Hox gene induction in the neural tube depends on three parameters: competence, signal supply and paralog group

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SUMMARY
It has been previously shown that Hox gene expression in the rhombencephalon is controlled by environmental cues. Thus posterior transposition of anterior rhombomeres to the r7/8 level results in the activation of Hox genes of the four first paralog group and in homeotic transformations of the neuroepithelial fate according to its position along the anteroposterior axis. We demonstrate here that although the anteroposterior levels of r2 to r6 express Hox genes they do not have inducing activity on more anterior territories. If transposed at the posterior rhombencephalon and trunk level, however, the same anterior regions are able to express Hox gene such as Hoxa-2, a-3 or b-4. We also provide evidence that these signals are transferred by two paths: one vertical, arising from the paraxial mesoderm, and one planar, travelling in the neural epithelium. The competence to express Hox genes extends up to the forebrain and midbrain but expression of Hox genes does not preclude Otx2 expression in these territories and results only in slight changes in their phenotypes. Similarly, rhombomeres transplanted to posterior truncal levels turned out to be able to express posterior genes of the first eight paralog groups to the exclusion of others located downstream in the Hox genes genomic clusters. This suggests that the neural tube is divided into large territories characterized by different Hox gene regulatory features.

Key words: spinal cord, hindbrain, Hox genes, chick, homeobox, transplantation, rhombomeres, induction

INTRODUCTION
In recent years the rhombencephalon has become the subject of intensive research. This stems from the fact that it is the only part of the vertebrate nervous system obviously metamereized into true segmental units characterized by cell lineage restriction (Fraser et al., 1990). Segmental organisation in this part of the brain is also reflected at the molecular level, since the expression domains of Hox genes respect boundaries between rhombomeres. These genes are sequentially activated from head to tail as development proceeds, according to their position from the 3’ to the 5’ end of the DNA molecule in the Hox gene clusters of the vertebrate genome (Krumlauf, 1994 for review). Thus, the genes of the first four paralog groups are the only ones to be expressed in the hindbrain according to a pattern that has been documented in the mouse (for review, see McGinnis and Krumlauf, 1992) and in the chick (Sundin and Eichele, 1990; Sundin and Eichele, 1992; Gaunt and Strachan, 1994; Prince and Lumsden, 1994; Morrison et al., 1995). Altering their expression in the mouse embryo either by targeted mutations (Krumlauf, 1994 and references therein; Zhang et al., 1994; Alexandre et al., 1996; Condic and Capecci, 1994; Manley and Capecci, 1995) or by retinoic acid treatment (Ruiz i Altaba and Jessell, 1991; Moriss-Kay et al., 1991; Marshall et al., 1992; Kessel, 1993; Gale et al., 1996) cause abnormalities in the development of the hindbrain and in the structures derived from the rhombencephalic neural crest.

Experimental mutations of Hox genes in the mouse clearly demonstrated that a strict control of Hox gene expression is required for a harmonious development of the rhombencephalic neural tube and associated neural crest. The problem was raised as to whether Hox gene expression is intrinsically programed in the neuroepithelium (NE) or subjected to environmental cues. Autonomy was supported by the fact that heterotopic transplantations of the anterior rhombomeres (r2, r4) carried out in the chick at stage HH9-10 (Hamburger and Hamilton, 1951) did not modify the normal expression pattern of the Hoxb-1 gene in this area (Guthrie et al., 1992; Kuratani and Eichele, 1993; Prince and Lumsden, 1994; Simson et al., 1995). This view was, however, challenged by Grapin-Botton et al. (1995) and Itasaki et al. (1996). Anterior rhombomeres transposed to the level of r8 acquired the capacity to express genes of the fourth Hox gene paralog group. This resulted in homeotic transformations such that the graft differentiated into neural structures corresponding to its new position. The developmental plasticity of rhombomeres was further demonstrated by Martinez et al. (1996) who showed that cerebellar structures can be induced in r3 if it is placed under the influence of the organizing activity of the midbrain-hindbrain junction area.

The problem of the source of an inductive signal able to induce posterior Hox genes in the rhombencephalon was raised by Grapin-Botton et al. (1995) and Itasaki et al. (1996). Both planar signals travelling in the plan of the NE (Grapin-Botton
et al., 1995) and vertical induction arising from the paraxial mesoderm (Itasaki et al., 1996) were proposed to account for the changes observed in Hox gene expression in transposition experiments. In the present work, we have considered both the problem of the competence of the cephalic neurectoderm to respond to the Hox gene inducing signal as well as the possible tissues able to convey this signal. We confirm that the signal can be conveyed by both the paraxial mesoderm and the NE. In addition, we find that forebrain and midbrain neurectoderms are capable of expressing hindbrain-type Hox genes. Moreover, we demonstrate that, at least for the genes tested in this work, the rhombencephalic NE, when transposed to various levels of the spinal cord is able to express only Hox genes of the 8 first parapalogue groups and not those located in a more 5’ position in the Hox gene clusters.

