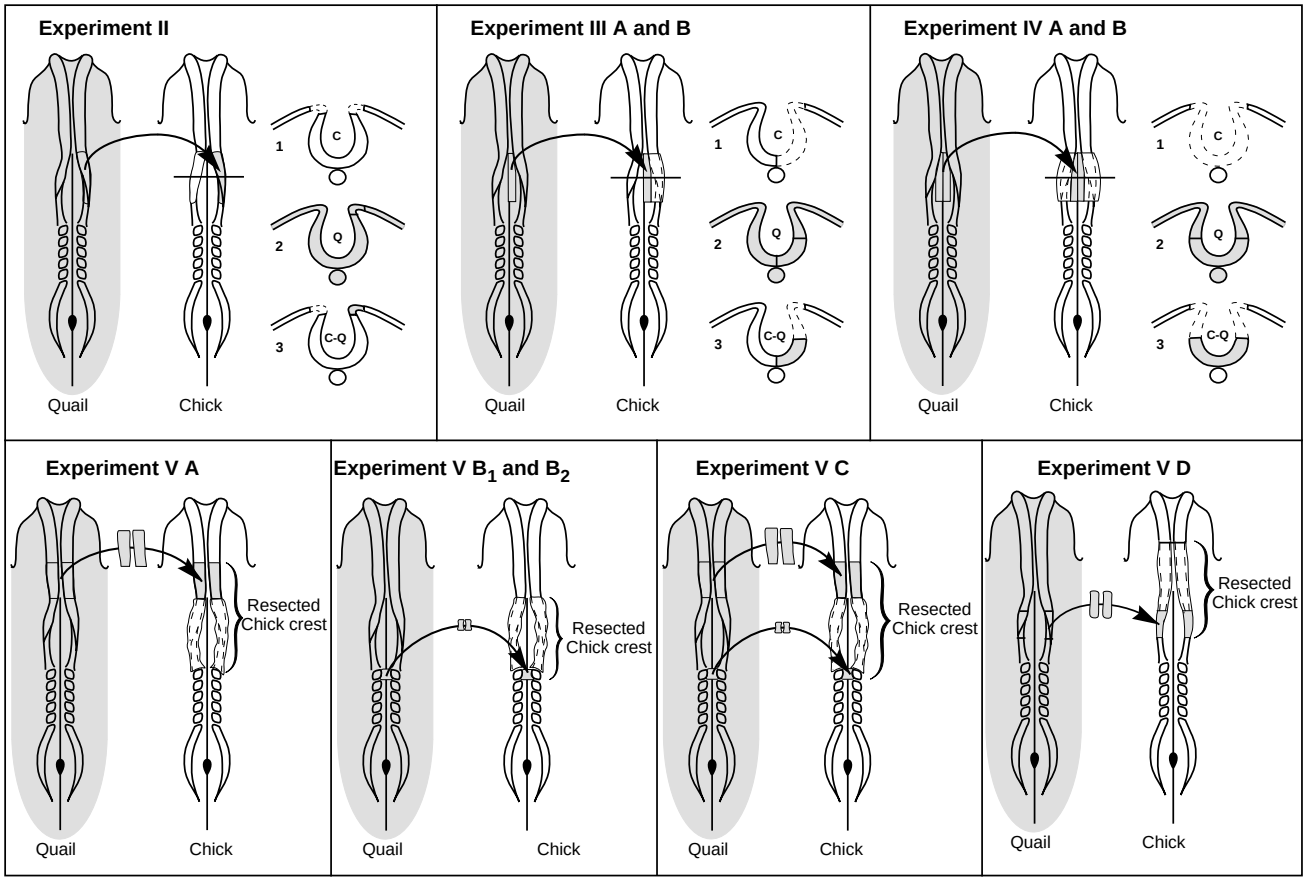

Fig. 1. Schematic representation of the various experiments performed (see explanations in Materials and Methods section). For experiments II, III and IV, schematic transverse sections (bar indicates the level) showing in 1 the chick (c) resected tissue, in 2 the fragment of quail (q) neural tissue which will be grafted in the chick host and in 3 the constructed chimera.

Hox gene. The neural crest cells of BA2, derived from r3 and 4, and to a lesser extent from r5 are *Hoxa-2*$^+$ as previously shown by Prince and Lumsden (1994). The neural crest cells of BA3, derived from r5 and 6 are *Hoxa-2*$^+$*Hoxa-3*$^+$. The neural crest cells of BA4 and BA5, derived from r7 and 8 are *Hoxa-2*$^+$*Hoxa-3*$^+$ and partly *Hoxb-4*$^+$. Similar results were obtained in quail and chick embryos.

II Effect of the excision of the neural fold on neural crest derivatives and branchial arch development (experiments II, III, IV) (Fig. 1)

1. Contact between the neuroepithelium and the superficial ectoderm is established soon after resection of the neural fold

One of the problems raised by these experiments was whether the neuroepithelium and superficial ectoderm establish a contact and fuse after the excision of the neural fold or of the dorsal half of the neural tube. In order to answer this question, ablation of the neural fold was performed unilaterally and that of the dorsal half of the neural tube bilaterally. The embryos were regularly observed and fixed soon after the operation. It was seen that in both types of experiments, a contact between the two tissues took place within the hour following the excision. Fig. 4 shows that the two tissues are fused in both types of experiments.

2. Bilateral excision of the neural folds at 5-somite stage followed by their unilateral replacement by a quail neural fold (Experiment II)

The experiment involved the bilateral resection of the chick anterior rhombencephalic neural fold at 5-somite stage (r1-r3) followed by the unilateral graft of the quail neural fold taken at the same level from a 5-somite stage donor. Four embryos were examined at 8- to 11-somite stage, 2 at 20- and 27-somite stage after immunostaining with QCPN mAb and 8 at E7.

Although the graft was carefully placed on the right or left side of the sectionned neural tube at 5-somite stage, a few hours later, at 8- to 11-somite stage, the grafted neural fold

