Plasticity of transposed rhombomeres: Hox gene induction is correlated with phenotypic modifications

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SUMMARY

In this study we have analysed the expression of *Hoxb-4*, *Hoxb-1*, *Hoxa-3*, *Hoxb-3*, *Hoxa-4* and *Hoxd-4* in the neural tube of chick and quail embryos after rhombomere (r) heterotopic transplantations within the rhombencephalic area. Grafting experiments were carried out at the 5-somite stage, i.e before rhombomere boundaries are visible. They were preceeded by the establishment of the precise fate map of the rhombencephalon in order to determine the presumptive territory corresponding to each rhombomere. When a rhombomere is transplanted from a caudal to a more rostral position it expresses the same set of Hox genes as in situ. By contrast in many cases, if rhombomeres are transplanted from rostral to caudal their Hox gene expression pattern is modified. They express genes normally activated at the new location of the explant, as evidenced by unilateral grafting. This induction occurs whether transplantation is carried out before or after rhombomere boundary formation. Moreover, the fate of the cells of caudally transplanted rhombomeres is modified: the rhombencephalic nuclei in the graft develop according to the new location as shown for an r5/6 to r8 transplantation.

Transplantation of 5 consecutive rhombomeres (i.e. r2 to r6), to the r8 level leads to the induction of *Hoxb-4* in the two posteriormost rhombomeres but not in r2,3,4. Transplantations to more caudal regions (posterior to somite 3) result in some cases in the induction of *Hoxb-4* in the whole transplant. Neither the mesoderm lateral to the graft nor the notochord is responsible for the induction. Thus, the inductive signal emanates from the neural tube itself, suggesting that planar signalling and predominance of posterior properties are involved in the patterning of the neural primordium.

Key words: hindbrain, rhombomeres, Hox genes, Hox code, chick embryo, transplantations

INTRODUCTION

Segmentation of the nervous system of the vertebrate embryo is visible during a transient phase of development in the rhombencephalon where it appears as undulations in the internal surface of the neural tube designated as rhombomeres (r). Although first described more than a century ago (Orr, 1887; Vaage, 1969) their biological significance remained uncertain until recent cellular and molecular data demonstrated they were true segmental units of organisation in the hindbrain (reviewed by Wilkinson, 1993). As a part of rhombomere formation it was noted that homologs of the *Drosophila* Hom-C homeotic selector genes, the Hox gene clusters displayed segment-restricted expression. The anterior limits of Hox gene expression in the mouse rhombencephalon coincide with the limits between rhombomeres and presents a two-segment periodicity. Thus, for the Hox B complex the anterior limit of expression is between r2 and r3, r4 and r5, and r6 and r7 for *Hoxb-2*, *Hoxb-3* and *Hoxb-4* respectively, illustrating the rule

of colinearity according to which the sequence of expression of the genes of a given cluster from rostral to caudal parallels their respective position from 3′ to 5′ on the chromosome (for reviews, McGinnis and Krumlauf, 1992; Wilkinson, 1993; Krumlauf, 1994). Thus each pair of rhombomeres expresses a specific combination of Hox genes generating a 'Hox code' which could provide a molecular identity to each segment of the hindbrain. In support to this, ectopic expression of *Hoxa-1* causes a transformation of rhombomere 2 to an r4 identity showing that Hox genes can regulate segmental identity (Zhang et al., 1994). Also consistent with the molecular interpretation of segmentation, several other genes have been shown to have a segmentally restricted expression: those of zinc finger regulators such as *Krox-20* (Wilkinson et al., 1989), of secreted signalling molecules such as *fgf-3* (Wilkinson et al., 1988) and of protein kinases such as *sek* (Nieto et al., 1992).

Although the avian Hox homeobox genes have not been studied as extensively as their mammalian counterparts, several chicken or quail Hox genes have been isolated and, in

their majority, they were shown to have a similar pattern of expression as in the mouse (Prince and Lumsden, 1994; Kuratani and Eichele, 1993; Guthrie et al., 1992; Izpisua-Belmonte, 1991). Recently, we have cloned all the members of the chicken Hox-a, -b and -d complexes and examined their expression in the hindbrain. All have identical anterior rhombomere boundaries of expression, but there is variation in the relative levels between segments (McNaughton et al., in preparation). This suggests conserved expression domains in vertebrates coupled to hindbrain segmentation.

The avian embryo was used in a number of investigations demonstrating the metameric nature of the transient segments defined as rhombomeres (Keynes and Lumsden, 1990; Lumsden, 1990; Fraser et al., 1990; Guthrie et al., 1993). Moreover, this model was crucial in the analysis of the important morphogenetic processes occurring in the rhombencephalon during embryogenesis. The rhombencephalic neural crest provides most of the mesenchymal cells which are at the origin of the hypobranchial region (see Le Douarin, 1982 for a review; Lumsden et al., 1991; Graham et al, 1993; Sechrist et al., 1993).

It was claimed, on the basis of heterotopic transplantation experiments, that the patterning of the skeletal branchial arches is, at least to a certain extent, under the control of the neural crest-derived mesectodermal cells (Noden, 1978). This view was further substantiated by the observation that in most cases the neural crest cells and the rhombomere from which they migrate express the same 'Hox code' (Hunt et al., 1991). Finally, the biological significance of the 'Hox code' in specifying the identity of both the rhombomeres and their neural crest derivatives was established in the mouse by the targeted mutation method. Null mutations of several Hox genes (*Hoxa-1*: Carpenter et al., 1993; Mark et al., 1993) (*Hoxa-2*: Gendron-maguire et al., 1993; Rijli et al., 1993) (*Hoxa-3*: Chisaka and Capecchi, 1991; Condie and Capecchi, 1994) result in severe anomalies or deficiencies in the development of the hindbrain in some cases and also of the mesectodermal or neural derivatives of the corresponding neural crest (for reviews, McGinnis and Krumlauf, 1992 and Krumlauf, 1994). It was therefore of interest to see to what extent the 'Hox code' is intrinsically determined in the neuroepithelium of a given rhombomere during the early stages of neurogenesis. Previously, grafting experiments in the chick suggested that rhombomeres behaved as cell autonomous units in ectopic locations, maintaining their proper patterns of Hox expression (Kuratani and Eichele, 1993; Guthrie et al., 1992; Simon et al., 1995), and we wanted to examine this in more detail. For this purpose, we have performed heterotopic transplantation experiments of the presumptive territory of defined rhombomeres within the area of the hindbrain and medulla oblongata (from r1 to somite 7). We have taken advantage of the quail-chick chimera system (Le Douarin, 1969; Le Douarin et al., 1984) to identify precisely the grafted tissue in the chimeras and used the probes for *Hoxb-1, b-3, b-4, a-3* and *d-4* to assess its capacity to express a given set of Hox genes. Most of the experiments were done at the 5-somite stage i.e. before the formation of rhombomere boundaries. In order to know as precisely as possible the anteroposterior limits of the presumptive rhombomeres at these early stages, we have first established a prospective map of the rhombencephalic neuroepithelium by using DiI and carbon particles marks.

It appears that the transplantation of a given presumptive rhombomeric territory from a caudal to a rostral level of the anteroposterior (AP) axis does not perturb the expression of the *Hoxb-4* gene in the grafts. In contrast, transposition of rhombomeres from a rostral to a caudal position results in the induction in the transplant of all the Hox genes tested, which are normally expressed in the graft site. This induction is still possible at stages following the establishment of rhombomere boundaries. We also show that the inductive signal diffuses tangentially in the neural epithelium. These results show that cell interactions play a significant role in maintaining the 'Hox code' at the levels of the neuroepithelium considered in this study.

MATERIALS AND METHODS

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-somite stage precisely unless otherwise indicated (about 30 hours of incubation in a humidified atmosphere at 38°C). A window was cut in the shell and India ink diluted 1:1 in PBS was injected into the sub-blastodermic cavity in order to make the embryonic structures more visible without using any 'vital' stain. The vitelline membrane was windowed and a gap corresponding to a certain number of hemi-rhombomeres (as indicated in Tables 1 and 2) was made in the neural tube using a microscalpel, made by sharpening a steel needle on an Arkansas stone. The limits of the grafted tissues and of the sites of implantation are defined according to a map in which the anteroposterior limits of the rhombomeres were established as described below. Hemineural plates corresponding to a defined number of rhombomeres were dissected out from a quail donor. In most experiments, the neural plate was not dissociated from its surrounding tissues by enzymatic digestion. In some instances, possible contamination by mesodermal cells was eliminated by dissociation of the neural primordium after treatment with pancreatin (GIBCO). The notochord was left intact in the host embryos and in no case was it included in the graft.

In certain experiments, supernumerary notochord grafts were carried out. A fragment of either vagal, truncal or rhombencephalic notochord from a 10-somite donor was grafted in a lateroventral position, with respect to the rhombencephalon, by insertion into an incision along the full length of the rhombencephalic neural plate of a 5-somite recipient.

The operated embryos were killed in most cases at stage 14-15 according to Hamburger and Hamilton (HH; 1951). In some experiments, they were allowed to develop up to stage 20 or 34 (3 and 8 days of incubation respectively: E3 and E8). The fixative used was Carnoy's fluid. Paraffin sections were cut frontally at 5 μm for the early stages (stages 14-15 and 20) and at 6.5 μm transversally for E8 embryos. Young embryos were then treated for in situ hybridization whereas alternate sections of E8 embryos were treated using the Feulgen-Rossenbeck (1924) technique, which enables a better visualization of quail nuclei, and cresyl violet, which gives a better definition of the structures.

Mapping of the rhombomere presumptive territories at the 5-somite stage
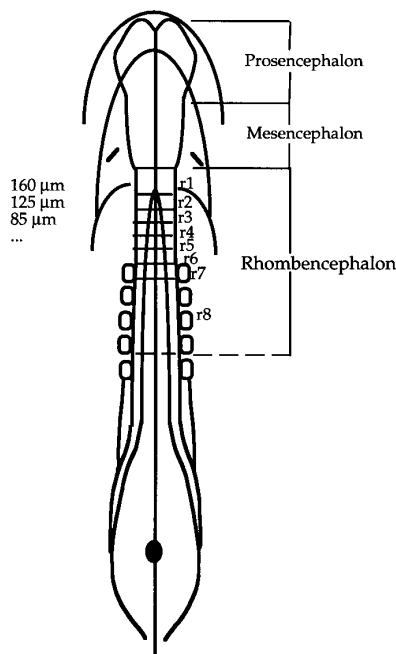
Mapping of the rhombencephalon was carried out in both quail and chick embryos. 20 chick and 12 quail embryos were used. Although the embryos were carefully staged at 5 somites, they differed slightly in size. To obtain reproducible results despite this fact, the magnification was set in order to place the top of the head and the rostral limit of the first somite at defined postitions on a micrometric scale. The

distance between these two markers was of 1600 µm±10%. The limit between r1 and the mesencephalon, already visible at the 5-somite stage as a notch in the neural tube, was located at 980 µm±10% from the tip of the head and 620 µm±10% from the anterior limit of the first somite (Fig. 1). Several incisions were then made in the neural tube in defined positions, measured on the scale with an resolution of 20 µm (length of half a graduation at this magnitude). Carbon particles or DiI (at a 0.05% concentration in 3 M sucrose in PBS) were then used to label the cells in the incision. Embryos were observed 24 hours later and the markers were assigned to the now formed rhombomeres. The limit between rhombomeres 6 and 7 corresponded always to the anterior limit of somite 1. Presumptive r1 had a size of 160 µm±20%. Presumptive r2 had a size of 125µm±20%. Presumptive r3 to r7 had a size of 85µm±25%. r8 whose posterior limit is undefined was not mapped (Fig. 1).