MATERIALS AND METHODS

Microsurgery

Microsurgery was performed as described previously (Grapin-Botton et al., 1995). Different grafts were carried out involving heterotopic transplantations of quail neuroepithelium into chick recipients at various levels of the neural axis. The transplants were of various lengths according to the experimental series considered but always involved only one side of the neural tube, the floor plate being excluded from the operation.

1) Heterotopic grafts of pairs of rhombomeres in the rhombencephalic area

The stage chosen for the operations was precisely the 5 somite stage (5-ss) for both donors and recipients. The location of the grafted territories along the anteroposterior (AP) axis was determined according to the rhombomeric fate map of the rhombencephalon established at that stage by Grapin-Botton et al. (1995). A fragment of neural epithelium of the same length, corresponding to pairs of rhombomeres but coming from a different AP level was dissected out from a quail embryo and grafted into the chick recipient (Fig. 3A, D).

2) Long rhombencephalic grafts

In one experimental series, the graft involved the presumptive territory corresponding to 4 rhombomeres (r2-8 inclusive). In this case, the experiment was performed at the 9- to 10-ss, when the rhombomeric boundaries are visible.

3) Heterotopic exchanges of mesencephalic, prosencephalic and rhombencephalic territories

Diencephalic and mesencephalic territories were reciprocally exchanged with rhombomeres (Figs 1A, 2A) at the 5- to 8-ss. To match the sizes of the host and donor territories, only the presumptive alar plate of the mes-diencephalon was involved in the graft. The length of the graft was about 150-200 µm. The neural tissue was not cleared by examination by mesodermal cells by means of enzymatic digestion.

4) Heterotopic grafts in the spinal cord

Two series of experiments were performed depending on the genes under investigation. For analysis of Hoxb-4, Hoxb-8 or Hoxb-9 expression, rhombomeres 5 and 6 or neural tube fragments at the levels of somites 3-4 or 5-6, removed from 5- to 8-ss donor quail embryos, were transplanted into 10- to 29-ss chicks at the level just caudal to the last somite formed (Fig. 4E). The anteroposterior location of the graft was therefore different according to the developmental stage of the host. For analysis of Hoxa-10 and Hoxd-11 expression the same fragments were transplanted into stage HH16-18 chicks at the level of the posterior limb.

5) Graft of somites within the paraxial mesoderm at the rhombencephalic level

Somite pairs originating from 6- to 10-ss quail donors were dissociated surgically from surrounding tissues and grafted laterally to the rhombencephalon of 5- to 9-ss hosts after removing the endogenous paraxial mesoderm and ectoderm (Fig. 5A). Superficial ectoderm overlying the somites was included in the transplant.

6) Observation of the operated embryos

The operated embryos were harvested between stage HH14-15 and E12. The fixative used was Carnoy’s fluid for embryos subjected to histology in section and was 3.5% formaldehyde in PBS+EGTA (2 mM) for in toto hybridization. Embryos were cut frontally in 5 µm paraffin sections. Serial sections of each embryo were then treated alternately for in situ hybridizations and for immunostaining using the QCPN monoclonal antibody (mAb). The QCPN mAb (Developmental Studies Hybridoma Bank) recognizes a common species-specific antigen carried by quail but not by chick cells. Embryos at E8-12 were treated on alternate sections with QCPN or BEN mAbs (Pourquié et al., 1990), cresyl violet, and in situ hybridization with a Hoxb-4 probe. At these stages the BEN mAb labels the neurons of the inferior olivary nucleus (Chédotal et al., 1995) and, at lower levels, the motoneurons.

In situ hybridization

To generate suitable probes for the in situ analyses, we used chicken cDNA fragments described elsewhere: Hoxb-4 (Sasaki and Kurouwa, 1990), Hoxa-2 (Prince and Lumsden, 1994), Hoxa-3, Hoxb-9 (Grapin-Botton et al., 1995), Hoxb-8, Hoxa-10, Hoxd-11 (Burke et al., 1995). The RNA probes were labelled either by incorporation of [35S]UTP as described by Grapin-Botton et al. (1995) or incorporation of dig-UTP (Boehringer Mannheim). Radioactive hybridizations were carried out as previously described (Grain-Botton et al., 1995). The whole-mount non-radioactive in situ hybridizations were performed according to Henrique et al.’s method (1995) on HH20-stage neural tubes which had been dissected out from surrounding tissues.