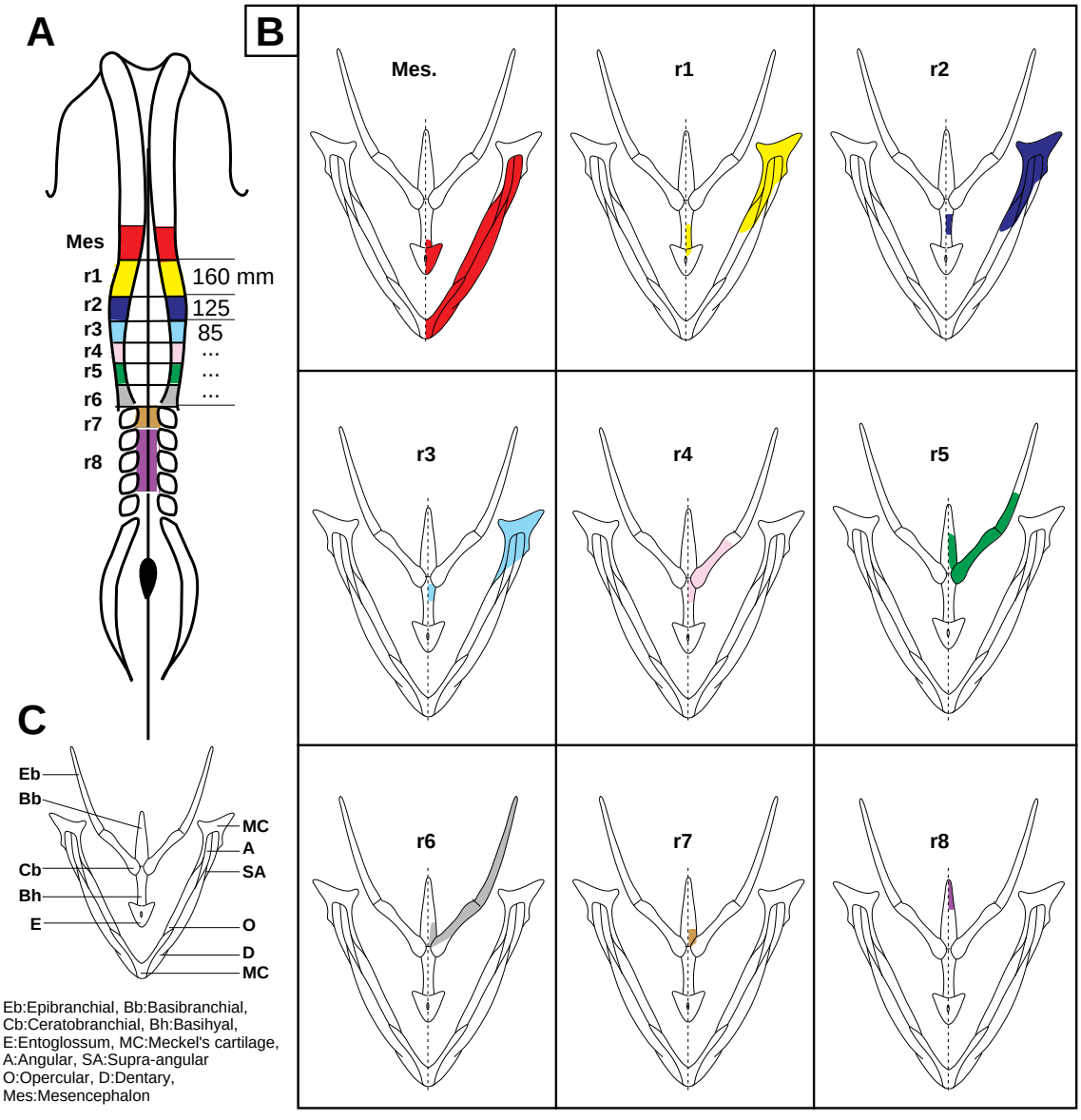

Fig. 2. Construction of the fate map of the mesencephalic (Mes) and rhombencephalic (r1-r8) neural fold (A) on the basis of the results of experiment I (B). (C) Lower jaw and hyoid bones with the corresponding legends.

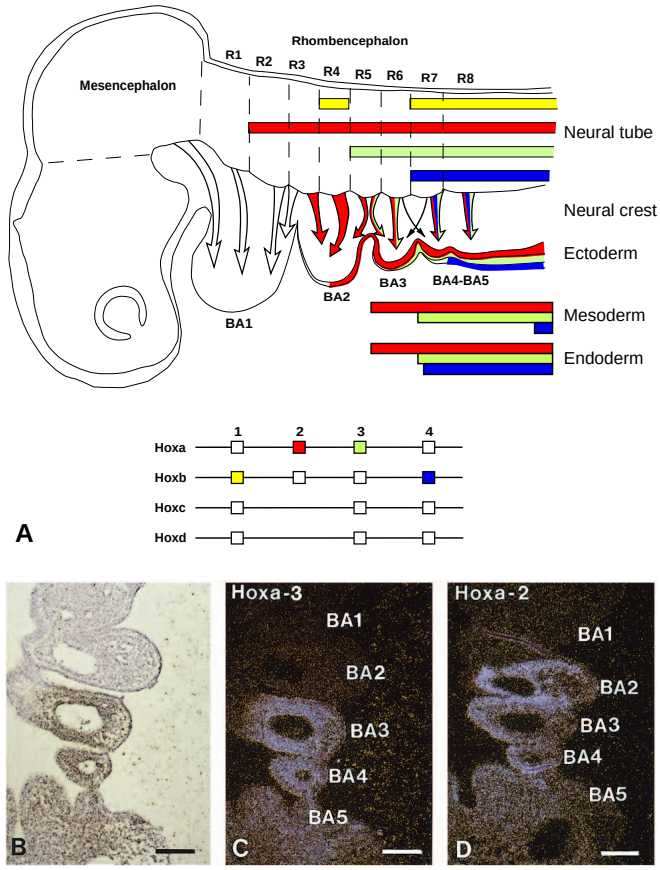

Fig. 3. (A) Schematic representation of Hox gene expression in the chick and quail embryo at E3 when the branchial arches (BA) are being colonized by neural crest cells originating from the posterior half of the mesencephalon and the rhombomeres (r1-r8). The arrows indicate the AP origin of the neural crest cells migrating to each BA. Expression of Hox genes is also indicated in the superficial ectoderm, the endoderm and mesoderm. (B) Frontal section of a chick embryo at E3 showing *Hoxa-3* (B,C) and *Hoxa-2* (D) expression in the branchial arches: (B) In situ hybridization with *Hoxa-3* seen in bright-field illumination. (C,D) Dark-field pictures of *Hoxa-3* (C) and *Hoxa-2* (D). Scale bar, 100 μm.

cells, which have strikingly increased in number, occupy the whole dorsomedial area between the dorsal aspect of the neural tube and the healing superficial ectoderm which rapidly covers the graft. Thus, the unilaterally grafted neural fold has formed a neural crest which is virtually undistinguishable from the normal crest at that stage (Fig. 5A) and which begins a bilateral migration.

Interestingly, if the chick neural fold is removed unilaterally and replaced by its quail counterpart, crest cell migration is essentially ipsilateral with, however, some contralateral migration occurring from both quail and chick sides (Fig. 5B).

In the case of bilateral neural fold excision followed by a unilateral quail graft, one can see at the 27-somite stage that the quail neural crest cells have migrated into the branchial arches according to the normal pattern in a strictly identical bilateral manner (Fig. 5C). Moreover, staining of adjacent sections with either HNK1 or QCPN mAbs confirms the neural crest origin of the QCPN-positive cells in embryos at 10- 11- and 27-somite stage. Interestingly, if the chick neural fold is ablated unilaterally at the same level and the same stage and not replaced by an equivalent quail graft, one can see that the contralateral neural fold flops over to cover the ablated side within the minutes following the ablation. This process progresses from rostral to caudal. Crest cell migration from the unique neural fold left in situ then proceeds bilaterally.

Eight embryos subjected to the replacement of the neural fold by their quail counterpart were analyzed for chimerism at E7 after Feulgen-Rossenbeck's staining. The same 1st branchial arch-derived structures recorded in the fate map analysis described above (experiment I) were labelled in a bilateral situation.

3. Graft of the ventral half of a quail neural tube at the r1-r3 level (Experiment III A and B)

Two experimental series were performed as illustrated in Fig. 5D. In one (experiment IIIA), the quail fragment of neuroepithelium was removed surgically from the donor and grafted immediately; in the other (experiment IIIB) it was treated with pancreatin before grafting in order to remove the mesenchymal cells adhering to the neuroepithelium.

At 22-27-somite stages (n=3), serial sections (experiment IIIA) treated with either the Feulgen-Rossenbeck's technique or the QCPN mAb, revealed that the graft was well incorporated into the host neural tube. Quail cells were seen in the mesenchyme and located dorsally in the vicinity of the neural tube, often integrated in the host endothelium and labelled by the MB1 mAb specific for an endothelial cell antigenic determinant of the quail (see Fig. 5G-I, similar experiment involving bilateral grafting of the ventral neural tube). None of them migrated to the branchial arch.