In situ hybridization

Probes

To generate suitable probes for the in situ analyses, we used the following cDNA fragments: a 500 bp *Bgl*II-*Eco*RI fragment of chicken *Hoxb-1* subcloned in pGem3, a 400 bp *Eco*RI-*Sph*I fragment of chicken *Hoxb-3* subcloned in pGem4, a 1170 bp fragment of chicken *Hoxb-4* subcloned in pBluescript, a 1300 bp fragment of chicken *Hoxd-4* subcloned in pBluescript, a fragment of chicken *Hoxa-4* subcloned in pBluescript, a 900 bp *Kpn*I-*Eco*RI fragment of chicken *Hoxa-3* subcloned in pBluescript KS+ (McNaughton et al., unpublished data). The RNA probes were labelled either by incorporation of ^{35}S-UTP (Amersham, 1000 Ci/mmol) during synthesis (Promega, Riboprobe Gemini II) or incorporation of dig-UTP (Boehringer Mannheim). The radioactive probes were then hydrolysed to generate 150 nt fragments. The sizes of probe fragments were checked by gel electrophoresis. The non-radioactive probes were used as full length transcripts.
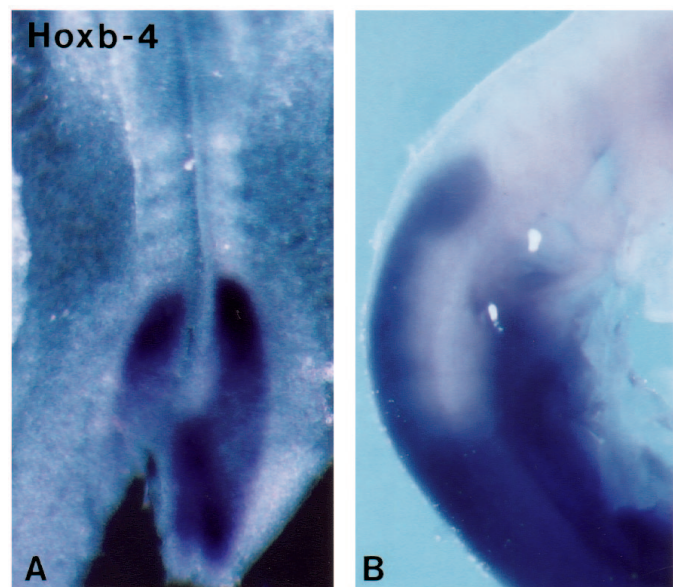
Hybridization

Radioactive hybridizations were carried out as previously described (Eichmann et al., 1993). Probes were used at a minimum concentration of 10^4 counts/min/µl. In order to obtain a well defined signal, the time of exposure was 7 days for *Hoxb-4*, 10 days for *Hoxb-1*, *Hoxb-3* and 15 days for *Hoxa-3*. The sections were counterstained with Gill's hematoxylin. Whole-mount non-radioactive in situ hybridization was performed exactly according to Wilkinson et al. (1991).

RESULTS

Hox gene expression in control embryos

Previously we had established the expression pattern in the neural primordium of the 6 Hox genes used in this study (Morrisson et al., submitted; McNaughton et al., unpublished data) and we have verified their expression in quail in comparison with chick embryos from 5 somites to 3 days. The precise points of the expression pattern which relate to the study are summarised below.

At grafting time (5-somite stage) *Hoxb-4* is strongly expressed in the mesoderm and more weakly expressed in the neural primordium at the AP level of the unsegmented region (Fig. 2A). At stage 14-15 (20-26 somite), the rostral limit of expression has spread forward up to the boundary between r6 and r7 (Fig. 2B), as recently shown also in the chick for its paralogue *Hoxd-4* (Gaunt and Strachan, 1994). As in the mouse, it is noticeable that the expression is lower in r7 than in the rest of the tube. At stage 20 (3 days of incubation) the labelling in the external layer of the neuroepithelium in r7 and r8 becomes higher than at stage 14-15 (20-26 somite) (Fig.

Fig. 1. Fate map of the presumptive territories of the rhombomeres at the 5-somite stage. The size of the presumptive rhombomeres is measured in quail (*Coturnix coturnix japonica*) and chick (*Gallus gallus* strain JA57) under a micrometric scale by placing carbon and DiI markers at defined positions on the neural tube. One day later, the position of the markers is assigned to a particular rhombomere (see Materials and Methods section). The size of the rhombomeric segments is about the same for r3 to r7. In contrast, r1, r2 and r8 are larger. The sizes of r1, r2, r3 are given on the left.

Fig. 2. Distribution of *Hoxb-4* transcripts in wholemount preparations of 5-somite (A) and 14-15 HH (B) stages chick embryos. A is photographed from its dorsal aspect whereas B is photographed from its lateral aspect. (A) *Hoxb-4* is expressed in the unsegmented paraxial mesoderm and in the neural tube at the same level. (B) Expression of *Hoxb-4* has reached more anterior levels (r6/7 limit) in the neural tube.
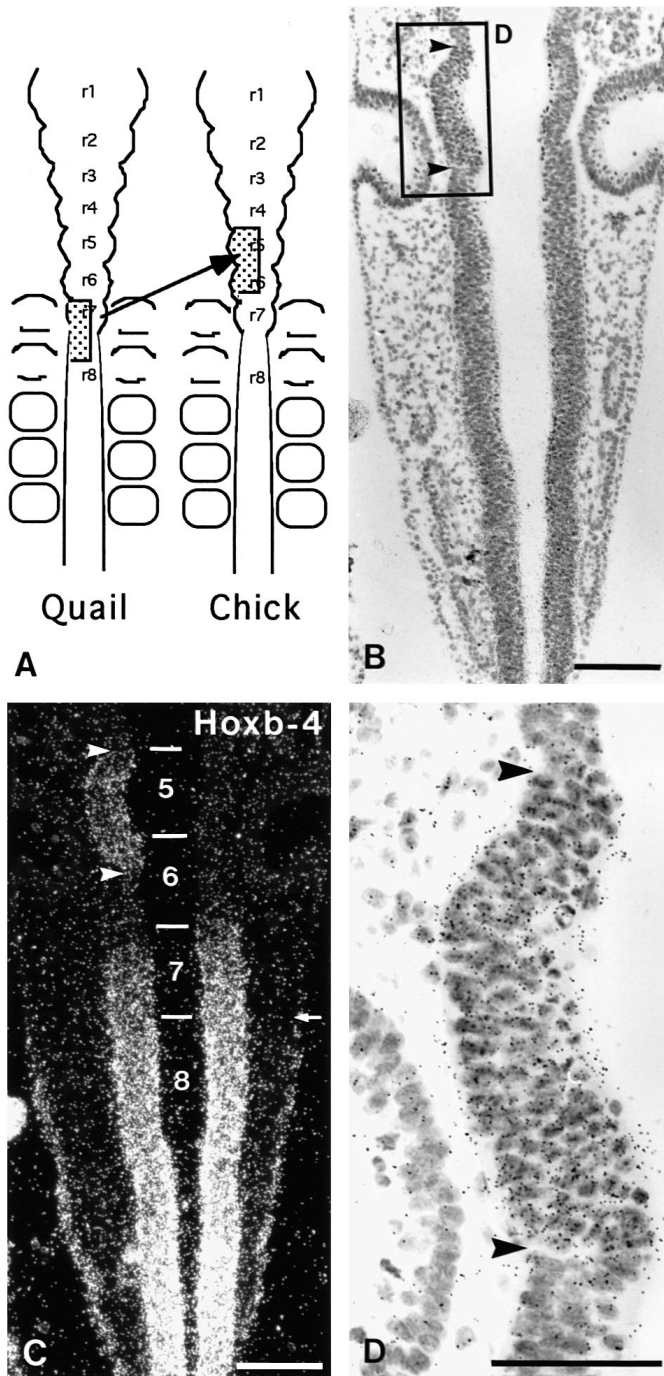
Fig. 3. In situ analysis of *Hoxb-4* expression after transplantation at the 5-somite stage of r7/8a in r5/6 on the left side (A). The embryo has been sectioned frontally, 1 day after the graft, at stage 15. The grafted tissue facing the otic vesicule covers r5 and a part of r6 as revealed by an hematoxylin staining in B and at a higher magnification in D, where one can distinguish quail nuclei and silver grains in the same territory which corresponds to the graft. (C) In the chick host territory, *Hoxb-4* is expressed in a gradient from r7 downward. In the superficial ectoderm, *Hoxb-4* is expressed from the level of r8 downward (arrow). On the grafted side (left), the transplant is clearly labelled. In this case, the mesodermal cells have been removed from the explant using pancreatin. Scale bars in B and C 100 µm, and in D 50 µm. Arrowheads indicate the limits of the graft. Numbers correspond to the host's rhombomeres.

Table 1. Summary of two-rhombomere transplantations

Level of origin	Level of transplantation	Label	Stage of operation	Number of embryos
r7-8 or r8	r5-6	Hoxb-4	5-somite	6+2*+2†
r7-8 or r8	r5-6	Hoxb-4	9-11 somite	3
r8	r3-4 or 1-2	Hoxb-4	5-9-somite	9‡
r5-6	r6-7	Hoxb-4	5-somite	2
r5-6	r7-8	Hoxb-4	5-somite	2
r5-6	r7-8	Hoxb-4	9-11 somite	3
r5-6	r8	Hoxb-4	5-somite	5+2*+14†
r3-4	r7-8 or r8	Hoxb-4	5-somite	5
r1-2	r7-8 or r8	Hoxb-4	5-somite	11+3‡
r1-2 or r3-4	r8	Hoxa-3	5-somite	5
r1-2 or r3-4	r8	Hoxb-3	5-somite	5
r5-6	r8	Hoxb-1	5-somite	3
r5-6	r8	Hoxd-4	5-somite	3†
r5-6	r8	Hoxa-4	5-somite	3†

(*) indicates an enzymatic dissociation of the transplant.
(†) indicates that instead of radioactive in situ hybridization, whole-mount in situ hybridization was performed.
(‡) indicates fixation at stage 20 instead of 14-15.

10E). Moreover, the external layer of the alar plate is also labelled in r6 dorsally. The labelling patterns are identical in quail and chick.