RESULTS

Evidence for a rostrocaudal gradient of competence to respond to Hoxb-4 induction in rhombencephalic neural epithelium (NE)

Hoxb-4 is normally expressed in the neural tube from r7 caudalward. Expression of Hoxb-4 was tested in pairs of anterior rhombomeres (i.e. r1/2, r3/4, r5/6) transposed to the level of the posterior half of r8 (r8p), corresponding to the level of somites 3/4 as described in Materials and Methods. The embryos were analysed at regular times after grafting from 10 to 48 hours (stage HH12 or 14-15-ss to stage HH18-20). The results, in Table 1, show that the more anterior the rhombomeres, the more time is required for Hoxb-4 expression. The competence to respond to the inductive signal received in r8p decreases in the anterior hindbrain NE according to a caudorostral gradient.

Hox gene induction in the midbrain and forebrain territories

These results prompted us to see whether competence to respond to Hoxb-4 induction exists in the neural epithelium of the diencephalon and mesencephalon.
(1) Promesencephalic grafts to the r8p level

The alar plate of the mesencephalon and the diencephalon of a quail donor was grafted at the level of r8p into a stage matched chick host (Table 2). In all the mesencephalic ($n=5$) and diencephalic ($n=24$) transplants analysed at stages HH14-15 to 20 by in situ hybridization on sections or whole mount, Hoxb-4 was induced in the graft (Table 2; Fig. 1A-C). Most of the inductions were limited to the extremities of the transplant, either rostrally, caudally or both. Forebrain and midbrain NE, which never express any Hox genes have the capacity to express them when subjected to the appropriate inductive signals.

Of the embryos that had received a diencephalic graft at the level of r8p, 21 were hybridized with an Otx2 probe (Table 2). Otx2 transcripts were present in all the transplants although in 12 out of 21 embryos the expression was lower than its endogenous level in the mes-diencephalon area (Fig. 1A,B,D, see Fig. 2C for comparison). In embryos where in situ hybridization was performed for both Hoxb-4 and Otx2 on adjacent sections ($n=13$), Otx2 expression was uniformly decreased in the graft while in most cases Hoxb-4 induction occurred only at the ends of the transplant. Therefore, at least in certain areas, the transposed NE expressed simultaneously anterior and posterior homeobox-containing genes.

(2) Rhombencephalic grafts at the promesencephalic level

The ability of the anterior neural plate to express...
Table 2. Transplantations between the hindbrain and the mid-forebrain

<table>
<thead>
<tr>
<th>Transplantation</th>
<th>Labeling</th>
<th>Expression in the graft</th>
<th>Induction in adjacent tissues</th>
</tr>
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<tbody>
<tr>
<td>Mesencephalon→r8</td>
<td>Hox-4</td>
<td>2/3 at HH20</td>
<td>2/2 at HH14-15</td>
</tr>
<tr>
<td>Diencephalon→r8</td>
<td>Hox-4</td>
<td>16/36 at HH20</td>
<td>3/3 at HH14-15</td>
</tr>
<tr>
<td></td>
<td>Oct2</td>
<td>16/21 at HH20</td>
<td>0/13 at HH20</td>
</tr>
<tr>
<td>r8→mesencephalon</td>
<td>Hox-4</td>
<td>5/5 at HH20</td>
<td>3/5 at HH20</td>
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<tr>
<td></td>
<td>Oct2</td>
<td>0/2 at HH20</td>
<td>0/2 at HH20</td>
</tr>
<tr>
<td>r8→diencephalon</td>
<td>Hox-4</td>
<td>15/15 at HH20</td>
<td>7/9 at HH20</td>
</tr>
<tr>
<td></td>
<td>Oct2</td>
<td>0/15 at HH20</td>
<td>0/1 at HH14-15</td>
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*Number of embryos in which induction is observed in the graft.

| *Number of embryos observed. |

for grafts into the mesencephalon and the diencephalon respectively). Moreover, Hox-4 expression spread in the host tissues adjacent to the graft in 9 out of 14 cases (64%). This could not be distinguished after in situ hybridization since the graft limits were not visible, but was apparent on sections where the quail territories could be recognized (Fig. 2A,B,D). Induction of Hox-4 gene expression occurred not only in the host NE rostrally or caudally to the graft but also, in one case, in the contralateral dorsal side of the host forebrain (Fig. 2B). Oct2 expression was examined on adjacent sections (n=5) and was always limited to the host tissues, meaning that neither inductive signal was present or the r8 NE was not competent to express Oct2 in these conditions (Table 2; Fig. 2A,C,D). The absence of Oct2 expression was also confirmed by in situ hybridization on sections of transplants which had been dissected out with pancreatin prior to grafting, and were therefore devoid of mesodermal contamination. Therefore, as previously demonstrated for Hox genes, no anteriorization of the NE was ever observed. The induction of Hox-4 and the decrease of Oct2 expression indicate that mes-diencephalic transplants can be posteriorly grafted after being transplanted rostrally and when subjected to the influence of hindbrain grafts. NE grafted more rostrally than its level of origin on the AP axis retains Hox-4 expression at least up to E10-12 (n=3/3).

