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The caudal limit of *Otx2* gene expression as a marker of the midbrain/hindbrain boundary: a study using in situ hybridisation and chick/quail homotopic grafts

Sandrine Millet[1], Evelyne Bloch-Gallego[1], Antonio Simeone[2] and Rosa-Magda Alvarado-Mallart[1]

[1]INSERM U106, Hôpital de la Salpétrière, Bâtiment de Pédiatrie, 47, Bd de l'Hôpital, 75651 PARIS Cédex 13, France
[2]International Institute of Genetics and Biophysics, CNR, Via Marconi, 12, 80125 Naples, Italy

SUMMARY

Segmentation of the neural tube has been clearly shown in the forebrain and caudal hindbrain but has never been demonstrated within the midbrain/hindbrain domain. Since the homeobox-containing gene *Otx2* has a caudal limit of expression in this region, we examined, mainly in chick embryos, the possibility that this limit could represent an interneuromeric boundary separating either two cerebellar domains or the mesencephalic and cerebellar primordia. In situ hybridisation with chick or mouse *Otx2* probes showed the existence of a transient *Otx2*-negative area in the caudal mesencephalic vesicle, between stages HH10 and HH17/18 in chick, and at embryonic day 9.5 in mice. The first post-mitotic neurons of the mesencephalon sensu stricto, as labelled with an anti-β-tubulin antibody, overlay the *Otx2*-positive neuroepithelium with a perfect match of the caudal limits of these two markers at all embryonic stages analysed (until stage HH20). Chick/quail homotopic grafts of various portions of the midbrain/hindbrain domain have shown that the progeny of the cells located in the caudal mesencephalic vesicle at stage HH10 are found within the rhombomere 1 as early as stage HH14. Furthermore, our results indicate that the cells forming the HH20 constriction (coinciding with the caudal *Otx2* limit) are the progeny of those located at the caudal *Otx2* limit at stage HH10 (within the mesencephalic vesicle). As a result, the *Otx2*-positive portion of the HH10 mesencephalic vesicle gives rise to the HH20 mesencephalon, while the *Otx2*-negative portion gives rise to the HH20 rostral rhombomere 1. Long-survival analysis allowing the recognition of the various grisea of the chimeric brains strongly supports the view that, as early as stage HH10, the caudal limit of *Otx2* expression separates mesencephalic from isthmo/cerebellar territories. Finally, this study revealed unexpected rostrocaudal morphogenetic movements taking place between stages HH10 and HH16 in the mediodorsal part of the caudal *Otx2*-positive domain.

Key words: homeobox-containing gene, interneuromeric boundary, morphogenetic movement, neural tube segmentation, chick, quail, *Otx2*

INTRODUCTION

The occurrence of hindbrain neuromeres and their possible morphogenetic role in neural specification are commonly accepted. The rhombomeres (the neuromeres of the rhombencephalon), already visible in vivo, have boundaries that coincide with the arrest of expression of the homeobox-containing genes of the Hox family. These genes are believed to act as transcription factors involved in the rhombomere specification (for reviews see McGinnis and Krumlauf, 1992; Wilkinson, 1993). Moreover, cell clonage studies performed before and after the formation of rhombomeres have shown that these morphogenetic units are separated by clonal restriction boundaries (Fraser et al., 1990). Finally, the cells constituting the interneuromeric boundaries of the rhombencephalon are connected by gap junctions with reduced permeability properties compared to those linking cells in the walls of the rhombomeres (Martinez et al., 1992). A segmental subdivision of the forebrain has also been described, although it has been subject to controversy. Recently, morphological features and the expression of candidate regulatory genes (*Emx-1*, *Emx-2*, *Dlx-1*, *Dlx-2*, *Pax-6*, *Wnt-3*, *Gbx-2* among others) has led to the identification of six prosomeres (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Rubenstein et al., 1994). Also, in the prosencephalon, clonal restriction boundaries (Fidgor and Stern, 1993) and reduced permeability properties at the interneuromeric boundaries (Martinez et al., 1993) have been reported.