The other genes were studied at stages 14-15 (20-26 somite) and 20 (3 days). *Hoxd-4* and *Hoxa-4* are expressed in the neuroepithelium like their paralogue *Hoxb-4* at stage 14-15 (Fig. 6G,H). At stage 14-15, *Hoxb-3* is expressed at a very low level in r5 and r6 and at a higher level more posteriorly. At stage 20 *Hoxb-3* labelling is still low in r5 and r6, and higher in r7 and r8 and the spinal cord. The message is essentially concentrated in the external layer of the neuroepithelium at all levels (Fig. 10F). At stage 14-15, *Hoxb-1* is expressed from r7 downward and in addition in a single band in r4 at a very high level (Maden et al., 1991; Sundin and Eichele, 1992). At stage 20, the labelling becomes more intense in the external layer of the neuroepithelium at all levels (Fig. 10G). *Hoxa-3* is expressed

Fig. 4. Posteriorization of pairs of rhombomeres as shown by *Hoxb-4* expression. (A) Transplantation of r5/6 to the position of r7/8a. (B) Bright-field photomicrograph of a frontal section of this embryo. The grafted territory is framed, and enlarged in C where the quail cells are recognizable from the neighbouring chick host tissues. (D) Dark-field photomicrograph of the same section as in B. *Hoxb-4* is induced in the graft, on the right side. Notice the anteroposterior gradient of expression. (E) Transplantation of r5/6 to the position of r6/7. (F) Bright-field photomicrograph of a frontal section of this embryo. (G) Higher magnification of the graft. (H) Dark-field photomicrograph of the same section as in F. *Hoxb-4* is induced in the r7 position but not in r6, thus reconstituting a normal *Hoxb-4* pattern. (I) Transplantation of r3/4 at the level of r8. (J) Bright-field photomicrograph of a frontal section of this embryo. (K) Higher magnification where the graft is visualized among the chick host tissues. (L) Dark-field photomicrograph of the same section as in J. *Hoxb-4* is induced in the graft as in the contralateral control side. (M) Transplantation of r1/2 to the level of r8p. (N) Bright-field photomicrograph of a frontal section of this embryo. (O) Higher magnification of the graft. (P) Dark-field photomicrograph of the same section as in N. The posterior part of the graft expresses *Hoxb-4* messenger whereas its anterior part doesn't. Scale bars 100 µm. Arrowheads indicate the limits of the grafts. Numbers correspond to the host's rhombomeres.
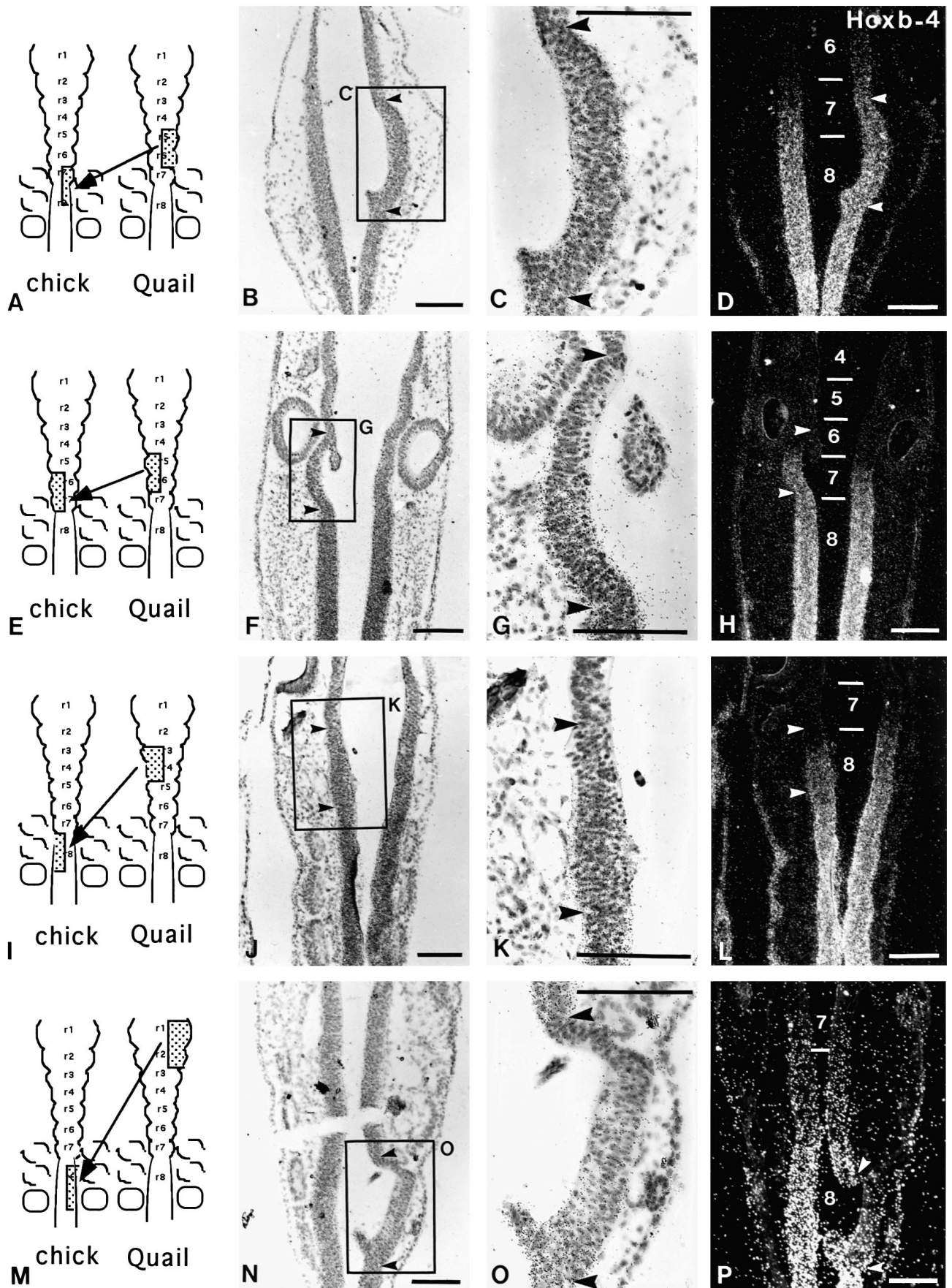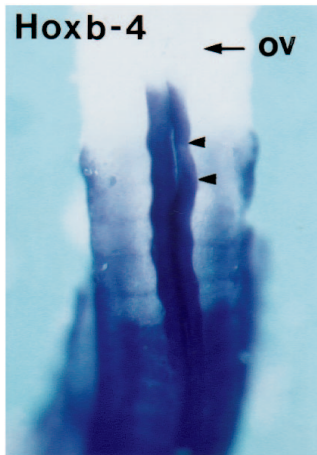
Fig. 5. Whole-mount in situ hybridization with *Hoxb-4* of an embryo which has received, in r8p, a r5/6 graft on the right side at the 5-somite stage. Regions to which the probe has hybridized are labelled in blue. *Hoxb-4* is induced in the graft (arrowheads) at a level similar to the contralateral side. The otic vesicle (ov) marks the position of r5/6.

at a high level in r5 and r6 and at a lower level from r7 to the tail at stage 14-15 (Fig. 6D).

Expression of *Hoxb-4* in rostrally transposed rhombomeres

Since the chick probe cross-reacts with quail mRNA, and since chick and quail *Hoxb-4* expressions are identical, we made use of the quail-chick chimera system to investigate the expression of *Hoxb-4* in heterotopic transplants of pairs of rhombomeres. The quail territory corresponding to r7 and the anterior part of r8 lateral to somite 2 (r8a), which will normally express *Hoxb-4*, was transplanted into a chick host to the position of r5 and r6, where expression of this gene is never observed. The grafts were unilateral so that the unoperated side serves as control (Table 1). Fig. 3 shows that the transplanted rhombomeres r7/8a exhibit a level of *Hoxb-4* expression identical to control r7 and r8a. Therefore, at the 5-somite stage, this territory already has the information to express the *Hoxb-4* gene and its expression is not negatively influenced by surrounding anterior tissues, which do not express this gene. The expression of *Hoxa-4* and *Hoxd-4* is also maintained in r7/8a transplanted in r5/6 (data not shown). These 3 genes were also maintained in homospecific transplantations, i.e. chick r7/8a grafted into chick at the r5/6 level (data not shown).

It has to be noted that in 20% of the cases observed 48 hours after the graft (n=9) *Hoxb-4* was induced in the adjacent host epithelium.

Inducibility of *Hoxb-4* in caudally transplanted rhombomeres

It led us to see whether *Hoxb-4* can be regularly induced in territories rostral to the normal limit of expression of this gene after transplantation to a more posterior level.

In order to answer this question, rhombomeres 5 and 6 were transplanted unilaterally at the transverse level of r7 and r8a (Table 1). These rhombomeres are normally not expressing *Hoxb-4*, but were found to express this gene as soon as 16 hours after the graft, i.e. at stage 14 (Fig. 4A-D). The level of expression

was the same as in the controlateral side, i.e, high in r6 (r8 position) and lower in r5 (r7 position). When the r5/6 transplant was transferred more posteriorly to the level of r8p (r8 posterior, facing somites 3 and 4) (Table 1), *Hoxb-4* was up-regulated in the transplant as in the contralateral side, i.e. at a high level in the whole transplant (data not shown). Whole-mount in situ hybridization was performed on 14 embryos that had received quail or chick transplants and they were then observed 24 hours after grafting (i.e. at stage 14-15) (Table 1). In 11 of them a blue coloration corresponding to the presence of *Hoxb-4* in the graft appeared. The intensity of the coloration in the graft compared to the control side was the same in 3 cases (Fig. 5) and lower in 8 cases. In the 3 remaining cases there was no *Hoxb-4* induction. The latter observation may reflect the incomplete incorporation of the graft within the host's neuroepithelium, a situation difficult to appreciate in whole-mount staining. The induction was not different in chick and quail transplants. In some cases the observation was made 48 hours after the operation. Induction of *Hoxb-4* was seen in all embryos. In addition, r5/6 grafted in r6/7 position became positive in r6 (r7 position) and remained negative in r5 (r6 position) like the contralateral control side (Table 1; Fig. 4E-H). Induction of *Hoxb-4* in the grafted rhombomeres therefore respects the normal anterior boundary of expression of this gene, and expression of *Hoxb-4* follows the same pattern as in the host's rhombencephalon.

Treatment of the transplant with pancreatin prior to the graft in order to remove possible adherent mesenchymal cells has no effect on the induction (Table 1). However, the induction of *Hoxb-4* was only observed in cases where the graft was perfectly integrated in the host neural tube, and when its anterior and posterior limits were in contact with the sections made in the host neuroepithelium, making its limits not visible except by referring to the nuclear structure of the implanted quail cells. This finding suggests that expression of *Hoxb-4* is regulated within the neurectoderm by signals coming from surrounding tissues, i.e. from anterior, posterior or from lateral neuroepithelial cells. These rostral to caudal transpositions reveal that Hox expression does not always behave in a cell autonomous manner since the grafted rhombomeres express *Hoxb-4* according to a pattern consistent with their novel position along the AP axis.

Induction and non-autonomy of anterior rhombomeres

In order to know whether *Hoxb-4* was also inducible in anterior rhombomeres, we transplanted r1/2 and r3/4 pairs into r7/8a or r8p (Table 1). In the case of r3/4 transplantations (Fig. 4I-L), *Hoxb-4* was always induced in the transplant whereas for r1/2 the induction occurred in 7 out of 11 cases and was restricted to the posterior part of the graft when observed 24 hours after the graft (Fig. 4M-P). However, a complete induction of *Hoxb-4* occurred in all the r1/2 to r8p grafts when the chimeric embryo was killed 1 day later, i.e. at stage 20 (3 days of incubation).

Then we addressed the question as to whether the induction can be obtained on grafted tissue taken at later developmental stages. Transplantations of r5/6 to r7/8a or r8p after the formation of rhombomere boundaries gave similar results to those described above (Table 1), showing that both the stimulus responsible for the induction and the capability to respond are still present at these stages.

Inducibility of other Hox genes

The transplantation of rhombomeres 5/6 into the r8 position also leads to the activation of *Hoxd-4* (Fig. 6G), *Hoxa-4* (Fig. 6H) and *Hoxb-1* (Table 1). Transplantation of r3/4 or r1/2 into r8p leads to the activation of *Hoxa-3* (Fig. 6B-E) and *Hoxb-3* (data not shown, Table 1). This shows that induction of all the Hox genes tested, which are normally expressed at this level, is dependent on the position along the body axis.

However, *Hoxb-1* was never induced in quail r2 rhombomeres put into r4 (data not shown), as previously shown by others for chick to chick grafts (Kuratani and Eichele, 1993; Guthrie et al., 1992).

Origin of the inducing signal

Our transplantation studies demonstrate that Hox expression patterns and possibly their segmental identity are at least partly determined by adjacent tissues. As schematized in Fig. 7, the induction may originate from the neuroepithelium either through transversal (Fig. 7A) or longitudinal signals (Fig. 7B). The lateral (Fig. 7C) or ventral mesoderm, i.e. the notochord (Fig. 7D) might also be responsible for providing the graft with new positional information.