(3) Further development of the rhombencephalic and promesencephalic transposed territories

Alar plates of the posterior half of the mesencephalon were removed from quail 5- to 8-ss donors and grafted at the level of r8p after removal of the lateral wall of the neural tube (Fig. 1A). 4 embryos that reached E12 were examined in transverse sections. In two cases, the graft gave rise to recognizable tectal structures on the operated side (Fig. 1G, H). In the other two cases, the graft did not generate an ectopic tectum (Fig. 1H-K). Neither in these 4 embryos nor in embryos which had received a diencephalic transplant (n=4 at E8-12) could the inferior olivary nucleus be detected in the grafted territory (Fig. 1G, J). In the 8 embryos where columns of motoneurons were interrupted by the graft (Fig. 1K), large neurons of quail could be recognized intermingled with chick motoneurons at the limit between graft and host territories as illustrated for nucleus supraspinalis (Fig. 1E,F). Therefore although the fate of the transplant is not drastically transformed, significant changes in certain cell fates are observed in these experiments.

In the reverse grafts, half of the neural tube from r8p (level of somites 3/4) of 5- to 8-ss quail donors was substituted for the alar plate of the diencephalon of 5- to 8-ss chick recipients (Fig. 2A). Two chimeras were observed at E10, one at E11. At E3, similar grafts were shown to keep their posterior molecular traits as attested by Hox-4 expression and absence of Otx2 transcripts. Accordingly, the inferior olivary nucleus developed in all the transplants (Fig. 2E,F). No other structure could be reliably recognized in the transplants with the methods we used.

Efficiency of the inductive signal along the AP axis

The next question raised in this work concerned the presence and efficiency of the inductive signal along the AP axis. First, we asked whether the preotic and otic levels in which Hox genes of the first three paralogue groups are expressed corresponds to an inducing zone for these genes.

Since the anterior limit of Hoxa-3 expression is between r4 and r5, we examined the capacity of the NE of r1 to r4 to express this gene if transposed to the level of r5/6. As a control, r1/2 or r3/4 were also transplanted to r7/8 levels. The results presented in Fig. 3D and E show that Hoxa-3 induction does not occur when the transplant was placed at r5/6 AP level. This is true for observations at both stages HH14-15 (n=4/4) and HH20 (n=7/7). In contrast, Hox-a-3 was rapidly induced when transplanted to r7/8 levels (Fig. 3D,F).

Hoxa-2 is normally expressed in the neural tube from r2 downward. Transplantation of r1 was made to various positions down the axis from r2 to r8p. Expression of Hoxa-2, observed at stage HH20, never occurred in r1 transplanted below the levels of r2 and r6 (n=12) (Fig. 3A,B). It was, however, induced in all cases when it was transplanted into the r7/8 territory (n=3) (Fig. 3A,C).

Therefore, induction of Hox genes of the first three paralogue groups tested in this study occurs at the level of r7-8 and not r2-6, although these genes are normally expressed in the latter domain.

In order to see whether the inductive signal responsible for the expression of anterior genes is also operative more posteriorly in the spinal cord, a hemi-neural tube of r5/6 was transplanted to the upper thoracic level (somites 15-16) according to the procedure described in Materials and Methods (Fig. 4E). Two days later, at stage HH20, Hoxb-4 was induced in r5/6. Moreover, the level of expression was higher in the grafted rhombomeres than the surrounding spinal cord (n=2/2) (Table 3; Fig. 4J,K). After transposition of the same graft at more anterior levels (r7/8 or r8p, see Grapin-Botton et al., 1995 and Table 1), the amount of Hox-4 expression was not higher than in surrounding tissues. This strongly suggests that inductive signal available in the spinal cord is higher than it is in the r7/8 area.