In contrast, the presence of interneuromeric boundaries within the midbrain/hindbrain (MHB) domain has not yet been clearly demonstrated. To our knowledge, neither clonal restriction boundaries nor gap junctions with reduced permeability have ever been reported within the MHB domain. Fate map experiments using the chick/quail model (Le Douarin, 1969) have revealed that, in the avian embryo of 10-12 somites (stage 10 of Hamburger and Hamilton, 1951, HH10), the cerebellar primordium is located on both sides of the so-called 'midbrain/hindbrain' constriction (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990; Alvarez Otero et al., 1993; Hallonet and Le Douarin, 1993). Thereby the cerebellum, a

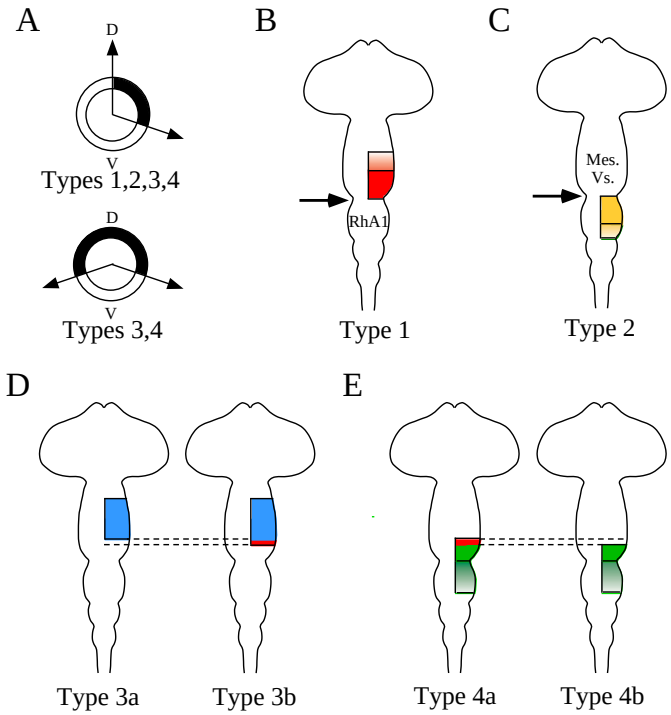

Fig. 1. Schematic representation of grafting experiments. All embryos are schematised at stage 10-12 somites (HH10). (A) Section of the neural tube representing the dorsoventral extent of the area taken as a graft (in black). D, dorsal; V, ventral. Note that for types 3 and 4 grafts some transplantations were made bilaterally. (B) Grafting of the caudal mesencephalic vesicle (in red). Note that the caudal graft border always coincides with the constriction (arrow) separating the mesencephalic vesicle (Mes. Vs., in C) and the prorhombomere A1 (RhA1). Conversely, the rostral border of the graft, which was not pertinent to our analysis, was variable inside the graduated shaded area. (C) Grafting of the RhA1 (in yellow). Note that the rostral border of the graft coincides again with the constriction (arrow). The caudal border varied inside the graduated shaded area. (D-E) Grafting of *Otx2*-positive (type 3, D) or *Otx2*-negative (type 4, E) territories (the grafted areas are schematized in blue and green, respectively, whether they include the red area or not). The important border of these two types of grafts (the caudal graft border in type 3 and the rostral one in type 4) is located at the level of the caudal *Otx2* limit. Because this limit was not visible in vivo, we have scanned a region (in red, between dashed lines) which should enclose the oblique *Otx2* caudal arrest. Subtypes 3a and 4a define the transplantations with the common graft border at its most anterior position, and subtypes 3b and 4b define transplantations with a common graft border at its most posterior position. Note also that type 4 grafts can concern either only the caudal mesencephalic vesicle or extend within RhA1 (their limits being the rostral and caudal end of the graduated shaded area, respectively).

structure presenting an amazingly homogeneous cytoarchitecture, seems to originate from two distinct neuromeres: one 'mesencephalic' and the other rhombencephalic. This possibility is supported by studies demonstrating anterior/posterior differences in the cerebellar cortex. For instance, several murine mutations affect the anterior or posterior cerebellum differently (Ross et al., 1990; Herrup and Wilczynski, 1982). The genes *En-2*, *En-1*, *Gli*, *Wnt-7* (see Millen et al., 1995), *Otx1* and *Otx2* (Frantz et al., 1994; Millet et al., 1994) have been described as having transient and different expressions in the anterior or posterior cerebellar cortex. The external granular layer, the secondary cerebellar neuroepithelium, develops exclusively from the rhombencephalic portion of the cerebellar anlage (Hallonet et al., 1990; Alvarez Otero et al., 1993; Hallonet and Le Douarin, 1993).