To test a possible role of the contralateral neural tube, we performed bilateral grafts of transverse neuromeric fragments from the level of r5/6 to that of r8p (Table 2). The transcrip-
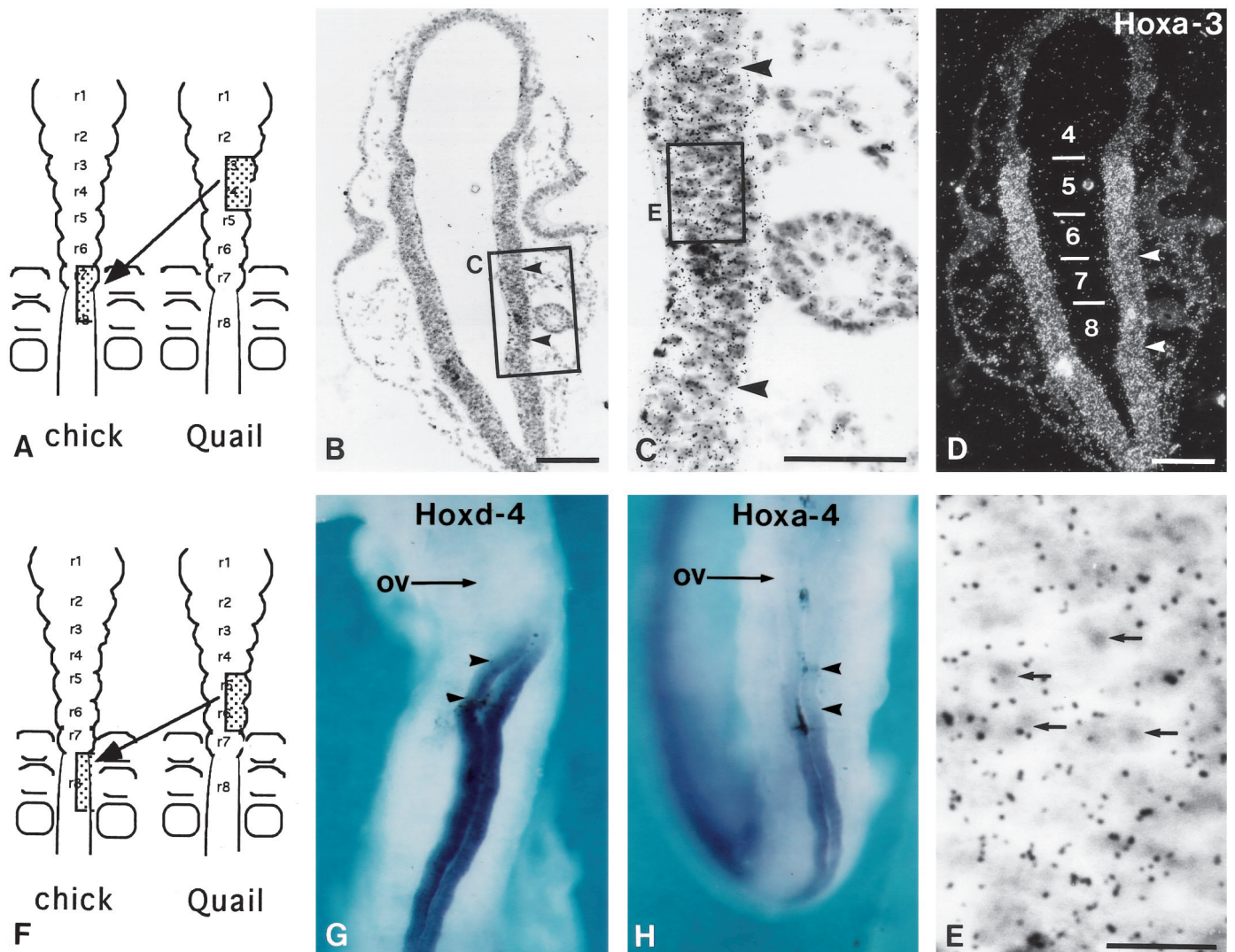
Fig. 6. Induction of various Hox genes after caudal transplantation. (A) Transplantation of an r3/4 at the level of r8 on the right side at the 5-somite stage. (B) Frontal section of an embryo treated as in A which has received an r3/4. Part of the ectodermic presumptive territory of the otic placode has been transplanted and gives rise to a small extra otic placode. (C,E) Higher magnifications show the quail neurectoderm with the chromatic condensations of quail nuclei well visible (arrows). (D) Dark-field photomicrograph of the same section as in B showing strong *Hoxa-3* expression in r5/6 and weaker expression posteriorly both in the graft and the contralateral control side. (F,G,H) Transplantations of r5/6 at the level of r8 at the 5-somite stage. (G) An embryo grafted on the left side hybridized with a *Hoxd-4* probe. (H) An embryo grafted on the right side hybridized with a *Hoxa-4* probe. The Hox genes that are not expressed in the transplanted rhombomeres at the time of the graft are induced in all cases at the same level as in the control non-operated contralateral side. The otic vesicle marks the position of r5/6. Scale bars, 100 μm in B and D; 50 μm in C, and 10 μm in E. Arrowheads indicate the limits of the grafts. Numbers correspond to the host's rhombomeres.

tion of the *Hoxb-4* gene was found to be up-regulated in the whole graft as it was in unilateral transplantations (Fig. 8A-D). Thus, a source other than the contralateral neuroepithelium must operate in these circumstances.

It was previously shown that the anterior notochord is able to induce *En-2* expression in the neurectoderm more efficiently than the posterior notochord in explant association experiments (Hemmati-Brivanlou et al., 1990). The notochord might thus be at the origin of the induction of genes containing a homeodomain. In order to test its possible role in reprogramming *Hoxb-4* expression in the grafted neural ectoderm, we implanted a supernumerary notochord fragment from the level of somites 1 to 8 lateroventrally to the presumptive level of r1 to r6 at the 5-somite stage (Fig. 8E-G). The presence of the extra piece of notochord originating from the region where *Hoxb-4* is strongly expressed in the neural tube did not induce the activation of this gene in the anterior rhombomeres (Fig. 8H). The notochord by itself at this stage does not seem to be responsible for *Hoxb-4* expression in transplanted rhombomeres.

Alternatively, *Hoxb-4* could be induced by signals coming either laterally or longitudinally from the mesoderm or the neural tube located anterior or posterior to the graft. To distinguish between these hypotheses we grafted large rhombomeric territories extending from r2 to r6, both included in the position of the host's somites 1 to 4 or 2 to 5 (Table 2; Fig. 8I-L). In these embryos, *Hoxb-4* was induced in r5 and r6 (now placed at the level of r8) whereas r2,3,4 remained negative. This result shows that the inducing signal does not come from the tissues lateral to the graft (mesoderm, superficial ectoderm) since *Hoxb-4* is inducible in short grafts of r2,3,4 placed in the same position. Therefore, the obvious interpretation of this result is that the inductive signal spreads in the graft along the AP axis from the posterior neural tube, inducing high levels of *Hoxb-4* in r6 and lower levels in r5. This pattern schematized in Fig. 9, is the same when the chimera is observed at E3 (i.e. 2 days after grafting) (Fig. 10E). Although the host's neuroepithelium expresses *Hoxb-4* cranially to the graft, the signal in this case does not spread in a rostrocaudal direction since r2-4 remained negative. However, when the r2-6 fragment was grafted more posteriorly, lateral to somites 3-6 or 4-7 (Fig. 9), *Hoxb-4* was induced in both r5 and r6 in all the 5 cases observed but also in the anterior part of the graft after 2 days (3 cases). Among these 3 cases, in 2 cases a central zone devoid of *Hoxb-4* expression was observed in the explant. In the remaining case, transcripts of the gene where present in the whole grafted rhombomeres but with a central region where the transcripts were sparse. This indicates that the inducing signal(s), is present in sufficient amounts from the level of r8 downward. In r7 however, where *Hoxb-4* is expressed at a lower level than posteriorly, the inductive capacity is not sufficient to induce *Hoxb-4* expression in the grafted neuroepithelium.

The r2-6 grafted embryos, labelled with a *Hoxb-3* probe show an enhancement of the signal in r6 and the posterior part of r5 (Fig. 10F). Moreover, whereas the expression of *Hoxb-1* is maintained in r4, its posterior domain of expression does alter and is expanded rostrally to the posterior half of r5 and is up-regulated in r6 (Fig. 10G). There is thus an enhancement of the expression of the 3 genes described.

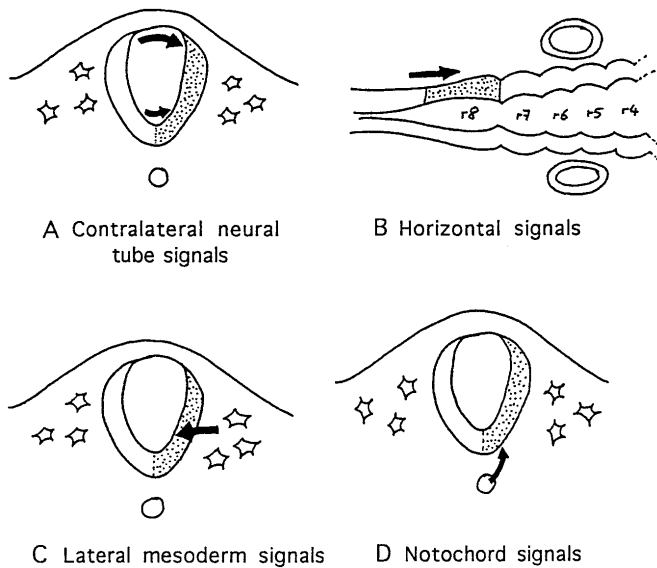## Correlation of Hox gene induction with phenotypic changes in further differentiation of the rhombomere

There are indications that the Hox code is responsible for generating segment identity. Since the Hox code was changed in our grafts, we wondered whether this change in gene expression pattern resulted in a change in rhombomere identity and further differentiation. We grafted r5/6 in the r8p position unilaterally and compared the nuclei of the hindbrain in the grafted and the control side at stage 34 (E8) on transverse sections stained with cresyl violet. The position of the graft was recognized on adjacent sections stained with Feulgen-Rossenbeck technique by the centronuclear mass of heterochromatin characteristic of the quail nuclei. In order to know the rhombomeric origin of the nuclei of the hindbrain, we previously performed 3 homotopic replacements of r5/6 at 5-somite stage and fixed the embryos at day 8 of incubation (Fig. 11A). The quail cells, i.e. cells of r5/6 origin, are found in particular in nuclei tangentialis (11F,G), laminaris (11C,D) and magnocellularis (11C,E) for the sensory nuclei, and nVI, nVII and nIX-X for the motor nuclei. Quail cells were never observed in the inferior olivary nucleus nor the nucleus of nerve XII and nucleus supraspinalis. These results are in agreement with previous observations made for motor nuclei (Keynes et al., 1990).

In chimeras where quail r5/6 were placed in the r8p position of a chick rhombencephalon (Fig. 11B), we investigated whether the nuclei in the graft were of r5/6 or r8 type. It appears that at the level of the graft the nuclei already formed at E8 (Harkmark, 1954; Tan and Le Douarin, 1991) are identical to the contralateral chick nuclei. The overall shape of the neural tube is identical bilaterally from the rostral (Fig. 11H) to the caudal (Fig. 11I) part of the graft. The Xth nerve roots are located at the same level on both sides at the rostral end of the graft (Fig. 11H) and XIIth ventral nerve roots at the caudal end of the graft (Fig. 11I). Nuclei of nerves IX-X and XII, nucleus supraspinalis (Fig. 11J,K,N,O) and both ventral and dorsal inferior olivary nuclei (Fig. 11L,M,P,Q) can clearly be distinguished. Moreover, nuclei characteristic of a r5/6 origin: n. angularis, n. tangentialis, n. laminaris, n. magnocellularis and n. abducens, are never observed in the graft. The phenotype of the transplanted rhombomeres is thus modified in the graft and converted from r5/6 to r8p. The phenotypic modification is thus in agreement with the genetic conversion.