The notion that the signal is distributed in a decreasing caudorostral gradient is also supported by results obtained in a different experimental paradigm. In our previous work (Grapin-Botton et al., 1995) we showed that the posterior transposition of a large NE territory corresponding to r2 to r6 inclu-
respectively, resulted in different Hoxb-4 induction patterns according to the level at which the graft was inserted. The more posterior the graft, the faster and more complete was the induction. For anterior grafts at the AP level of somites 2-5, Hoxb-4 was induced after 24 hours or 48 hours, only in the posterior end of the transplant, corresponding to r5/6. For transplants at the level of somites 3-6 or 4-7, the induction occurred in a larger area including not only its posterior end (r5/6) but also, after 48 hours exposure to the inductive field, in the anterior end of the explant. When the orientation of the transplanted r2-6 NE fragment was inverted by 180° along the AP axis, strikingly, after only 24 hours, radioactive in situ hybridization on sections revealed a uniform and dense hybridization signal over the whole graft (n=6). This result can be accounted for by the fact that the lower level of competence of the anterior rhombencephalon has been compensated by the higher strength of the signal available at the posterior end of the transplant.

**COMPETENCE OF THE RHOMBENCEPHALON TO EXPRESS POSTERIOR SPINAL CORD GENES**

We have further investigated the capacity of the rhombencephalic NE to express genes located in a more 5′ position on the Hox gene clusters. We chose Hoxb-8, Hoxb-9, Hoxa-10 and Hoxd-11 whose anterior limits of expression normally lie within the spinal cord itself. From stage HH14-15 and at least up to E5, Hoxb-8 is expressed in the neural tube from the level of somite 8 caudalward (Fig. 4A). From stage HH14-15, Hoxb-9 is expressed from the level of somite 11 caudalward (Fig. 4B). Hoxb-10 and Hoxd-11 are respectively expressed from the levels of somites 25 and 30 caudalward (Fig. 4C,D).

Transplantation of hemi-neural tube was carried out from the levels of r5/6 (n=4), somites 3/4 (n=20) and somites 5/6 (n=5) to various AP positions between somite 11 and somite 30 (Table 3; Fig. 3H). Hoxb-8 was induced in all cases at E3, 4 or 5 (Fig. 3E,F,J,L). In most cases, the inductions were partial when investigated by in toto hybridization both at E3 and E4 (Fig. 4F). Using the more sensitive technique of radioactive in situ hybridization on sections, inductions were found to be complete in 9 out of the 10 cases observed (Fig. 4J,L). Hoxb-8 is therefore inducible in the rhombencephalic NE.

Hoxb-9 expression was investigated in the same grafts on alternate sections and in toto on other grafted embryos (Table 3). The Hoxb-9 gene was never induced at E3, E4 (Fig. 4E,G,J,M), E5 or E7, whatever the in situ hybridization method used or the territory of origin of the transplant, which varied from r5/6 to the spinal cord at the level of somites 5/6. The graft position which extended from the level of somite 11 to that of somite 30 had no influence on the outcome of the experiment. Since the observations have been done over a long period of development (up to E7), the time of exposure to a putative inductive signal was long enough for the induction to occur if the grafted territory had been competent to respond to it. The results indicate that this is not the case. When the hybridizations were done on adjacent sections for Hoxb-8 and Hoxb-9, Hoxb-8 was induced but not Hoxb-9.

An hemi-neural tube originating from the level of r5/6 to somites 5/6 was transplanted at stage HH16-18 caudally to the posterior limb, i.e. at the level of somites 26-34 (Table 3). Two days after the graft, at E4.5, neither Hoxa-10 (Fig. 4H) nor Hoxb-9 (Fig. 4I), which are normally expressed at this level, were induced in the grafts.

Therefore, when transposed to the trunk level, the cephalic and upper cervical regions of the neural primordium were not able to perform a complete posterior conversion of their Hox code. The competence of the rhombencephalon and the cervical spinal cord to express Hox genes is limited under these conditions to genes belonging to the first eight paralogue groups.

**THE SOURCE OF INDUCING SIGNAL**

Since Hox genes are expressed in both the neural tube and the paraxial mesoderm, what are the respective roles of these structures in patterning the nervous system via Hox gene expression?

**The neuroepithelium delivers an inducing signal**

To confirm the involvement of the neural tube in Hox gene induction, we have transplanted r8p between r2 and r6 and looked at Hoxb-4 expression in surrounding tissues. Induction was found to occur in 7/19 (36%) cases at stage HH20. Similar transplantsations were performed within the mes- and diencephalic vesicles (see above). Induction in the host occurred in 64% of cases when observed at stage HH20 (Fig. 2B). It should be noted, as seen in Fig. 2B, that the induction of Hoxb-4 in the host occurs in a gradient, with higher induction next to the graft. Examination of serial sections revealed that the induction in the contralateral side proceeded via a dorsal route. This therefore demonstrates that Hox gene induction is propagated within the NE itself.