Observation of the embryos at E7 (n=2) allowed the fate of the quail cells located in the mesenchyme to be followed. They contributed to mesodermal derivatives: meninges of the anterior rhombencephalon, chondrocytes in the orbitosphenoid and otic capsule (Couly et al., 1992). In contrast, no quail cells were present in the 1st branchial arch derivatives. Thus, neither the dorsal part of the Meckel's cartilage nor any of the bones of the lower jaw and posterior part of entoglossum and bashyal which are of r1/r3 crest origin were derived from the graft in these experiments.

When the quail neuroepithelial graft had been treated with pancreatin prior to implantation (experiment IIIB: observation at 27-30-somite stage, n=5) and was thus devoid of contaminating mesenchymal cells of mesodermal origin, no donor cells were seen in the host mesenchyme. The only quail cells were those of the neuroepithelial graft (Fig. 5E). In all cases observed, the superficial ectoderm was in close contact with the graft. This contact had in fact been established from the time of excision of the dorsal neural tube since the superficial ectoderm had been carefully put back onto the dorsal surface of the neuroepithelium. This however did not generate the production of neural crest cells by the ventral neural tube (Fig. 5F).

We conclude that the ventral half of the neural tube wall is not able to produce neural crest cells. The quail cells present in the host mesenchyme in experiment IIIA are the progeny of mesodermal cells adhering to the neuroepithelial graft. Those cells are removed by pancreatin treatment (experiment IIIB).

4. Bilateral graft of the ventral half of the quail neural tube at the level of r1-r3 (Experiment IVA and B)

This experiment is similar to that just described except that the host neural tube is totally removed surgically at the r1-r3 level and replaced by the ventral half of the equivalent quail neural tube taken from a stagematched embryo. It was performed to see whether deficiencies in neural crest cell supply to the BA1 was observed in the absence of neural crest emigration from r1,2,3. In experiment IVA, the donor neuroepithelium was surgically dissected and directly implanted, while in experiment IVB, it was treated with pancreatin prior to implantation. The results obtained show that in certain cases the grafted neural epithelium contacts the host's surface ectoderm and tends to form a tube (Fig. 5G,H).

A few QCPN-positive quail cells were seen again in the mesenchyme proximal to the graft in experiment IVA (Fig. 5G,H) whereas no such cells were present when the quail neuroepithelial graft had been treated with pancreatin prior to implantation in the host (experiment IVB).

No neural crest cells have ever been found exiting from the grafted tube (as observed at 25- to 32-somite-stage) and no derivatives of the graft were present in the BA1 in any of these experiments. The mesodermal nature of the mesenchymal quail cells found in experiment A was further confirmed by using the anti-MB1 mAb which is specific for quail vascular endothelial cells and which labels endothelial cells forming the perineural vascular plexus (Fig. 5I).

In none of these experiments (III and IV) where the chick neural tube was replaced by only the ventral half of its quail counterpart, which was not able to yield neural crest cells, did the branchial arches of the host show mesectodermal deficiencies. The problem was thus raised of the origin of the neural crest cells which replaced the deficiencies after ablation. To answer this question the capacity of the remaining neural crest, located rostrally or caudally to the ablation to substitute for the removed material was tested.

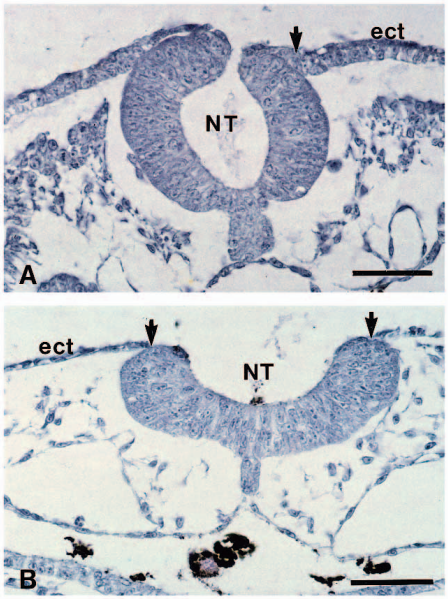

Fig. 4. Establishment of contact between the neuroepithelium and the superficial ectoderm soon after resection of the neural fold. (A) Transverse section of a chick embryo after resection of the neural fold unilaterally (right) at the rhombencephalic level, from r1 to r6. Fixation 2 hours after the operation. The fusion between superficial ectoderm (ect) and epithelium of the neural tube (NT) has occurred (arrow). (B) The embryo seen in transverse section was subjected to bilateral removal of the dorsal neural tube and the neural fold. Fixation after 5 hours shows healing at the ectoderm/neuroepithelium junction (arrows). Scale bar: 50 μm.

III Rostral and caudal regeneration of the neural crest after resection of the neural fold at the mesencephalic and rhombencephalic level (Experiment V, Fig. 1)

In the first experiment of this series (VA), the neural fold was removed bilaterally from the level of the mid-mesencephalon down to the 1st-somite in a 5-somite chick embryo. This was followed by the bilateral isotopic graft of a 160 μm neural fold in the mesencephalic area (Fig. 6A).

Two embryos were observed at 28-29-somite stage, 13 at E3, 4 at E4 and 2 at E6. The embryos examined at E3 and E4 were cut in 5 μm frontal sections and treated on alternate sections with the QCPN mAb and for in situ hybridization to reveal the Hox mRNAs.

At E3, BA1 was colonized by QCPN-positive cells (except the mesoderm core which was of host origin) which were therefore derived from the posterior mesencephalon. In 9/13 cases, quail cells were also found in BA2. They were numerous in 2/13 cases and limited to a stream of cells in the rostral part of the BA2 in 7/13 cases. The 3rd, 4th and 5th branchial arches were normal in appearance and contained chick host cells. At E4, the population of quail cells in BA2 had increased (Fig. 6B,D), and completely filled the arch in 2/3 cases (except for the mesoderm). In one case however, the crest cells were of chick origin in the caudal part of the arch.

No expression of *Hoxa-2* was found either at E3 or at E4 in the quail cells which arose from the mesencephalon and colonized BA1 and BA2 (Fig. 6C). As a result, the domains of expression of *Hoxa-2* and *Hoxa-3* have the same rostral limit which is, in most cases, located between BA2 and 3 (Fig. 6C,E) although in some cases [both at E3 (4/13) and E4 (1/3)] a stream of *Hoxa-2*$^+$ and *Hoxa-3*$^+$ cells is found in the caudal part of BA2. Note that in control embryos BA2 is colonized by cells arising from r3,4,5 which express *Hoxa-2*.

At E6 the neural crest derivatives, which have developed from the grafted cells, that have colonized BA1 and BA2 (Fig. 6F,G) are found in the mandibular skeleton (Meckel's cartilage and primordium of membrane bones) in its totality, the entoglossum and the basihyal. Quail cells were also forming the complete trigeminal ganglion and were found in the rostral half of the acoustico-facial and geniculate ganglia. The geniculate ganglion received a nerve anastomosis from the mandibulary branch of the trigeminal nerve. Few quail cells were located in the jugular ganglion and conotroncus of the heart (Fig. 6H).

Comparison of this situation with the normal fate map (Figs 2B-6F) shows that the neural crest cells arising from the posterior mesencephalon yield the derivatives which normally originate from r1 to r3 and r4 (partly) without acquiring the Hox code that they should express according to their novel position.

In experiment VB (Figs 1, 7A), the same type of excision (from mid-mesencephalon to either r7 or the anterior region of r8 -'r8a'- inclusively) was done as in VA but it was

followed by the isotopic bilateral graft of a quail neural fold at the level of r7 or r7/8a. At E3 the extent of quail crest cell invasion included BAs 3, 4 and 5 (n=4/4) (Fig. 7B) with few cells invading the ventral and caudal part of BA2 (n=3/4) (not shown on this section). In one case BA2 was full of quail cells. At E4 (n=2) the quail cells were more numerous in the caudal part of BA2. Therefore the distribution of quail and chick cells in the BAs was exactly the reverse to that observed in experiment VA, showing that in this situation the behaviour of quail and chick cells in the chimeras is similar.