DISCUSSION

One of the aims of this study was to systematically investigate whether the Hox-code which characterizes the hindbrain segments during development is intrinsically determined or if it can be altered in heterotopically transplanted rhombomeres. It is of interest if the latter alternative proves to be the case, whether the influence of the *Hox*-code on the subsequent development of hindbrain structures could be challenged. However, current information has suggested that rhombomeres behave in a strictly autonomous manner (Guthrie et al., 1992; Kuratani and Eichele, 1993, Simon et al., 1995). The findings in this study clearly demonstrate differential responses to environmental changes depending upon the type of rostrocaudal graft

A Contralateral neural tube signals

B Horizontal signals

C Lateral mesoderm signals

D Notochord signals

Fig. 7. Possible origins of the inductive signal. The graft is represented by the dotted areas. Four hypotheses are proposed to account for the origin of the signal which induces *Hoxb-4* in the posteriorised transplants. In A the signal diffuses laterally from the contralateral neural tube, in B the signal diffuses tangentially in the plane of the neuroepithelium. The lateral mesoderm (C) or the notochord (D) could also induce the gene in the graft.

Table 2. Transplantations realised to determine the origin of the inductive signal

| Operation	Stage of operation	Label	Number of embryos
Bilateral transplantation of r5-6 into r7-8 or r8	5- and 9-somite	Hoxb-4	3
Supernumerary troncal notochord	5-somite	Hoxb-4	4
r2-6 into r7 and following neuroepithelium	9-somite	Hoxb-4	1
r2-6 into r8 and following neuroepithelium	9-somite	Hoxb-4	2+2*
r2-6 into r8 and following neuroepithelium	9-somite	Hoxb-1	2*
r2-6 into r8 and following neuroepithelium	9-somite	Hoxb-3	2*
r2-6 into r8 lateral to somite 3 and more posteriorly	9-somite	Hoxb-4	2+3*

Radioactive in situ hybridization was performed in all cases.
(*) Embryos killed 2 days after the graft instead of 1, i.e. at stage 20 (HH).

and suggest the presence of inducing signals which alter the identity of grafted tissue. This provides strong evidence for plasticity in the posterior hindbrain.

Caudal to rostral grafts demonstrate cell autonomy

The heterotopic transplantations of the presumptive rhombomeres have in most cases been performed precisely at the 5-somite stage for both donor and host embryos. Quail and chick combinations have ensured that host and donor tissues can be precisely delineated when the results are analyzed whatever the stage considered, either by in situ hybridization or, later in development, when neural structures are recognizable. Since at the 5-somite stage, rhombomeres have not formed morphologically or become lineage restricted, a careful mapping of the hindbrain territory was first established in order to localize the level of the presumptive rhombomeres in both host and donor embryos. The fate map represented in Fig. 1 could thus be constructed. Certain tranplantations were also made later in development, after the formation of rhombomere boundaries, in order to test the temporal extension of the neural plasticity evidenced in earlier grafts. Our investigations have concerned essentially the gene *Hoxb-4* and its paralogs *Hoxa-4* and *Hoxd-4*, and also *Hoxb-1, Hoxa-3* and *Hoxb-3*.

We found that expression of the *Hoxb-4* gene is already programmed at the 5-somite stage in the neuroepithelium of the quail embryo even in its anteriormost area of expression (i.e. that corresponding to r7 and 8a). In fact, in general agreement with previous evidence for cell autonomy of *Hoxb-1* in the tube and *Hoxa-2* in the crest, when transplanted at the presumptive level of r5 and r6, the neuroepithelium corresponding to r7/r8a expressed *Hoxb-4*, *Hoxd-4* and *Hoxa-4* in a cell-autonomous manner, that is in a pattern characteristic of site of origin not of the ectopic location.

It was also important to note the fact that the quail hemineural tube transplanted anteriorly did not induce the r5/6 contralateral chick neuroepithelium to express the *Hoxb-4* gene. Hence, signals that maintain Hox expression do not appear to move contralaterally in these conditions. It was easy to see that the *Hoxb-4* transcripts were strictly restricted to the quail cells. Since 1 somite is added every hour, this determination takes place at least 9 hours before transcripts can be detected. Moreover, it is interesting to consider that this determination is intrinsic to the neural tube since complete removal of the mesoderm from the explant by pancreatin has no influence on Hox gene expression. These results are also in agreement with those described by Gaunt and Strachan (1994) showing that implantation of a glass barrier between the posterior domain of expression and its definitive rostral boundary does not prevent the expression of *Hoxd-4* rostral to the barrier. Together, this indicates that if a posterior inducing signal is required to maintain or establish expression of the Hox genes it is in place very early before expression actually occurs.

Other regions of the chick neuroepithelium have been shown to maintain their expression when transplanted heterotopically. Such is the case for the *En2* gene which was still expressed in transplants displaced from the mes- to the diencephalic territory at the 10-somite stage (Itasaki et al., 1991; Gardner and Barald, 1991). The early commitment of the mes-metencephalic cells to express *En-2* was further attested by transplantations carried out at the 3-5-somite stage to the rhombencephalic level (Marc Hallonet, personnal communication).

Plasticity of hindbrain rhombomeres

The fact that the regions of the neuroepithelium which normally express *Hoxb-4* are committed early and behave autonomously when heterotopically transplanted to some regions, does not necessarily imply that expression of this gene is not inducible in other, normally non-expressing regions of the brain. In order to answer this second question, territories corresponding to the rostral hemi-rhombomeres 1/2, 3/4 and 5/6 were translocated caudally and grafted at the presumptive levels of the r7/8a or r8. Surprisingly, in all cases transcription of the *Hoxb-4* gene was induced in the transplant at levels
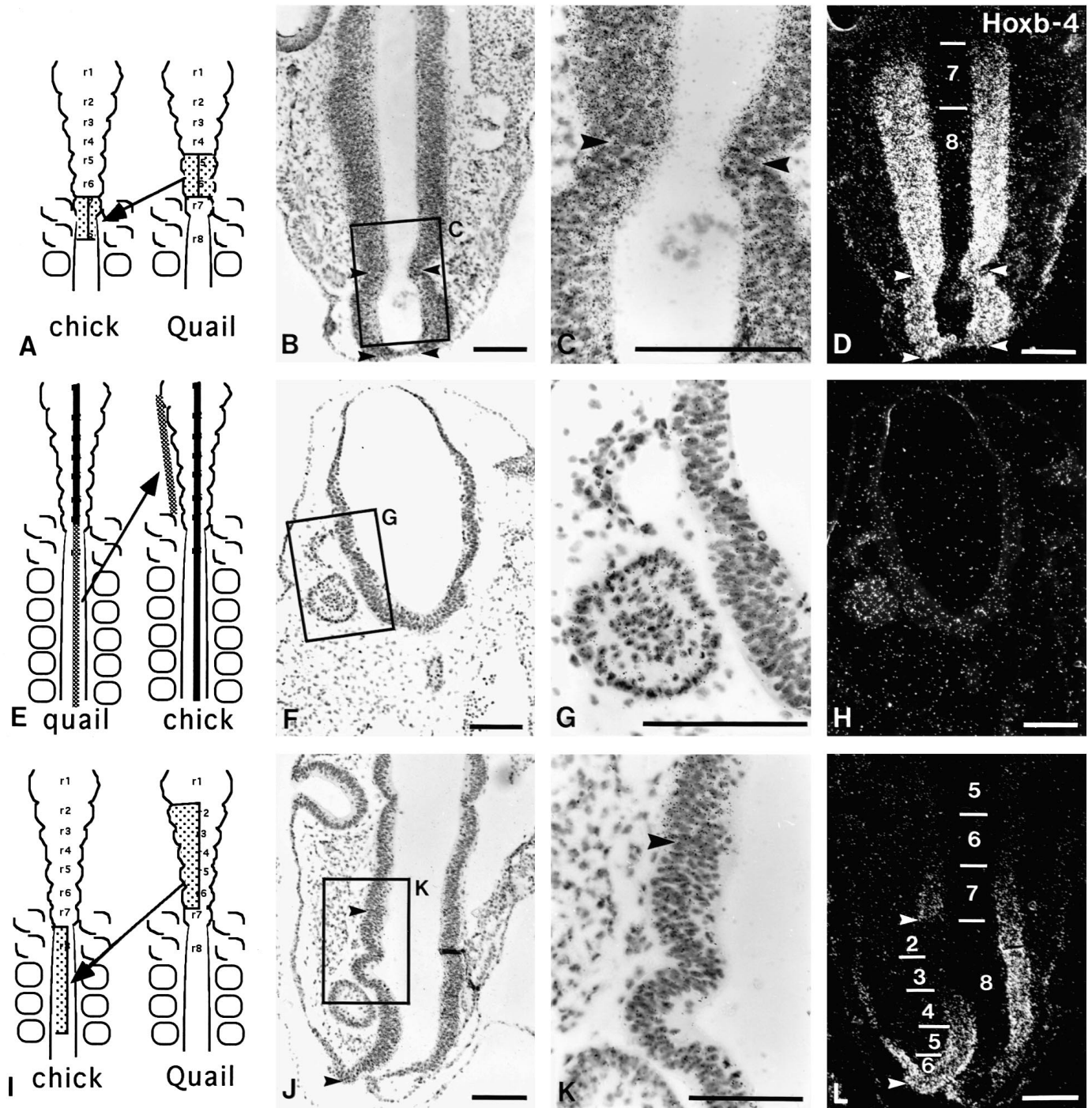
Fig. 8. Determination of the origin of the inducing signal. (A) r5/6 were grafted bilaterally into the r8p position at the 5-somite stage and the embryo was fixed 1 day later at stage 14-15. (B) Bright-field photomicrograph of such a grafted embryo sectionned frontally. (C) Higher magnification of B showing the rostral limit of the graft. (D) Dark-field photomicrograph of the same section as in B showing *Hoxb-4* labelling in the graft indicated by arrowheads. (E) A supernumerary troncal notochord has been placed laterally to the rhombencephalon at the 5-somite stage. (F) A transverse section at the level of r4, 1 day after the graft, shows the position of the supernumerary notochord of quail origin, framed and represented at a higher magnification in G. (H) A dark-field photomicrograph of the same section as in F showing that the neural tube lateral to the notochord does not express *Hoxb-4*. (I) r2-6 grafted into the r8 position in the same anteroposterior orientation at the 5-somite stage. (J) One day later, the graft has sealed and given rise to a supernumerary otic vesicle lateral to r5. (K) Higher magnification of the anterior limit of the graft shows clearly the quail nuclei. (L) The limits of the graft are indicated by arrowheads. The numbers in the center correspond to the host's rhombomeres and the numbers on the left correspond to the grafted ones. *Hoxb-4* is induced in r5 and 6 but r2-4 are still negative. The otic vesicule marks the position of r5/6. Scale bars, 100 μm.
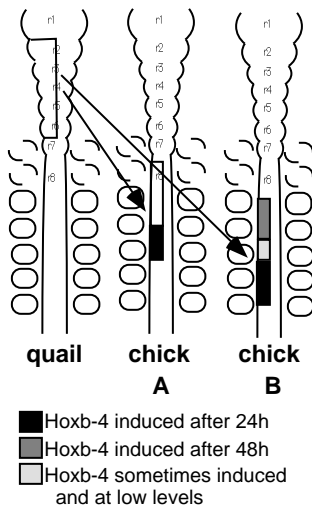
Fig. 9. Summary of the results of r2-6 posteriorizations. In A r2-6 are transplanted to the position of r8, lateral to somites 2-5, *Hoxb-4* is induced in r5/6 only. In B r2-6 are transplanted to r8 lateral to somites 3-6 or 4-7, *Hoxb-4* is induced in r5/6 as in A but also in r2/3 in 3 cases out of 5. In these 3 cases, *Hoxb-4* expression in r4 is variable, from nothing to a sparse signal.

similar to those observed in the contralateral unoperated side. In the case of transplantation of r1/2 however, a delay of about 12 hours was observed before the maximal level of induction of *Hoxb-4* was reached in the anterior region of the graft, indicating that competence to respond to inducing signals decreases in the anteriormost rhombencephalic neuroepithelium. These results clearly demonstrate that *Hoxb-4* expression does not always display a cell autonomous pattern, and can be ectopically induced in other regions of the hindbrain.