In both mouse (Simeone et al., 1992, 1993) and chick (Bally-Cuif and Wassef, 1994, 1995), the *Otx2* gene is expressed within the forebrain and the midbrain with a posterior limit at the MHB junction. The null mutation of *Otx2* gene in mice results mainly in a deletion of forebrain and midbrain but, in mutants with the strongest phenotype, it can also affect the cerebellar domain as suggested by loss of *En-2* expression and

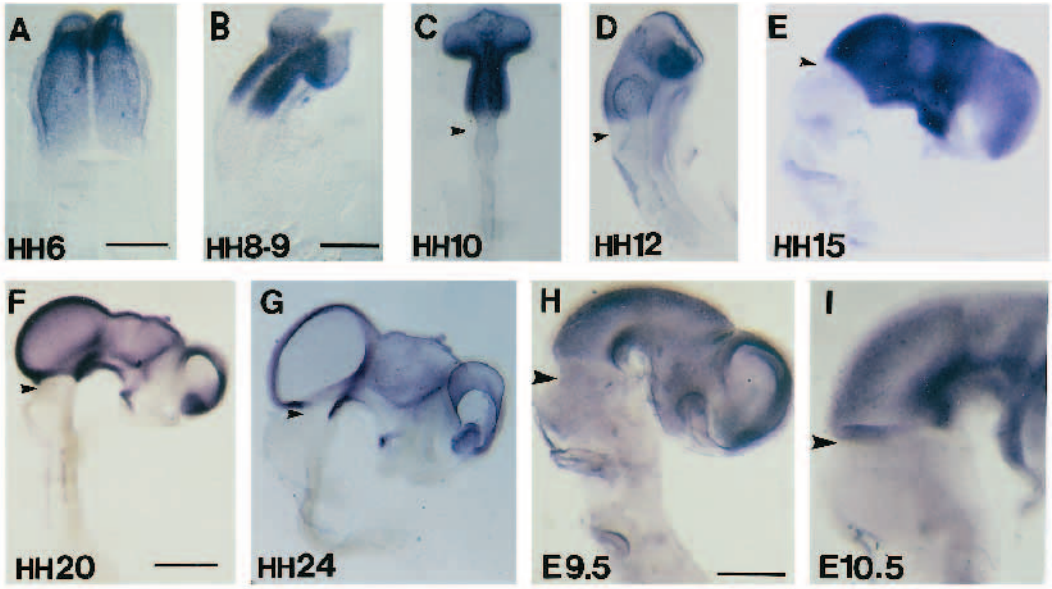

Fig. 2. Localisation of *Otx2* transcripts on whole-mount neural tubes of normal chick embryos (A-G) and mouse embryos (H,I). The developmental stage is specified for each figure. (A-C) Dorsal views; (D-I) lateral views and (G) lateral external view of an hemi-neural tube cut along the midline. The arrows point to the constriction separating the mesencephalic and the first rhombencephalic vesicles. Note that, at stage HH10, when this constriction is first recognised, the arrest of *Otx2* expression is clearly rostral to it. During the following stages, it becomes closer to the constriction and coincides with it at stage HH20. Note also that the *Otx2* arrest is rostral to the constriction in the mouse embryo at E9.5 and coincides with it at E10.5. Scale bars: A, 250 μm; B-E, H,I, 500 μm; F,G, 1 mm.

by the deletion of rhombomeres 1 and 2 (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). On the contrary, Bally-Cuif and Wassef (Bally-Cuif, 1994; Bally-Cuif and Wassef, 1995; Bally-Cuif et al., 1995) have shown that the perturbation of the boundary of *Otx2* expression by a non-functional *Wnt-1* protein results in a mixing of the cerebellar and mesencephalic territories. These studies have provided evidence in favour of the occurrence of an interneuromeric boundary separating the primordia of mesencephalon and cerebellum and which coincides with the boundary of *Otx2* expression.

The present study is aimed at analysing the relationship between the caudal limit of *Otx2* expression and the cerebellar primordia. By using in situ hybridisation (ISH) with an *Otx2* chick riboprobe, we have shown that the *Otx2* posterior limit varies during the early stages of neurogenesis with respect to the 'MHB' constriction. Immunostaining of postmitotic mesencephalic neurons and analysis of chick/quail chimeras with various types of homotopic grafts, analysed after different survival times, show that, from stage HH10 onwards, the caudal limit of expression of *Otx2* evolves in parallel with the boundary separating the mesencephalic and isthmocerebellar territories.

MATERIALS AND METHODS

Embryos

Fertilised White Leghorn Chick eggs (Haas, Strasbourg, France) and Japanese Quail eggs (La Caille de Chanteloup, Corps-Nuds, France) were incubated in a humidified atmosphere at 38±1°C. Mouse embryos were obtained from timed mating of outbred OF1 mice (IFFA-Credo, Lyon, France).