The neural crest cells that originate from r7/8a migrate normally in BA4 and BA5 (with only a few cells in BA3) and are *Hoxa-2*$^{+}$*Hoxa-3*$^{+}$ and partly *Hoxb-4*$^{+}$ (Fig. 3). In these experimental conditions the cells of r7-r8a origin which migrate very ectopically in BA3 and in the caudal part of BA2, are *Hoxa-2*$^{+}$ and *Hoxa-3*$^{+}$ (Fig. 7B-D) but *Hoxb-4*$^{-}$. *Hoxa-2* and *Hoxa-3* have thus the same anterior limit of expression in the caudal part of BA2 or between BA2 and BA3. *Hoxb-4* has however a normal expression pattern. As mentioned above, the crest cells arising from r7 and r8 express *Hoxb-4* in a decreasing caudorostral gradient and transcripts of this gene are in fact absent in the rostral part of BA4. One can therefore assume that the neural crest cells which fill the anterior BAs (BA3 and BA2) are mostly derived from a population of cells not expressing *Hoxb-4* . These results show therefore that the pop-

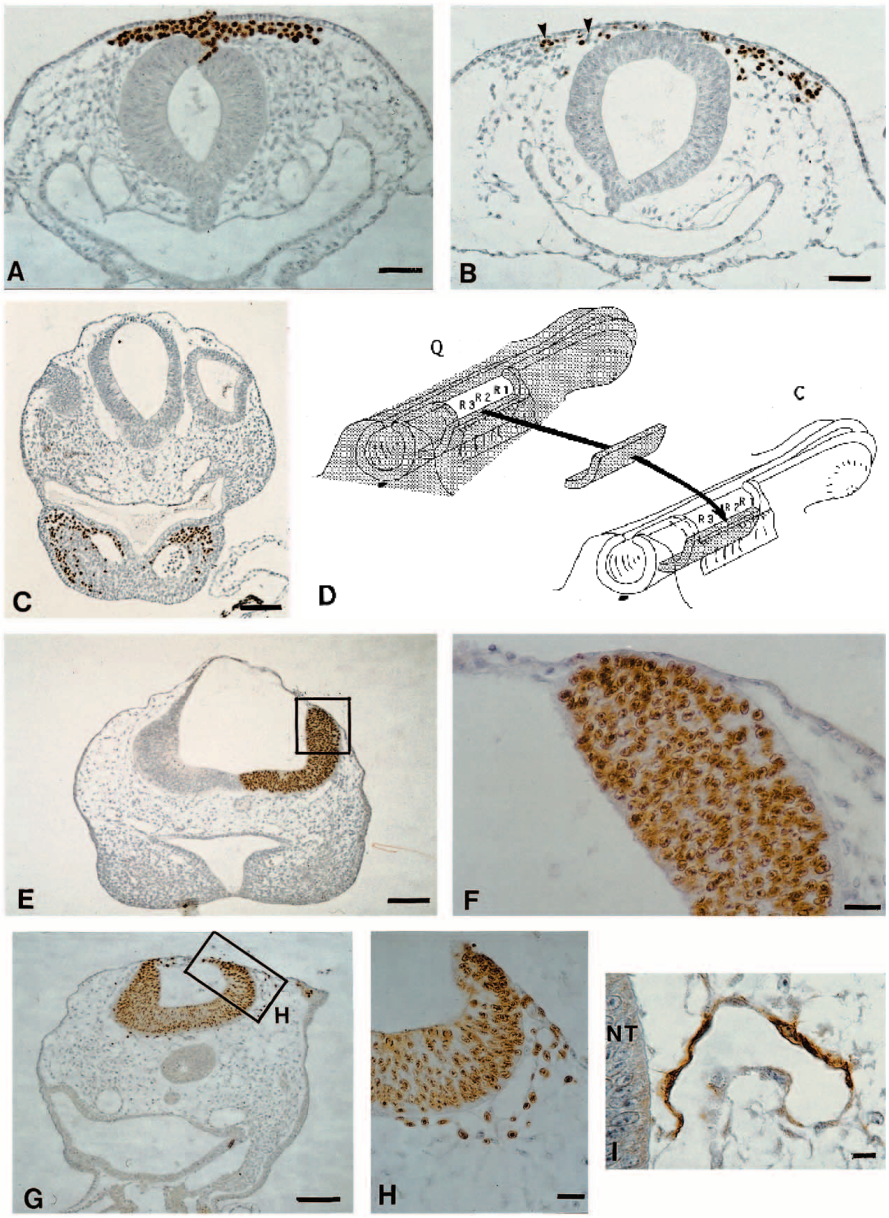

Fig. 5. Results of Experiments II (A-C), IIIB (D-F) and IVA (G-I). Experiment II: (A) Transverse section at the rhombencephalic level of an 8-somite stage chimeric embryo. After bilateral resection of the chick neural folds at 5-somite stage and isotopic grafting of a quail neural fold on one side only (right), the quail neural crest cells, labelled by QCPN mAb, occupy the whole dorsomedial area and migrate bilaterally. At 27-somite stage (C), the quail neural crest cells have invaded BA1 bilaterally. (B) Transverse section at the rhombencephalic level of a 10-somite stage chimera, treated as the embryo in A but the resection of the chick neural fold, performed at 5-somite stage was unilateral (right) and was also followed by its replacement by its quail counterpart. The quail neural crest cells migrate almost exclusively on the side of the graft with only a few quail and chick cells migrating contralaterally (arrowheads). Thus, in normal development crest cell migration is essentially but not exclusively ipsilateral. It becomes bilateral after resection of the neural crest on one side. Scale bar A-B: 50 μm and C: 100 μm. Experiment III: (D) Schematic drawing of experiments IIIA and IIIB showing the grafting of the ventral half of a quail neural tube into a chick embryo at 5-somite stage at the level of r1-r3. The dorsal ectoderm is put back to its normal position after graft completion. (E) Tranverse section at the rhombencephalic level of a 30-somite stage chimera. The neural epithelium was treated with pancreatin before grafting to remove contaminating mesodermal cells. The grafted half neural tube of the quail is perfectly integrated in the chick embryo. The graft clearly did not yield neural crest cells since no dispersed quail cells are seen in the chimera apart from the grafted neuroepithelium itself. Note the close contact between the graft and the host's superficial ectoderm (in E and F). Scale bar E: 100 μm and F: 20 μm. Experiment IVA: (G) Transverse section at the rhombencephalic level of a 25-26-somite stage chimera in which the neural tube of the chick was replaced isotopically at 5-somite stage by the bilateral half of a quail neural tube untreated with pancreatin. The quail grafted tissue is stained by QCPN mAb. No quail cells are present in the BAs but some QCPN-positive cells are dispersed in the dorsal region of the embryo (G and H). Most of these cells carry the MB1 epitope (I) specific for endothelial and hemopoietic cells of the quail, demonstrating their mesodermal origin. NT, neural tube. Scale bars, G,100 μm; H, 20 μm and I, 10 μm.

ulation which migrates anteriorly maintains the expression of *Hoxa-2* and *Hoxa-3*.