**Capacity of the paraxial mesoderm to induce Hox gene**

In this experimental series we have tested the capacity of somites which either do or do not express a given Hox gene to induce expression of the same gene in the NE of r2 to r6. We have transplanted somites 1/2 or 3/4, which do not express Hoxb-4 or somites 5/6 or 7/8 which do express it, laterally to
r2-6, and looked for Hoxb-4 induction in the rhombomeres after 48 hours, at stage HH20. We have observed that somite pairs 1/2 (n=6/6) and 3/4 (n=2/2) never induced Hoxb-4 in the adjacent neural tube (Fig. 5A-C). In contrast, somites 5/6 (n=4/8) (Fig. 5A,D,E) and 7/8 (n=2/2) (Fig. 5A,G,H) induced Hoxb-4 in the adjacent neural tube. Close contact between the grafted mesodermal cells and the neural tube was critical for the induction to occur, showing that the inducer has a very limited diffusion in mesenchyme. For somites 7/8, the level of expression in the induced rhombomeres (r5/6) was always higher than with somites 5/6. This result implies that a rostrocaudal gradient of inducer exists in the mesoderm as previously shown in the NE. Moreover, the high level of induction in r5/6 mediated by somites 7/8 is higher than the expression in the neural tube at the normal level of these somites. This implies that these rhombomeres are more competent than the NE at the level of somites 7/8. This is in agreement with the observation reported above that r5/6 express higher levels of Hoxb-4, when transplanted posteriorly, than the surrounding spinal cord (Fig. 4K).

The inducing domain corresponds to the normal domain of Hoxb-4 expression in both the NE and the paraxial mesoderm (Fig. 5F).

Fig. 2. Rhombencephalic grafts to the promesencephalic level. As schematized in A, the alar plate of the diencephalon of chick recipients is replaced by the posterior part of r8, facing somites 3 and 4, of quails at 5- to 8-ss. (B) 2 days after grafting, on frontal sections Hoxb-4 is expressed in the graft as well as in the adjacent and contralateral host neural tube. A decreasing gradient of expression is noticeable in the host tissues. (C) On adjacent sections, Otx2 is expressed in host but not grafted tissues. (D) The localization of the grafted tissue is revealed by the use of the QCPN antibody (arrowheads). (E) At E10, BEN immunostaining of a transverse section at the level of the graft, shows the presence of an inferior olivary nucleus only in the grafted side. The latter is indicated by dashed lines and enlarged in F. BEN-positive quail cells are evidenced by their dense nuclei. Scale bar, 100 μm in D and F; 200 μm in B, C and 1 mm in E.

Fig. 3. Incapacity of the otic and preotic level to induce Hox genes. (A) Transplantation of r1 to the position of r3/4 (B) or r8p (C). (B,C) Frontal sections hybridized with Hoxa-2 2 days after grafting. Hoxa-2 is induced in the graft (limits indicated by arrowheads), in r8p (C) but not in r3/4 (B). (D) Transplantation of r3/4 to the position of r5/6 (E) or r7/8 (F). (E,F) Frontal sections hybridized with Hoxa-3 2 days after grafting. Hoxa-3 is induced in the graft (limits indicated by arrowheads), in r7/8 (F) but not in r5/6 (E). Scale bar, 300 μm. Numbers correspond to the host’s rhombomeres.
Hoxa-2 was induced after grafting somites 5/6 (n=2/4) or 3/4 (n=2/7) but not somites 1/2 (n=0/3). Under similar conditions, Hoxa-3 expression was induced by grafting somites 5/6 (n=3/5) or somites 3/4 (n=1/5) but not of somites 1/2 (n=0/5).

The anterior domain of expression of these two genes is consequently devoid of inductive capacity in both the neurectoderm and the mesoderm.

**DISCUSSION**

In our previous work (Grapin-Botton et al., 1995), transposition of rhombomeres from a rostral to a more caudal position within the rhombencephalic domain resulted in reprogramming Hox gene expression in the NE and in homeotic transformation of rhombomere fate (Grapin-Botton et al., 1995). Itasaki et al. (1996) recently showed that the signals responsible for Hox gene induction in normally non-expressing territories can cross the avian/mammalian barrier, meaning that the mechanisms through which the NE assesses its identity along the AP axis have been evolutionary conserved.

In the present work, we wanted first to further investigate the variations in the presence of the inductive signal throughout the neurectoderm and also in the paraxial mesoderm, as well as the capacity of various regions of the NE to respond to this inducer. Our search for competence to respond to Hox genes of the four first paralogue groups, normally expressed in the rhombencephalon, was extended up to the di- and mesencephalic NE. The level of plasticity of the rhombomeres was further investigated by looking at their capacity to express posterior genes whose expression domains are normally restricted to the spinal cord.