Transplantation of rhombomeres 5/6 or 3/4 to r8 level were carried out to test if the expression of other Hox genes: *Hoxd-4*, *Hoxa-4, Hoxa-3* and *Hoxb-3* could also be induced and the induction of transcription in these grafts was observed for all of the Hox genes examined in a manner similar to *Hoxb-4*. This shows therefore that the neuroepithelium of the anterior rhombencephalon in which none of these genes is normally expressed has the competence to do so if subjected to the prevailing environment in the posterior hindbrain and spinal cord.

Properties of the inducing region: planar signalling

The question was then raised as to what in this environment is critical for Hox gene induction to proceed. The experiments described above rule out the possibility that the signal (or signals) acts transversally since in the caudorostral transplantation of hemirhombomeres from r7/8a to the r5/6 level the graft did not induce the contralateral unoperated rhombencephalic moiety to express *Hoxb-4*. Moreover, neither the notochord nor the paraxial mesoderm seem to be able to induce the changes in expression of the *Hox* genes involved in this study, at the stages considered. We noticed that one requirement is that the grafted fragment of tissue be perfectly incorporated within the plane of the host's neuroepithelium. This suggested that the inducing signal might be transmitted in the plane of the neuroepithelium itself. In agreement with this idea, large stripes of neuroepithelium encompassing the presump-

tive territory of 5 rhombomeres, implanted posteriorly at the level of the medulla oblongata convincingly demonstrated that the inductive signal is transmitted from caudal to rostral via the neuroepithelium itself. We found that in the grafts transplanted at the level of r8 (Figs 9, 10) the range of action of the signal extend over the length of 2 to 3 rhombomeres. But if the graft was transplanted more posteriorly, the area of induction was larger and in some cases included the whole explant. Nevertheless, some cases showed that the signal can pass in a rostrocaudal as well as in a caudorostral direction. This was observed when 5-rhombomere fragments were grafted lateral to somites 3-6 or 4-7 (Fig. 10). In r3 a central zone devoid of transcripts was observed while induction had occurred on both ends of the transplant. Nevertheless, the induction by the anterior end needed a longer period of time to occur since it was observed after 2 days. These data suggest that the signals are strongest in posterior regions of the hindbrain or spinal cord.

One can speculate on the nature of the signals responsible for Hox gene induction and maintenance in definite regions of the neuroepithelium and on their propagation. Our experiments indicate that the signal spreads from the host neural tube to the graft within the plane of the epithelium itself. The fact that Hox gene expression is limited anteriorly in both the normal and transplanted epithelium might result from the decrease in concentration of the inductive signal(s) along the AP axis. Thus, if more posterior grafts (from r8p) are transplanted anteriorly (levels ranging from r1 to r6) some induction of the host neuroepithelium can occur (in 20% of cases) whereas r7/8a anterior translocations do not have inductive effect on the host. Moreover, the r8p induction of *Hoxb-4* expression takes place only after a longer period of time (2 days) than in the experiments described above (data not shown). These results are in agreement with previous experiments (Alvarado-Mallart et al., 1990; Itasaki et al., 1991; Bally-Cuif et al., 1992, 1994) which show that transplanting the mes/metencephalon into the diencephalon leads to the acquisition of caudal characteristics by the neurectoderm surounding the graft. This supports the contention that the more posterior the fragments, the more inductive substance they contain. One can imagine that the cells induced to express Hox genes also acquire the capacity to produce the inductive signal and that a progressive decrease in the amount of substance generated at each step of its progression from caudal to rostral is responsible for fixing the anterior limit of Hox gene expression. Our experiments do not provide any insight concerning the nature of the signal. Retinoic acid could be a suitable candidate since it is expressed in a posterior to anterior gradient (Chen et al., 1994), it provokes anterior to posterior transformations of neural structures of *Xenopus* gastrulas (Durston et al., 1989) and mouse hindbrain segments (Marshall et al., 1992) and it has been shown to be a good inductor of Hox genes either directly or indirectly (for review, Mavilio, 1993). Furthermore, RARE in the *Hoxb-1* gene are required to establish normal expression in the neural ectoderm, suggesting RA has a normal role in specifying Hox expression in the hindbrain (Marshall et al., 1994).

Although the inducibility of several *Hox* genes is demonstrated in this work, the exclusive property of r4 for the anterior expression of *Hoxb-1,* already reported by others (Kuratani and Eichele, 1993; Guthrie et al., 1993), was confirmed in this study in the quail-chick system. Expression of *Hoxb-1* does not obey the rules followed by most of the other genes of the *Hox*
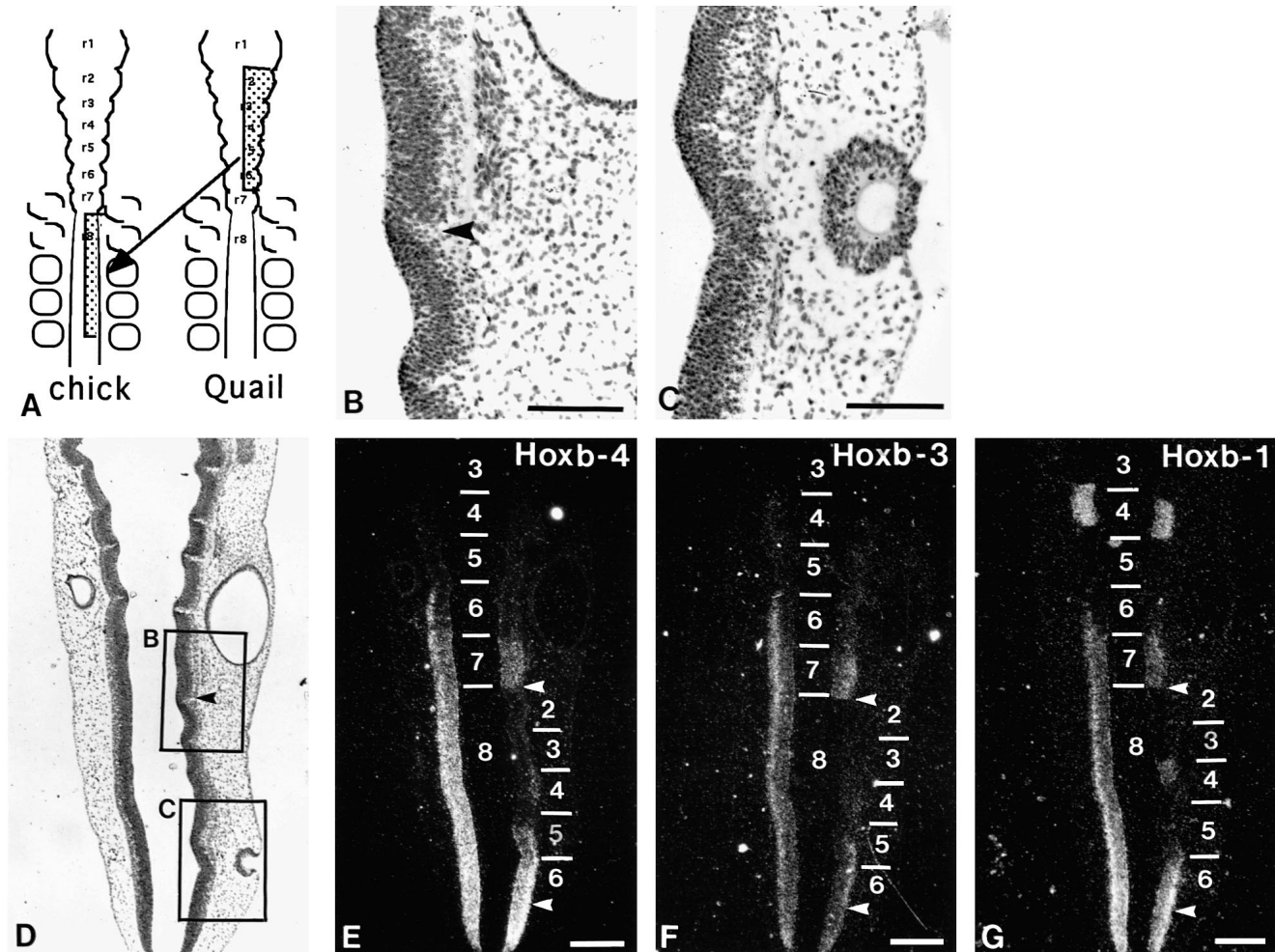
Fig. 10. (A) r2-6 graft in r8 position at 5-somite stage. Adjacent sections were hybridized with *Hoxb-4* (E) *Hoxb-3* (F) *Hoxb-1* (G) at 3 days (stage 20) i.e. two days after the graft. (D) Bright-field photomicrograph of the same section as in F. Higher magnifications of this section in B and C show the quail cells. In B, the arrowhead shows the rostral limit of the graft. In C, the region corresponding to r5/6 of the graft is represented and entirely made up of quail cells. Note the presence of an extra otic vesicle of graft origin. (E) *Hoxb-4* has been induced in r5/6, (F) *Hoxb-3* has been up-regulated in r5/6, (G) *Hoxb-1* is still expressed in r4 and has been induced in r6, whereas transcripts are absent in r5 as in normal embryos (see r5 of the host). Scale bars, 100 μm in B and D, and 200 μm in A and C. Numbers in the center correspond to the host's rhombomeres and numbers on the right to the grafted ones.

clusters since it is transcribed less rostrally than its position in the cluster would imply. Moreover, it is expressed from caudal up to r4 at the 5-somite stage and is subsequently strongly

enhanced in r4 while later on its expression progressively decreases from r5 down to the spinal cord. In our experiments, *Hoxb-1* can be induced in r5/6 transplanted to posterior

Fig. 11. Change in the identity of heterotopically grafted rhombomeres. r5/6 have been grafted on the left side either orthotopically from quail to chick at the 5-somite stage (A,C-G) or heterotopically at the level of r8p (B,H-M). The chimeras have been observed at E8. In both cases the nuclei that developed on the grafted side are identical to those of the contralateral side. In orthotopic grafts, quail cells, identified by a Feulgen staining (D-F) were found in nuclei laminaris (Lam) (C,D), magnocellularis (MCC) (C,E), tangentialis (Tan) (F,G) as well as the motor neurons of nerves VI and VII (A) as recognized after staining with cresyl violet (C,G). Note that the section represented in A is not strictly tranverse but examination of serial sections shows that the quail and chick territories develop according to a similar pattern. In heterotopic grafts the fate of the quail r5/6 has been profoundly changed since they

differentiate accordingly to their new position. Both the grafted and the host's sides have the same shape as seen in the rostral (Ba and H) and the caudal (Bb and I) sections of the medulla oblongata. Nerves X (Ba and H) and XII (Bb and I) are seen on both the normal and operated sides. At the rostral level one can distinguish the inferior olivary nucleus lamella dorsalis (IOld) and ventralis (IOlv) (Ba) and at the caudal level the inferior olivary nucleus (IO) (Bb). IO is shown in L and P for the operated quail side and in (M) and (Q) (see chick nuclei) for the host unoperated side. Panels Bb, J and K show the supraspinal nuclei (SS). In J and K one can see the mirror image of the two nuclei of graft (J) and host (K) origin. (N) Feulgen staining of a section adjacent to that in J showing the quail nuclei in the neurons (large nuclei) and glia (small nuclei). (O) A section adjacent to that in K stained with Feulgen and showing the chick nuclei.