Two chimeras were observed at E6 and E6.5: the posterior part of the basihyal, the ceratobranchial, the epibranchial as well as the anterior part of the basibranchial cartilages were of quail graft origin (Fig. 7E-G). Note that when the excision did not include r8, and therefore when the graft was placed into r7, the posterior region of the basibranchial cartilage was of host's type as expected. The neurogenic neural crest formed the jugular-superior ganglion and the caudal part of the acoustico-facial ganglia as well as the Schwann cells of the corresponding nerves. Some connective cells of quail origin were found in the rectus ventralis muscle meaning that they had migrated up to the level of the 1st BA. Therefore the neural fold of r7 alone has the capacity to replace the missing neural crest normally generated by the area corresponding to r6, 5-4 and partly r3.

In experiment VC, after resection as in VA and VB, the mesencephalic and r7 or r7/8a grafts were done on the same embryo (Fig. 7H). The results at E3 (n=6) (Fig. 7I, at E4 (n=1) and at E6 (n=1) (Figs 7J and 8) were those expected from the individual rostral and caudal grafts just described. Quail cells were found at E3 in BAs 1, 3, 4 and 5 in 6/6 cases and in BA2 in 5/6 cases. The population in BA2 is however always smaller than in normal development at this stage. Two populations are distinguishable in this arch: a caudal one that we assume is coming from r7/8a and a rostral one from the mesencephalon. On adjacent sections, the expression of *Hoxa-2* is normal in BA1 (no expression), BAs 3, 4, 5 (expression). The expression in BA2 is restricted to its caudal part. This is due to the fact that the cells which come from the mesencephalon remain *Hoxa-2* negative in spite of their posterior migration, whereas those originating from r7/8a normally express this gene. The expression of *Hoxa-3* is maintained in BAs 3, 4 and 5 and the

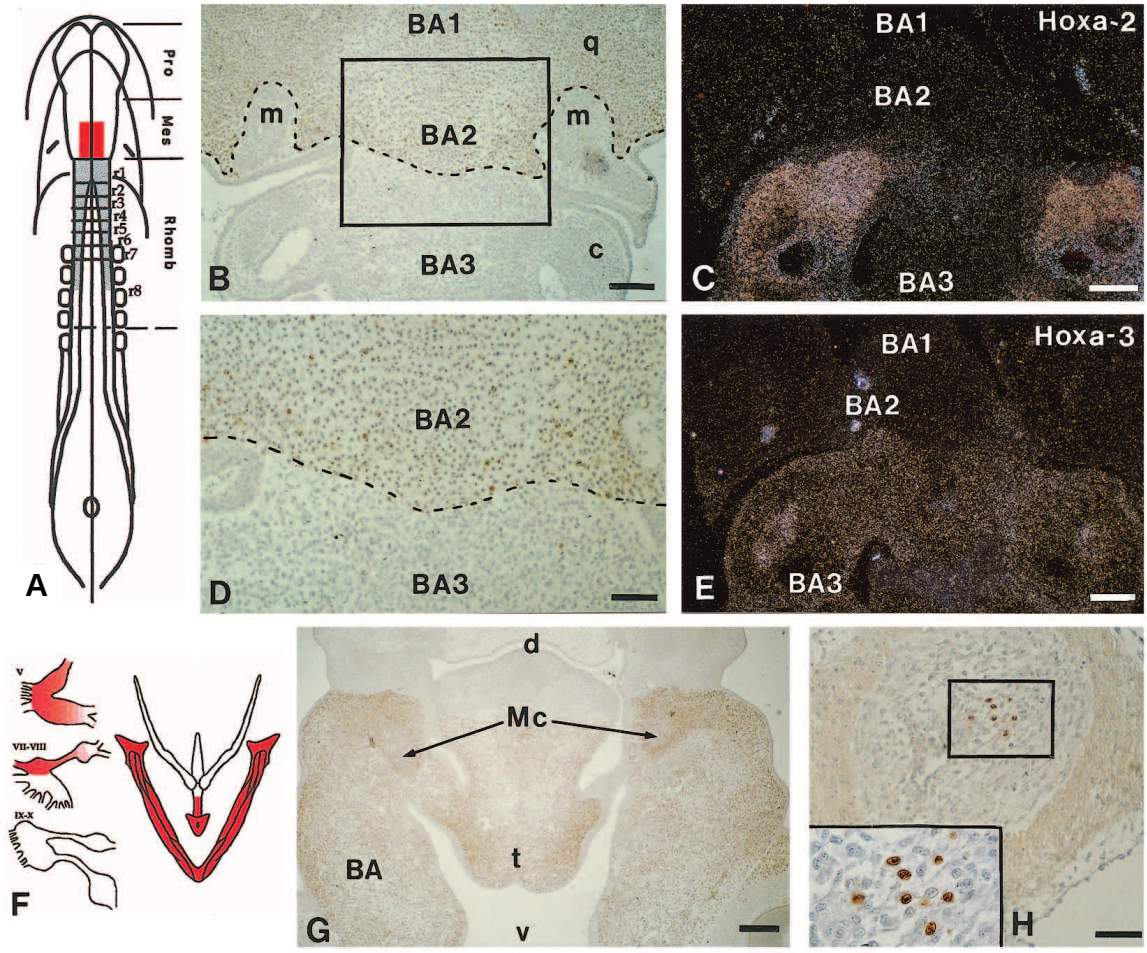

Fig. 6. Results of Experiment VA. (A) Schematic drawing of experiment VA in which the neural fold was bilaterally excised in a 5-somite chick embryo from the mid-mesencephalic level to somite 3 (grey zone). The resection was followed by the orthotopic bilateral graft of fragments of quail neural fold (red) corresponding to the posterior mesencephalon. (B-E) At E3, the quail neural crest cells labelled by QCPN mAb have filled up BA1 and BA2, and chick cells in BA2 correspond to the mesodermal core (B). The boundary between BA2 (quail) (q) and BA3 (chick) (c) is particularly obvious in D (enlargement of B). On adjacent sections, the quail cells derived from the grafted mesencephalic neural fold express neither *Hoxa-2* (C) nor *Hoxa-3* (E). (F) At E7, the quail neural crest cells form the Meckel's cartilage and membrane bones of the lower jaw, the entoglossum and basihyal (partly). They are also found in the trigeminal ganglion and in the rostral part of the acoustico-facial ganglion. (G) The boundary between chick and quail cells located in the tongue (t), BA1 and BA2, is perfectly evident. d,v indicate dorsoventral axis. (H) Some quail neural crest cells are located in the conotroncus. On the left, enlargment of the framed area. Pro., prosencephalon: Mes., mesencephalon; Rhomb., rhombencephalon. Scale bars, B-C and E: 100 μm; D and H, 50 μm and G, 200 μm.