**Competence of the NE to respond to Hox gene induction is present in the anterior encephalic vesicles and rhombomeres**

One of the main outcomes of this study is that when the NE of the promesencephalic vesicles and of the most rostral rhombomeres is subjected to an inducing field, it is able to express Hox genes.

First, the kinetics of Hoxb-4 induction was tested for transplantation of rhombomeres of different AP level into the r8p territory. We found that the more posterior the rhombomeres, the more rapid is Hoxb-4 expression in the transplant, meaning that competence to respond to the inductive signal decreases from caudal to rostral in the rhombencephalon. However, transplantation of more anterior territories belonging to the fore- and midbrain to r8p showed that competence to express Hox genes, far from disappearing rostrally, is still present beyond the rhombencephalic level. Moreover, anterior transplantation of r8p results in spreading of Hoxb-4 expression in the promesencephalic NE in situ.

In chick and mouse embryos, Otx2 is expressed before gastrulation in the whole epiblast and endoblast (Bailly-Cuif et al., 1995; Acampora et al., 1995). Later on, Otx2 transcripts become restricted to forebrain and midbrain only at a time when Hox gene expression is well established in the hindbrain. This may suggest that expression of these genes is mutually exclusive. Our experiments show that such is not the case since the prosencephalic and mesencephalic territories maintain
Otx2 expression while expressing Hoxb-4. It was previously shown that Otx2 expression in NE is regulated positively by anterior mesoderm and negatively by posterior mesoderm in mouse (Ang et al., 1994) and Xenopus (Pannese et al., 1995) at early stages of neural development. Moreover, Acampora et al. (1995) have demonstrated that expression of Otx2 in the anterior mesoderm is required for its activation in the ectoderm. The fact that anterior transposition of rhombomeres is not followed by induction of Otx2 may be due to the absence of inducer of this gene in the anterior mesoderm or to the loss of competence of the ectoderm at that stage. Whatever the reason may be, failure by rhombomeres to express genes in forebrain and midbrain confirms once more that anteriorisation of the neural territories cannot be achieved at that stage by changing positional cues (see Grapin-Botton et al., 1995).

The consequences of AP transpositions and changes in Hox gene expression for the specification of neural structures

Plasticity of diencephalic and mesencephalic alar plates has been extensively documented in the recent years (For reviews, Alvarado-Mallart, 1993 and Le Douarin, 1993; Marin and Puelles, 1994). When subjected to the isthmic organizing region these tissues express Engrailed 1-2 and Wnt1 genes and accordingly their differentiation program to tectal or cerebellar structures. This organizing region, when transplanted into the rhombencephalon was also able to induce En (Martinez et al., 1996).

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In the experiments described above, the alar plates of the di- and mesencephalon turned out to be able to express Hoxb-4 in two distinct experimental paradigms. In both cases, although expressing Hoxb-4, this anterior brain NE retained some Otx2 expression. Posteriorization of the forebrain and midbrain neural anlage was therefore incomplete at the molecular level.

Further development of these grafts showed that they retained most of their anterior characteristics, as attested to by their ability to form a tectum while being unable to form an inferior olivary nucleus. However, some cells of the diencephalic alar plate, which normally do not give rise to motoneurons, were recruited to the vicinity of the host motor nuclei and differentiated into motoneuron-like cells.

One can conclude that in forebrain to midbrain transplantations, expression of midbrain genes is dominant with respect to their effect on phenotype. In contrast, expression of hindbrain genes in forebrain to hindbrain transplantation is not since it does not result in obvious changes in phenotype.

Origin of the inducer and its distribution in a caudorostral decreasing gradient

The possibility that the specification of the AP neural patterning of the central nervous system could result not only from vertical signals of mesodermal origin, as proposed by Otto Mangold in 1933, but also from signals travelling in the plane of the neural epithelium itself, was suggested by experiments carried out in Xenopus embryos by Nieuwkoop (1952a-c), Nieuwkoop and Nater (1954) and Eyal-Giladi (1954). Nieuwkoop proposed that induction proceeds in two steps, the AP pattern resulting from the combined effect of an ‘activator’ which induces forebrain development and of a ‘transformer’ which modifies the anterior type specification into more posterior ones in a graded manner. This second step can be mediated by signals transmitted in the plane of the neuroectoderm. Doniach (1992, 1993) using Keller explants in which vertical signalling between ectoderm and mesoderm cannot take place, clearly demonstrated, by using molecular markers, that regionalization of the neuroepithelium can proceed in their absence. Our experiments in avians support the contention that both horizontal and vertical signals cooperate to establish the fine tuning of the AP neural pattern in the postotic hindbrain and spinal chord.