r5/6 → r8p
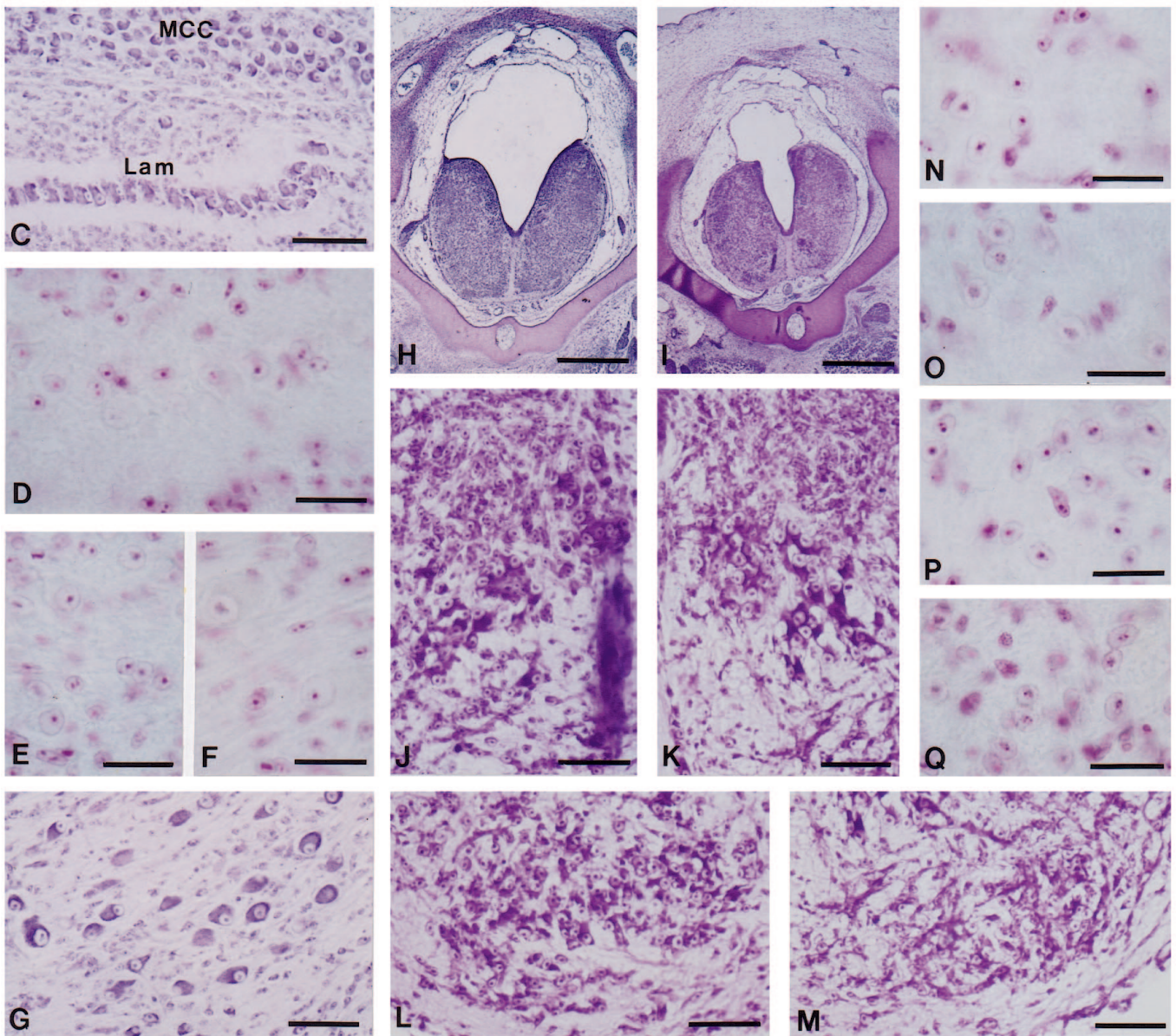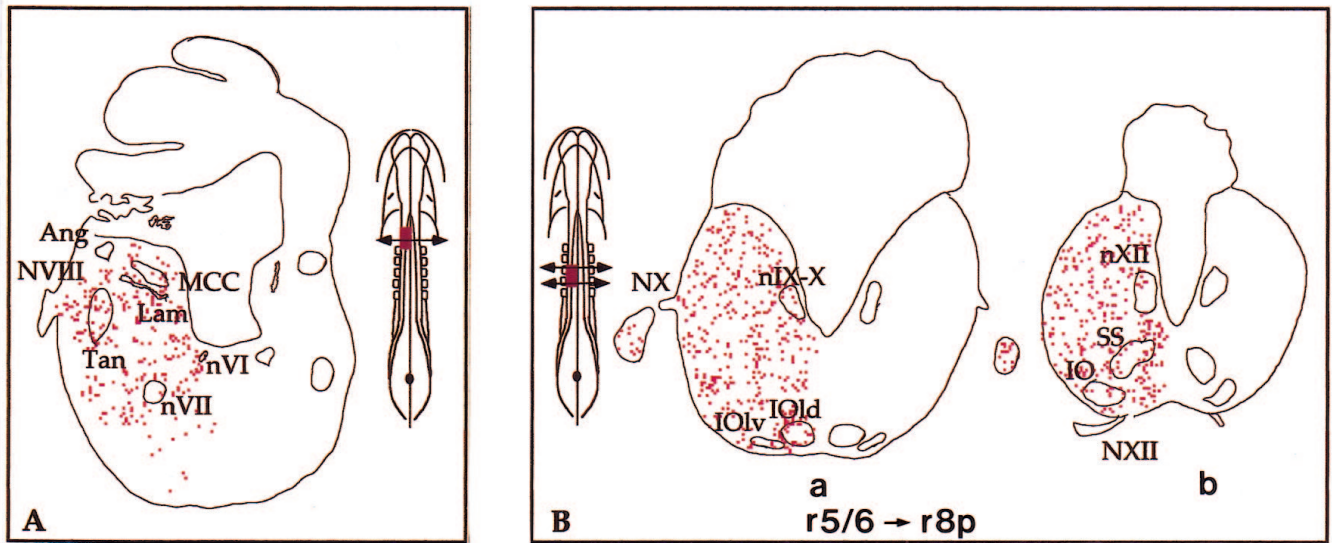
locations (r7/8) whereas transplantation of r2 into a r4 position is not followed by induction of *Hoxb-1* (Kuratani and Eichele, 1993; Guthrie et al., 1992; Simon et al., 1995). These differences in the anterior and posterior expression of *Hoxb-1* might be accounted for by the fact that transcription of this gene in r4 seems to be controlled by different promoter elements than in its posterior domain of expression (Marshall et al., 1994) and that specific repressors block its activity in rhombomeres adjacent to r4 (Studer et al., 1994). Different promoter elements also regulate the expression of *Hoxa-2* in anterior rhombomeres and r7/8 and spinal cord (Frasch et al., 1995) suggesting that the differential regulation between anterior and posterior rhombencephalon could be a more general feature. The possibility that the regulation of Hox gene expression in anterior and posterior rhombomeres follows different rules is currently being tested by transplantations.

Although homeogenetic induction had been noted in classical experimental embryology (Mangold and Spemann, 1927; Mangold, 1933) the most popular view has long been that neural induction proceeds essentially from vertical signal arising from the dorsal mesoderm (i.e. the notochord) to the neural plate. However, the experiments carried out by Nieuwkoop et al. (1952a,b,c), by Eyal-Geladi (1954) as well as the modern observation that neural genes are induced in the ectoderm of *Xenopus* exogastrulas (Kintner and Melton, 1987), rejuvenated the problem of planar induction in the neuro-epithelium. The reality of such a mechanism was definitively demonstrated in a number of experimental situations including Keller sandwiches and *Xenopus* exogastrulas (see Doniach, 1993 and Ruiz i Altaba, 1993 for reviews). Presently however, the respective role of planar versus vertical induction in normal development still remains to be defined. We provide here the evidence that maintaining the Hox code in the posterior rhombencephalon results from a posterior to anterior wave of induction which travels within the plane of the neuroepi-thelium itself.

The process of induction not only needs a signal but also competent cells. We have not yet looked for a possible anterior limit of competence in the neuroepithelium. Experiments are in progress to investigate this. It was also surprising from our results that older rhombomeric tissue was capable of plasticity. This suggested that the signals may be required throughout development and persist for some time.

Induction of Hox changes correlate with switch in anatomical identity

One of the first indications that the Hox code controls the specification of the anteroposterior structures in vertebrates was provided by the administration of retinoic acid to pregnant mice and its consequences on homeotic transformations of the vertebrae (Kessel and Gruss, 1991). Targeted mutations of several Hox genes and their effect on the development of hindbrain and branchial arch neural crest derivatives have fully confirmed this notion. Here we provide the evidence that a homeotic transformation can be induced in the organisation of the brain stem derived from the rhombomeres. By homotopic transplantation of the quail r5/6 into chick hosts we have first shown that the derivatives of these rhombomeres correspond to the sensory acoustic nuclei, nucleus laminaris, magnocellularis and tangentialis, and to the motor nuclei of nerves VI (abducens) and part of IX. In addition, the derivatives of the posterior half of r8 correspond to the motor nuclei of nerve XII

(hypoglossal) and X (vagus) partly. The inferior olivary nucleus is almost entirely contained in this region (Keynes and Lumsden, 1990 for a review and also Tan and Le Douarin, 1991). When r5/6 is transplanted at the level of r8p one sees the complete transformation of its presumptive fate into the neural structures characteristic of its novel position. Both the shape of the tube, the nerves which enter it and the nuclei are repatterned according to the level of the graft. As can be seen in Fig. 11 virtually no difference can be seen between the grafted and the control moiety of the brain stem at this level. The molecular changes induced by the graft thus result in a phenotypic change which is here compatible with the idea that a posterior prevalence operates in neural induction in vertebrates (Slack and Tannahill, 1992).

REFERENCES

Alvarado-Mallart, R.-M., Martinez, S. and Lance-Jones, C. C. (1990). Pluripotentiality of the 2-day-old avian germinative neuroepithelium. *Dev. Biol.* **139**, 75-88.

Bally-Cuif, L., Alvarado-Mallart, R.-M., Darnell D. K. and Wassef, M. (1992). Relashionship between *Wnt-1* and *En-2* expression domains during early development of normal and ectopic met-mesencephalon. *Development* **115**, 999-1009.

Bally-Cuif, L. and Wassef, M. (1994). Ectopic induction and reorganization of *Wnt-1* expression in quail/chick chimeras. *Development* **120**, 3379-3394.

Carpenter, E. M., Goddard, J. M., Chisaka, O., Manley, N. R. and Capecchi, M. R. (1993). Loss of Hox-A1 (Hox-1. 6) function results in the reorganization of the murine hindbrain. *Development* **118**, 1063-1075.

Chen, Y., Huang, L. and Solursh, M. (1994). A concentration gradient of retinoids in the early Xenopus laevis embryo. *Dev. Biol.* **161**, 70-76.

Chisaka, O. and Capecchi, M. R. (1991). Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene Hox-1. 5. *Nature* **350**, 473-479.