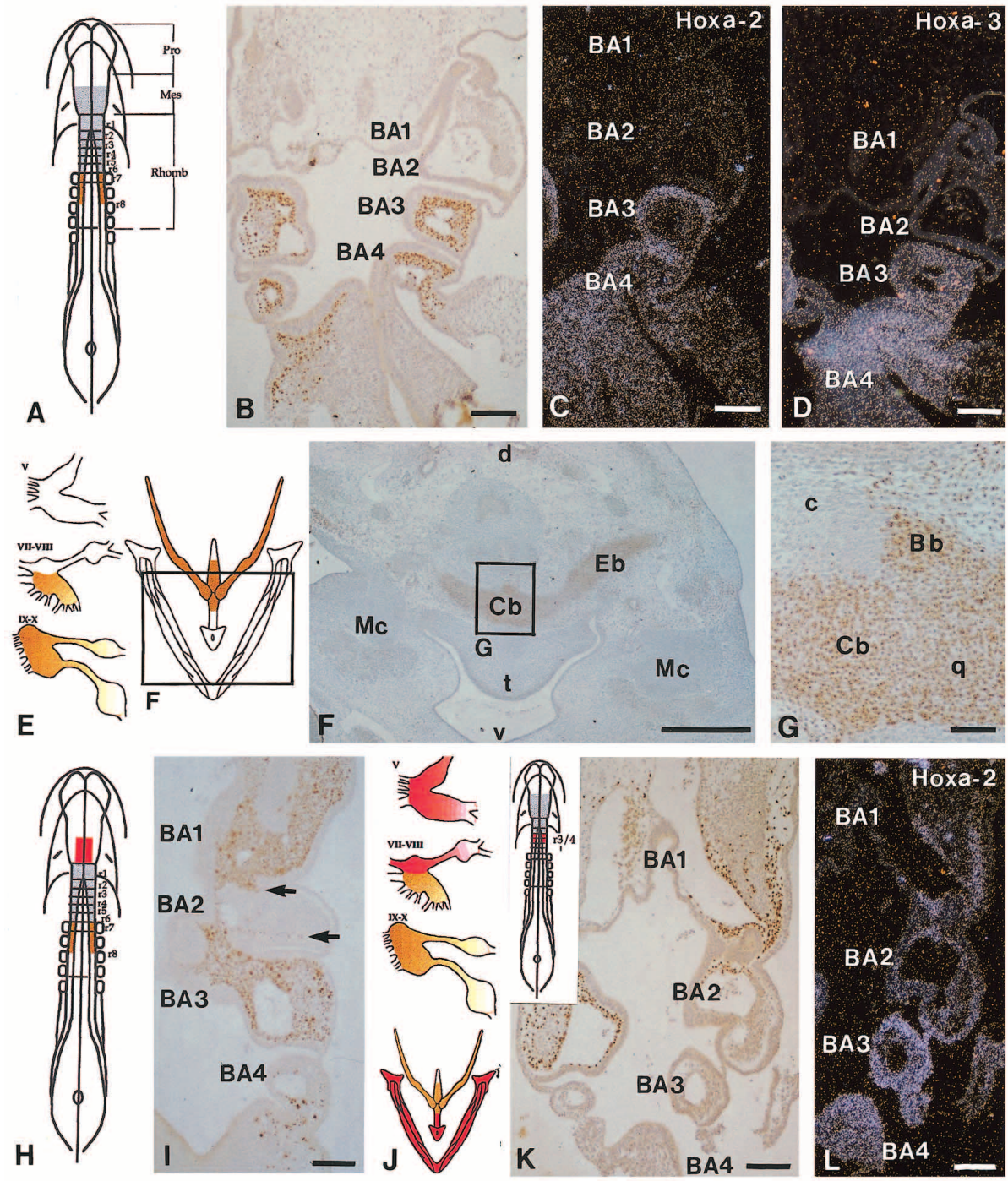

Fig. 7. Results of Experiments VB (A-G), VC (H-J) and VD (K,L). Experiment VB: (A) Bilateral resection of the mesencephalic and rhombencephalic neural fold including the posterior mid-mesencephalon plus r1 to r7 and r8a inclusively (grey). Excision was followed by the bilaterally orthotopic graft of fragments of the quail neural fold (brown) at r7-r8a level. (B) Frontal section of the chimera at E3. Quail neural crest cells labelled by QCPN mAb are located in BA3 and BA4/BA5. (C-D) *Hoxa-2* and *Hoxa-3* transcripts are present in BA3, and absent in BA2. (E) Schematic representation at E7 of the localization of the quail neural crest cells in the hyoid bone. F and G show the hyoid bone made up of neural crest cells (partly basihyal, partly basibranchial (Bb), epibranchial (Eb) and ceratobranchial (Cb) and the acoustico-facial and jugular-superior ganglia). Scale bars B-D, 100 μm; F, 500 μm and G, 50 μm. Experiment VC: (H) The same excision and grafts described in Figs 6A and 7A were performed on the same embryo. (I) All BAs are colonized by quail cells. Note that invading cells have not yet completely filled up BA2 (arrows). (J) Schematic representation of quail cell distribution in the cranial ganglia and the hypobranchial skeleton. Scale bar I, 100 μm. Results of Experiment VD: (K) Insert: schematic drawing of experiment VD in which the neural folds were removed bilaterally from the diencephalic/mesencephalic constriction down to r5. Excision was followed by the orthotopic bilateral graft of quail neural fold fragments corresponding to r3/4. The result is seen after QCPN mAb staining at E3 on frontal section: quail neural crest cells have invaded BA1 and BA2 and have maintained *Hoxa-2* expression in their heterotopic position (L). Scale bars K-L, 100 μm.

posterior cells which have colonized the caudal part of BA2 have maintained the expression of this gene although they have migrated to a position which is beyond its normal limit of expression. The expression pattern of *Hoxb-4* is normal. The embryo of this series which was examined at E6 presented no deficiencies in the branchial arch skeleton and in the neural derivatives of the crest. The distribution of crest cells of graft origin was as expected from the results of experiments VA and VB and is represented schematically in Fig. 7J and documented in Fig. 8 showing normal gross anatomy.

One can therefore conclude from these experiments (i) that the neural crest situated rostrally and caudally to the ablated neural fold replenishes the deficiencies following this operation by a longitudinal migration which fills up the BAs located at the excision level; (ii) the neural crest cells, while they migrate either rostrocaudally or caudorostrally, keep the Hox code corresponding to their level of origin. This was confirmed by a last experiment (experiment VD) in which the neural fold excision was more extended rostrally than before and reached the pro-mesencephalic constriction whereas it