The possibility that the paraxial mesoderm might be able to transfer the inductive signal to the NE has been investigated independently by Itasaki et al. (1996) and ourselves. We both show that only somites posterior to somite 5 can induce Hoxb-4. In addition, we demonstrate that only somites posterior to somites 3/4 can induce Hoxa-2 and Hoxa-3. We show that the capacity to induce a given Hox gene is independent of the expression of this gene in a somite. This suggests that expression of Hox genes in the NE and the mesoderm is regulated by different pathways. The signal which originates from the somites is short range in the mesoderm since it requires close contact with the NE to operate.

The fact that the inducer can be transferred in the neur ectoderm is further demonstrated here. After anterior transpositions of r8p in the fore-, mid- and hindbrain, Hoxb-4 is induced in the host NE. The results obtained in the rhombencephalon contrast with those of Itasaki et al. (1996). It may be because in most cases the induction extends only 10-20 cell diameters and therefore it might have been missed by these authors who used whole-mount in situ hybridization without a cell marker and so were unable to distinguish host and grafted cells. In our grafts, some mesodermal cells which stick to the neural tube might have been included. They can be recognized in some sections by the fact that they are of quail origin. Their extension is however very limited and by all means restricted to the close vicinity of the graft whereas the inducing field is much larger. Moreover, the somites lateral to the grafted NE are somites 3/4 which, alone, are not able to induce Hoxb-4 in r2-6. Transmission of this inductive signal can thus be accounted for only by a planar transduction via the NE itself. The involvement of a planar signal is also corroborated by the induction pattern of Hoxb-4 in the caudally transplanted fore- and midbrain. Hoxb-4 induction is sometimes observed at both ends, adjacent to the NE, and not in the center of the graft.

For Hoxb-4 there is a shift in the domains of expression and inducing capacities between neuroectoderm and mesoderm (Fig. 6). Ectodermal domains extend more anteriorly than their mesodermal counterparts. Ter Horst (1948) reached similar conclusions in experiments carried out in Triturus embryos.

Although expressing Hox genes of the three first paralogue groups such as Hoxa-2 and Hoxa-3, the pre-otic and otic rhombomeric level (from r2 to r6 inclusively) corresponds to a region of the AP axis where these genes are not induced in r1 or r2 after posterior transposition. Similarly, it was previously shown that Hoxb-1 is induced in a non-expressing rhombomere transplanted to the r7/8 level (Grapin-Botton et al., 1995) but not the r4 level (Guthrie et al., 1992; Kuratani and Eichele, 1993). Therefore the environmental cues capable of switching
on genes of the four first paralog groups can be present only from the level of r7 caudalward and not in the pre-otic and otic rhombencephalon. Interestingly three different regulatory elements have been identified in the Hoxa-2 promoter controlling respectively its expression in the r2, r4 and at the r7 to spinal cord levels. Regulation of the latter control element was shown to involve a retinoic acid response region. This suggests the participation of retinoic acid in the inductions that we observe, although additional cofactors could be involved. It is thus interesting to notice that expression of Hox genes in the...
otic and pre-otic rhombomeres is independently regulated in rhombomeres through different sets of promoter elements, meaning that cross talk between these metamic structures is not required (Sham et al., 1993; Marshall et al., 1994; Studer et al., 1994; Pöpperl et al., 1995; Frasch et al., 1995; Nonchev et al., 1996).

Two experiments support the contention that there is a gradient of inducer along the AP axis. Results of the transposition of long grafts after a 180° rotation to the level of r8 and anterior spinal cord differs from the results of grafts of the same territories in their normal AP orientation. This can be accounted for by the fact that the two gradients of competence and of inducer were inverted (see Fig. 7), thus making the inductive effect of the signal uniform throughout the graft. Moreover, we show that somites 7/8 induce Hoxb-4 better than somites 5/6.

**Limited competence of the rhombomeres to express posterior Hox genes**

We show that the rhombencephalon and cervical spinal cord is only competent to express Hox genes located at the 3′ end of the Hox gene clusters from the 1st down to the 8th paralogous group. From the 9th paralogous group onward this competence does not exist, at least for the genes tested in this study. The comparative analysis of Hox gene homeobox sequences has led Schubert et al. (1993) to distinguish 3 classes in the Antennapedia type homeobox genes from the early time of metazoan evolution. These authors consider that the genes of the 5′ end of the clusters (from paralogous groups 9 to 13) constitute a distinct class and were probably represented by a single gene up to the divergence between insects and vertebrates. The results obtained here suggest that their regulatory sequences, and as a consequence, the mechanisms which control their expression may also, at least to a certain extent, be evolutionary-inherited.

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