Condie, B. G. and Capecchi, M. R. (1994). Mice with targeted disruptions in the paralogous genes hoxa-3 and Hoxd-3 reveal synergistic interactions. *Nature* **370**, 304-307.

Doniach T. (1993). Planar and vertical induction of anteroposterior pattern during the development of the amphibian central nervous system. *J. Neurobiol.* **24**, 1256-1275.

Durston, A. J., Timmermans, J. P. M., Hage, W. J., Hendricks, H. F. J. de Vries, N. J., Heideveld, M., and Nieuwkoop, P. D. (1989). Retinoic acid causes an anteroposterieur transformation of the developing central nervous system. *Nature* **340**, 140-144.

Eichmann A., Marcelle C., Breant C. and Le Douarin N. M. (1993). 2 Molecules related to the VEGF receptor are expressed in early endothelial cells during avian embryonic development. *Mech. Dev.* **42**, 33-48.

Eyal-Giladi, H. (1954). Dynamic aspects of neural induction. *Arc. Biol.* **65**, 180-259.

Feulgen, R. and Rossenbeck, H. (1924). Mikroskopisch chemicher Nachweis einer Nucleinsaüre vom Typus der Thymenucleinsaüre und die darauf beruhende selektive Färbung von Zellkernen der mikroskopiscen präparaten. *Hoppe-Seyler's Z. Physiol. Chem.* **135**, 203-248.

Frasch, M., Xiaowei, C. and Lufkin, T. (1995). Evolutionary-conserved enhancers direct region-specific expression of the murine *Hoxa-1* and *Hoxa-2* loci in both mice and *Drosophila. Development* **121**, 957-974.

Fraser, S., Keynes, R. and Lumsden, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restriction. *Nature* **344**, 431-435.

Gardner, C. A. and Barald, K. F. (1991). The cellular environment controls the expression of engrailed-like protein in the cranial neuroepithelium of quail-chick chimeric embryos. *Development* **113**, 1037-1048.

Gaunt, S. J. and Strachan, S. (1994). Forward spreading in the establishment of a Vertebrate Hox expression boundary: the expression domain separates into anterior and posterior zones, and the spread occurs across implanted glass barriers. *Dev. Dynam.* **199**, 229-240.

Gendron-Maguire, M., Mallo, M., Zhang, Z. and Gridley, T. (1993). Hoxa-2 mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. *Cell* **75**, 1317-1331.

Gonzalez-Reyes, A., Macias, A. and Morata, G. (1992). Autocatalysis and phenotypic expression of *Drosophila* homeotic gene deformed: its dependence on polarity and homeotic gene function. *Development* **116**, 1059-1068.

Graham, A., Heyman, I. and Lumsden, A. (1993). Even-numbered rhombomeres control the apoptotic elimination of neural crest cells from odd-numbered rhombomeres in the chick hindbrain. *Development* **119**, 233-245.

Guthrie, S., Muchamore, I., Kuroiwa, A., Marshall, H., Krumlauf, R. and Lumsden, A. (1992). Neurectodermal autonomy of Hox-2. 9 expression reaveled by rhombomere transpositions. *Nature* **356**, 157-159.

Guthrie, S., Prince, V. and Lumsden, A. (1993). Selective dispersal of avian rhombomere cells in orthotopic and heterotopic grafts. *Development* **118**, 527-538.

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

Harkmark, W. (1954). Cell migrations from the rhombic lip to the inferior olive, the nucleus raphe and the pons. A morphological and experimental investigation on chick embryos. *J. comp. Neurol.* **100**, 115-209.

Hemmati-Brivanlou, A., Stewart, R. M. and Harland, R. M. (1990). Region-specific neural induction of an engrailed protein by anterior notochord in *Xenopus. Science* **250**, 800-802.

Hunt, P., Wilkinson, D. and Krumlauf, R. (1991). Patterning of the vertebrate head: murine Hox 2 genes mark distinct subpopulations of premigratory and migrating cranial neural crest. *Development* **112**, 43-50.

Itasaki, N., Ichijo, H., Hama, C., Matsuno, T. and Nakamura, H. (1991). Establishment of rostrocaudal polarity in tectal primordium-engrailed expression and subsequent tectal polarity. *Development* **113**, 1133-1144.

Izpisua-Belmonte, J.-C., Tickle, C., Dollé, P., Wolpert, L. and Duboule, D. (1991). Expression of the homeobox Hox-4 genes and the specification of position in chick wingdevelopment. *Nature* **350**, 585-589.

Kessel, M. and Gruss, P. (1991). Homeotic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. *Cell* **67**, 89-104.

Keynes, R. and Lumsden, A. (1990). Segmentation and the origin of regional diversity in the vertebrate central nervous system. *Neuron* **2**, 1-9.

Kintner, C. R. and Melton, D. A. (1987). Expression of Xenopus N-CAM RNA is an early response to neural induction. *Development* **99**, 311-325.

Krumlauf, R. (1994). Hox genes in vertebrate development. *Cell* **78**, 191-201.

Kuratani, S. C. and Eichele, G. (1993). Rhombomere transplantation repatterns the segmental organization of cranial nerves and reveals cell-autonomous expression of a homeodomain protein. *Development* **117**, 105-117.

Le Douarin, N. M. (1969). Particularités du noyau interphasique chez la Caille japonaise (Coturnix coturnix japonica). Utilisation de ces particularités comme marquage biologique dans les recherches. *Bull. Biol. Fr. Belg.* **103**, 435-452.

Le Douarin, N. M. (1982). *The Neural Crest* (Developmental and Cell Biology series; 12). Cambrige: Cambridge University Press.

Le Douarin, N. M., Teillet, M. A. and Fontaine-Perus, J. (1984). *Chimeras in Developmental Biology.* pp. 313-352. London: Academic Press.

Lumsden, A. (1990). The cellular basis of segmentation in the developing hindbrain. *Trends Neurosci.* **13**, 329-335.

Lumsden, A., Sprawson, N. and Graham, A. (1991). Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. *Development* **113**, 1281-1291.

Maden, M., Hunt, P., Eriksson, U., Kuroiwa, A., Krumlauf, R. and Summerbell, D. (1991). Retinoic acid-binding, rhombomeres and the neural crest. *Development* **111**, 35-44.

Mangold, O. (1933). Über die Induktionfähigkeit der verschiedenen Bezirke der Neurula von Urodelen. *Naturwiss.* **21**, 761-766.

Mangold, O. and Spemann, H. (1927). Uber Induktion von Medullarplatte durch Medullarplatte im jungeren Kiem, ein Beispiel homoogenetischer oder assimilatorischer Induktion. *Wilhem Roux Arch. EntwMech. Org.* **111**, 341-422.

Marshall, H., Nonchev, S., Sham, M. H., Muchamore, I., Lumsden, A. and

Krumlauf, R. (1992). Retinoic acid alters hindbrain Hox code and induces the transformation of r2/3 into r4/5 identity. *Nature* **360**, 737-741.

Marshall, H., Studer, M., Pöpperl, H., Aparicio, S., Kuroiwa, A., Brenner, S. and Krumlauf, R. (1994). A conserve retinoic acid response element required for early expression of the homeobox gene Hoxb-1. *Nature* **370**, 567-571.

Mark, M., Lufkin, T., Vonesch, J. L., Ruberte, E., Olivo, J.-C., Dollé, P., Gorry, P., Lumsden, A. and Chambon, P. (1993). Two rhombomeres are altered in Hoxa-1 mutant mice. *Development* **119**, 319-338.

Mavilio, F. (1993). Regulation of vertebrate homeobox-containing genes by morphogens. *Europ. J. Biochem.* **212**, 273-288.

McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.

Nieto, M. A., Gilardi-Hebenstein, P., Charnay, P. and Wilkinson, D. (1992). A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. *Development* **116**, 1137-1150.

Nieuwkoop, P. D. (1952a). Activation and organization of the central nervous system in amphibians. III. Synthesis of a new working hypothesis. *J. Exp. Zool.* **120**, 83-108.

Nieuwkoop, P. D. (1952b). Activation and induction of the central nervous system in amphibians. II. Differentiation and organization. *J. Exp. Zool.* **120**, 33-81.

Nieuwkoop, P. D. (1952c). Activation and organization of the central nervous system in amphibians I. Induction and activation. *J. Exp. Zool.* **120**, 1-32.)

Noden, D. (1978). The control of avian cephalic neural crest cytodifferentiation I: Skeletal and connective tissues. *Dev. Biol.* **67,** 296-312.

Noden, D. (1983). The role of the neural crest in patterning of avian cranial skeletal, connective and muscle tissues. *Dev. Biol.* **96,** 144-165.

Orr. x. (1887). Contribution to the embryology of the lizard. *J. Morph.* **1**, 311-372.

Prince, V. and Lumsden, A. (1994). Hoxa-2 expression in normal and transposed rhombomeres: independent regulation in the neural tube and neural crest. *Development* **120**, 911-923.

Rijli, F. M., Mark, M., Lakkaraju, S., Dierich, A., Dollé, P. and Chambon, P. (1993). A homeotic transformation is generated in the rostral branchial region of the head by disruption of Hoxa-2, which acts as a selector gene. *Cell* **75**, 1333-1349.

Ruiz i Altaba, A. (1993). Induction and axial patterning of the neural plate: planar and vertical signals. *J. Neurobiol.* **24**, 1276-1304.

Slack, J. M. W. and Tannahill, D. (1992). Mechanism of anteroposterior axis specification in vertebrates. *Development* **114**, 285-302.

Sechrist, J., Serbedzija, G., Schershon, T., Fraser, S. and Bronner-Fraser, M. (1993). Segmental migration of the hindbrain neural crest does not arise from segmental generation. *Development* **118**, 691-703.

Simon, H., Hornbruch, A. and Lumsden, A. (1995). Independent assignment of antero-posterior and dorso-ventral positional values in the developing chick hindbrain. *Cur. Biol.* **5**, 205-214.

Studer, M., Popperl, H., Marshall, H., Kuroiwa, A. and Krumlauf, R. (1994). Role of a conserved retinoic acid response element in rhombomere restriction of *Hoxb-1. Science* **265**, 1728-1732.

Sundin, O. H. and Eichele, G. (1992). An early marker of axial pattern in the chick embryo and its respecification by retinoic acid. *Development* **114**, 841-852.

Tan, K. and Le Douarin, N. M. (1991). Development of the nuclei and cell migration in the medulla o,blongata. *Anat. Embryol.* **183**, 321-343.

Vaage, S. (1969). The segmentation of the primitive neural tube in chick embryo (*Gallus domesticus). Adv. Anat. Embryol. Cell Biol.* **41**, 1-88.

Wilkinson, D. G., Peters, G., Dickson, C. and McMahon, A. P. (1988). Expression of the FGF-related protooncogene int-2 during gastrulation and neurulation in the mouse. *EMBO J.* **7**, 691-695.

Wilkinson, D. G., Bhatt, S., Chavrier, P., Bravo, R. and Charnay, P. (1989). Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. *Nature* **337**, 461-464.

Wilkinson, D. G. and Green, J. (1991). *In situ* hybridization. In *Postimplantation Mammalian Embryos. The Practical Approach* (ed. Copp, A. J and Cockcroft, D. L.), pp 155-167. Oxford : IRL Press.

Wilkinson, D. G. (1993). Molecular mechanisms of segmental patterning in the vertebrate hindbrain and neural crest. *BioEssays* **15** (8), 499-505.

Zhang, M., Kim, H.-J., Marshall, H., Gendron-Maguire, M., Lucas, D. A., Baron, A., Gudas, L. J., Gridley, T., Krumlauf, K. and Grippo, J. F. (1994). Ectopic Hoxa-1 induces rhombomere transformation in mouse hindbrain. *Development* **120**, 2431-2442.