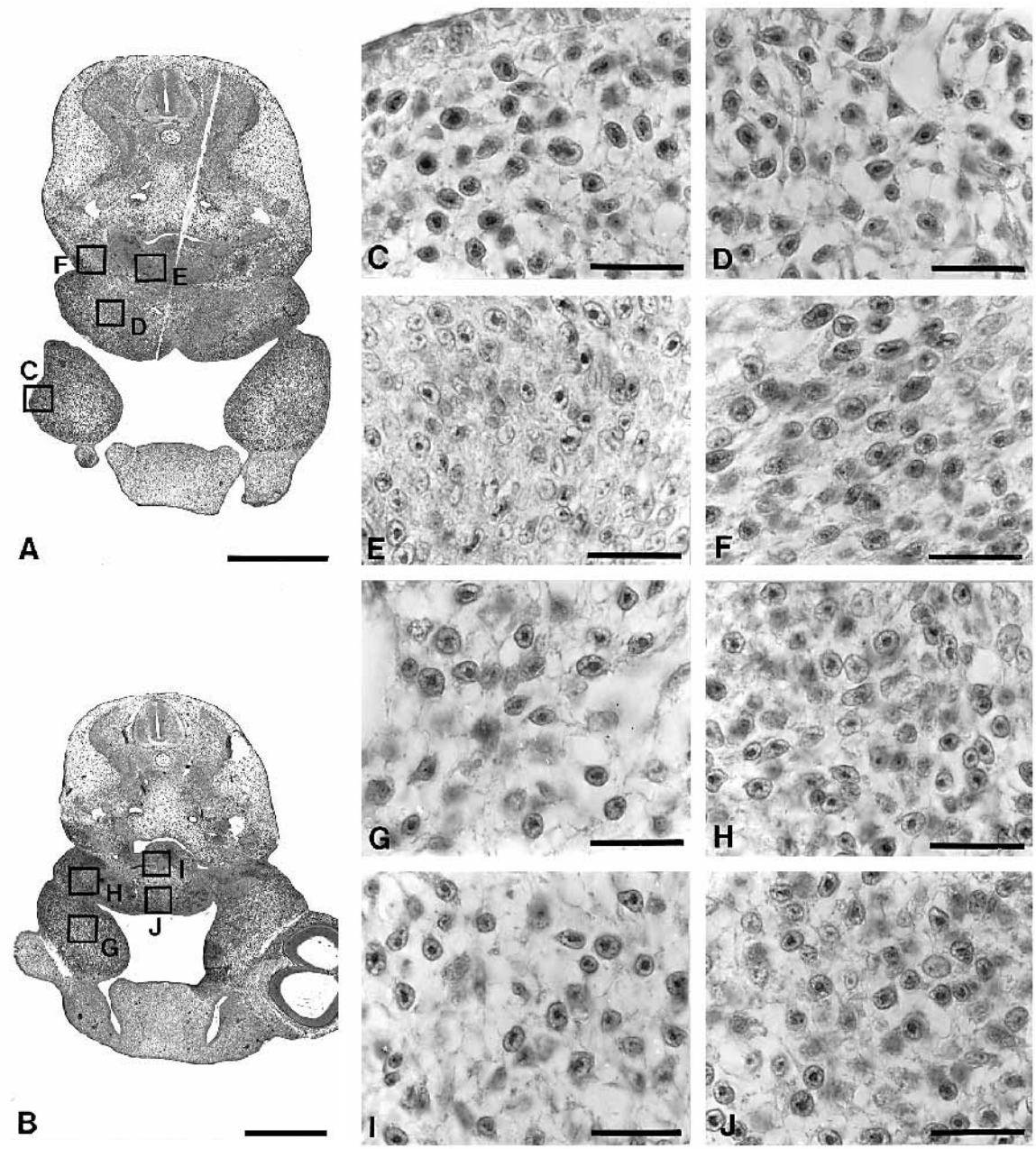

Fig. 8. Results of experiments VC: bilateral resection of the neural fold from the posterior mid-mesencephalon to r7 inclusively. Excision was followed by the bilaterally orthotopic graft of fragments of the quail neural fold at the mesencephalic and r7 levels. (A,B) Coronal sections of the head of E6.5 chimeras. The dermis of the maxillary bud (C), the chondrocytes of the basibranchial (D) and ceratobranchial (E), the dermis of the second branchial arch (F), the mesenchyme of the maxillary bud (G), the proximal part of the Meckel's cartilage (H), the chondrocytes of the basihyal (I) and the mesenchyme of the first branchial arch (J) are of neural crest quail origin. Scale bars, A,B, 5 mm and C-J, 20 μm.

ended caudally at the r4/r5 limit. This excision was followed by the isotopic bilateral graft of the quail neural folds at the r4, r3 level. The posteroanterior migration of cells originating from the graft resulted in the colonization of BA1 and BA2 (Fig. 7K) as seen on two embryos observed at E3. In both cases the cells which migrated anteriorly maintained their expression of *Hoxa-2*. This resulted in both cases in the expression of *Hoxa-2* in BA1 where normally no Hox gene transcripts are present (Fig. 7L).

DISCUSSION AND CONCLUSIONS

In these studies we have addressed the question of the capacity of the intermediate and ventral regions of the mesencephalo-rhombencephalic neural tube to regenerate neural crest cells in vivo at the early somitic stages of the avian embryo. The fact that removal of large segments of the dorsal neural tube did not result in major defects in the neural crest-derived hypobranchial derivatives had been recently established by several authors (Scherson et al., 1993; Sechrist et al., 1995; Hunt et al., 1995). However, explants derived from prospective ventral and intermediate regions of the neural plate isolated from the caudal region of stage 10 (HH) chick embryos were shown capable of expressing dorsal markers (e.g. *Msx1*, *Msx2* and *Slug* genes) and to produce $HNK1^+$ neural crest cells if subjected to certain members of the TGFβ family (Dsl1, BMP4 and BMP7) normally expressed in the dorsal neural tube and (for the BMPs) in the midline dorsal superficial ectoderm (Basler et al., 1993; Liem et al., 1995). Moreover, the juxtaposition of non-neural and neural ectoderm either in vitro or in vivo was also shown to induce a series of dorsal genes (*Wnt1*, *Wnt3a*, *Slug*) in ventral neural plate cells and, in certain circumstances, to result in the emergence of neural crest cells (Selleck and Bronner-Fraser, 1995; Dickinson et al., 1995).

It was therefore of interest to know if in the dorsoventral patterning of the neural plate such a plasticity, observed in definite experimental situations, could also account for the regulations observed at the mid- and hindbrain level in the embryos in ovo at the early somitic stages (see Scherson et al., 1993 and Hunt et al., 1995). Another alternative which remained to be carefully considered was the possible role in this process of the neural fold cells left in situ rostrally and caudally to the ablation. This was particularly important since, according to their rhombomeric origin, hindbrain neural crest cells express a definite Hox code which is thought to define their fate. We decided therefore to investigate what is the source and Hox gene expression of the cells which regenerate the ablated crest at the mid-hindbrain level of chick embryos at the 5-somite stage.

The capacity of the neural tube to regenerate neural crest cells

Extirpation of the neural fold and even of the dorsal moiety of the neural tube but variable lengths at the 5-somite stage was always, in our hands, followed by regeneration of neural crest cells which filled up the BAs and was followed by an apparently normal development of the hypobranchial and facial neural and skeletal structures. The ablations extended from the length of three rhombomeres to that of nearly all the rhombencephalon plus the posterior mesencephalon. In this respect, we thus confirm the results of previous authors (Scherson et al., 1993; Sechrist et al., 1995; Hunt et al., 1995). However, this regeneration capacity contrasts with the results of Hammond and Yntema (1964) who observed large depletions of pharyngeal cartilages following extirpation of cranial neural crest in chick embryos at stages ranging from 4 to 12 somites. The excisions in these cases were extended far further caudally (they included “the hindbrain plus posteriorly the length corresponding to 10 or more segments”, see p. 22).

In the present work the problem of the source of the regenerating cells has been studied with the help of cell markers. First, we demonstrate that when the neural fold is removed unilaterally at 5-somite stage in the rhombencephalon (and it is true as well at 3 to 7-somite stage, data not shown), the contralateral fold flops down and takes a mediodorsal position. This process is rapid and can be seen within the minutes following the excision just by simple observation under the dissecting microscope. By removing the neural folds bilaterally in the rhombencephalon of chick embryos and replacing one of them by its quail counterpart, we could observe the behaviour of the neural crest cells produced by the graft. In all cases, the quail neural crest cells rapidly extended bilaterally, thus producing virtually twice as many crest cells as in normal development. Examination of these embryos when organogenesis is completed showed that the structures corresponding to the level of the graft (according to the fate map that we have established) were quail cells bilaterally.

Interestingly, if the quail neural fold is grafted unilateraly while the contralateral host’s neural fold is left in situ, the distribution of the cells of each species is essentially ipsilateral with only a small contralateral migration on both sides (see Fig. 5B). Therefore, the unilateral deletion of the neural fold at these early stages is followed by a considerable (and not a small, see Scherson et al., 1993; Sechrist et al., 1995) compensation of the deficiency by the contralateral neural fold.

The capacity of the intermediate (and even ventral) parts of the neural tube to regenerate a neural crest (Scherson et al., 1993) was tested by removing the chick neural tube uni- or bilaterally at the rhombomeres level and replacing it with the ventral moiety (floor plate and presumptive basal plate) of the quail, uni- or bilaterally. No migratory cells were seen exiting from the dorsal side of the grafted hemineural tube even though it was in close contact with the superficial dorsal ectoderm of the chick from soon after the operation (see Fig. 4). In the embryos subjected to the replacement of the ventral moiety of the neural tube from the quail, two points are of interest. First, no deficiencies were noted in the colonization of the BAs by neural crest cells at the level of excision, showing that a regeneration of crest cells has actually occurred. Secondly, when the grafted neuroepithelium had not been subjected to trypsinisation to remove contaminating mesodermal cells which adhere to the neural tube, some quail cells were recorded in the cephalic mesenchyme. Their mesodermal nature was assessed first by their position within the territory normally occupied by the mesoderm and also by the fact that some of them are vascular endothelial cells as attested by their reactivity with the Mb1 mAb. We think that in the neural fold ablation experiments carried out by Scherson et al. (1993), the few cells carrying the Dil label initially applied on the dorsal aspect of the remaining neural tube may have been either contralateral neural crest cells in the unilateral excision experiments or,

perhaps, mesodermal cells which are in close contact with the neuroepithelium and disperse later on within the mesenchyme.

In view of the above results, the source of the massive compensation of neural crest cells occurring in the above described experiments (experiment IV) had to be found. The more likely possibility is that longitudinal migration of neural crest elements was occurring into the extirpated area from the ends of the operative field. In order to test this hypothesis we proceeded to the graft of quail neural fold segments on the length of one or two rhombomeres or of the posterior mesencephalon located at the extremities of the bilateral excisions.

When the neural crest was ablated from the mid-mesencephalon to r8 and replaced by quail territories either rostrally, caudally or both, a spectacular filling of the BAs took place from rostral and caudal. Quail cells are found very early (20-25-somite stage) lateral to the unreplaced ablated region at more than 100 μm of the graft. This result shows that crest cells can migrate along non-segmental AP pathways lateral to the tube soon after they have left the neural primordium. In addition, between E3 and E4, the quail population in BA2, which is the most distant BA from the limits of the excision, increases dramatically. Therefore, crest cells keep on migrating axially late after their emergence. When the quail neural fold was implanted only at one extremity of the excised territory the same longitudinal migrations took place and the waves of crest cells of anterior and posterior origins could be easily visualized by using the QCPN mAb. Interestingly the AP extension of the longitudinal crest cells migration was similar whether they belonged to quail (grafted neural fold) or to chick (the neural fold left in situ) according to the position of the graft, thus showing that quail and chick cells have a similar behaviour in the chimeras (see experiments VA and VB). Moreover, the permanence of the quail cell marker made it possible to follow the fate of the regenerating cells. Thus, after an ablation from the posterior mesencephalon to r8 and a mesencephalic replacement, quail cells were found not only in their normal position in BA1 but also in BA2 as soon as stage HH20. This BA is normally filled by cells coming from r3/4/5, located more than 200 μm caudal from the mes-metencephalic limit. Later, some cells were even found more caudally into the conotroncus of the heart, i.e. in a territory normally colonized by the crest of r7/8. The ectopic migration observed in the BAs is confirmed later by the participation of the quail cells which form the mandibular skeleton in its totality, the entoglossum and the basihyal, the complete trigeminal ganglion and the rostral half of the acoustico-facial ganglion. These structures are normally derived from the mesencephalon, r1, r2, r3 and part of r4, from which crest cells migrate in BA1 and BA2. The structures located more posteriorly which are normally derived from the ablated portion of the rhombencephalic crest are made up of chick cells. In fact, they result from AP longitudinal migration from host territories as shown by the complementary experiment, in which, the same ablation was made and followed by the replacement of the r7/8 neural ridge by its quail equivalent. The quail cells were then found in BA4 and BA5 and ectopically in BA3 and the caudal part of BA2. They, later on, gave rise to the basibranchial, ceratobranchial, epibranchial and to the posterior part of the basihyal, as well as to the jugular-superior and caudal part of the acoustico-facial ganglia, structures normally derived from r8/7/6/5/4. When quail replacements were performed both rostrally and caudally in the mesencephalon and r7/8, the complete crest-derived territories in between were reconstituted by quail cells.

In the experiments described by Hammond and Yntema (1964) the length of the excised territory was probably too large to completely fill the gap by longitudinal migration.

Our results show therefore that, at the 5-somite stage, the ventral neural plate is no longer able to produce a neural crest. Therefore, the cells which compensate for the lacking territories originate from the nearest point where the neural crest precursors have not been removed: the contralateral side if present or another AP level if not. They also suggest that the regulation of neural crest cell proliferation is strictly controlled by an autologous cell population pressure: if no neural crest cells are present in embryonic spaces that they normally occupy, the nearest crest cells proliferate accordingly to colonize the empty sites. In contrast, the presence of crest cells must have a negative feed back effect on their own proliferation.

The Hox-code in AP regenerating neural crest cells

We have previously shown that transposition of rhombomeres along the AP axis results in a posterization of the Hox code and in a profound change in cell fate and patterning of hindbrain segments (Grapin-Botton et al., 1995). It was therefore important to assess Hox gene expression of the regenerating crest cells with respect to both their level of origin and their definitive location after their longitudinal migration.

The most important result, is that, whatever the extent of their longitudinal migration and their AP position in the embryo, the regenerating neural crest cells keep the Hox code corresponding to their level of origin. Thus, after a mesencephalic to r8 ablation and the graft of the quail posterior mesencephalic neural folds, the quail cells which are found in BA2 do not express *Hoxa-2* as can be predicted from their level of origin and in contrast to the normal situation where r3/4 crest cells fill up this arch. Therefore *Hoxa-2* expression is not induced in mesencephalic crest even though they are in contact with CNS structures which normally express it.

In these experiments the Hox code of the BAs is thus modified. These results are in contradiction with those of Hunt et al. (1995) who show that after a bilateral deletion extending from r1 to r6 or r7, the expressions of *Hoxa-3* and *Hoxb-4* are normal. This discrepancy might come from the stage at which the experiments were done. If performed after the onset of neural crest cell migration, the excision cannot pertub Hox gene expression in the BAs. Another possibility is that the extent of the excision followed by longitudinal regeneration was such that the territory of expression of the gene tested is not significantly modified (see for example *Hoxa-3* expression in our experiment VC).

In the experiments involving the removal of the neural fold from the mid-mesencephalon down to r8 with replacement of the crest by homotopic quail segments on both ends, the hindbrain neural crest derivatives are normal thus showing that the lack of *Hoxa-2* in BA2 has no noticeable consequences on neural crest development. More striking is the result of the experiments where the neural fold was removed from the diencephalic-mesencephalic constriction down to the r4/r5 limit and replaced by a r3/4 quail neural fold. In this case, BA1 and BA2 were filled up by r3/4 quail crest cells expressing *Hoxa-2*. We do not yet know what will be the consequences of the

posteriorization of the Hox code on lower jaw development but experiments are in progress to answer this question.

In conclusion, the intermediate and ventral regions of the neural plate at the mid- and hindbrain level is not able to regenerate a neural crest at the early somitic stages. In contrast, the neural folds anterior and posterior to the excision yield neural crest cells which fill up the uncolonized BAs by longitudinal migration accompanied by an increase in cell proliferation. Interestingly, the longitudinally migrating cells keep the Hox code characteristic of their level of origin in the brain. Three characteristics of the chimeras make this a promising method for further study of the role of Hox genes in the development of facial and hypobranchial structures: first the fact that a significant number of the operated embryos can survive until completion of morphogenesis, second the possibility of identifying host and donor cells any time after the operation, third the fact that by removing the chick neural folds and replacing them by their quail counterparts at various levels of the mid-hindbrain areas, a large array of possibilities is offered with respet to Hox gene expression by neural crest cells in every particular BA.

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