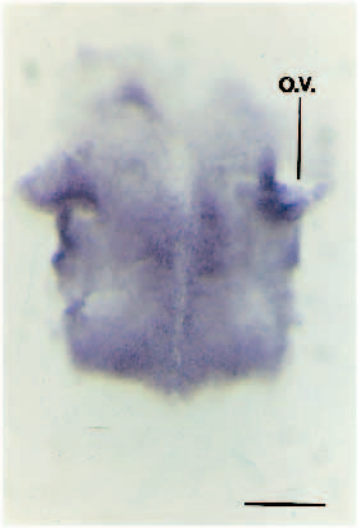

Fig. 3. Flat-mount neural tube of an HH10 normal chick embryo stained by ISH with the chick *Otx2* probe. The neural tube has been opened along the ventral midline. Note that the caudal limit of *Otx2* expression is sharp and forms an oblique line with a caudalward oriented 'beak'. Note also that the cells on the dorsal midline are *Otx2*-negative. o.v., optic vesicle. Scale bar, 250 μm.

Chick/quail grafting experiments

Four types of homotopic transplantations have been carried out (Fig. 1). Chick embryos were used as the host and quail embryos as the donor. The transplantations were performed at the stage of 10-12 somites (HH10). A detailed description of the microsurgical procedure, using hand-made microscalpels, has been described previously (Alvarado-Mallart and Sotelo, 1984). As schematised in Fig. 1A, all types of transplantation concerned exclusively the alar portion of the neural tube and were usually unilateral. For type 3 and 4 grafts, some bilateral transplantations were also performed.

As schematised in Fig. 1B and C, types 1 and 2 transplantations concern the caudal portion of the mesencephalic vesicle (area in red in Fig. 1B) and the first rhombencephalic vesicle (at this stage, the prorhombomere A1, RhA1, see Vaage, 1969, area in yellow in Fig.

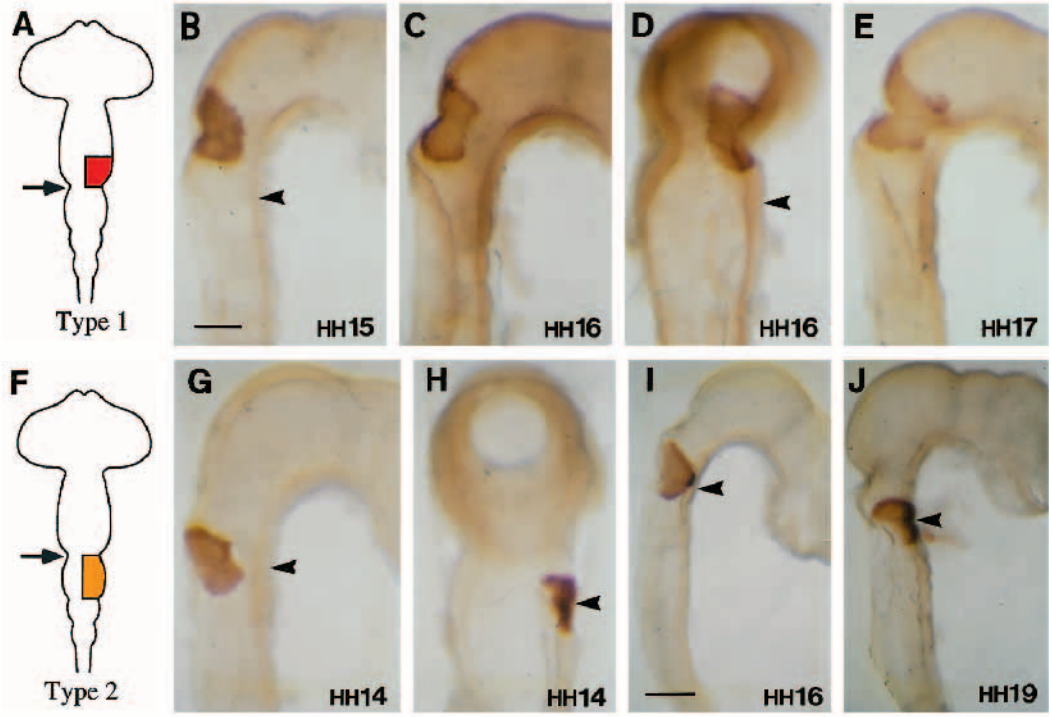

Fig. 4. Chimeric neural tubes bearing type 1 (B-E) and type 2 (G-J) grafts stained in toto with QCPN anti-quail antibody. The areas taken as a graft at transplantation are schematised in A (red) and F (yellow). The developmental stages at fixation are given for each case. Note that from surgery to fixation the grafts have undergone a relative caudalward shift with respect to the constriction separating the mesencephalic and first rhombencephalic vesicles. The original caudal border of type 1 grafts and the rostral border of type 2 grafts, corresponding to the HH10 constriction (arrows in A and F) are located caudally to the constriction at fixation. Also note that, in B-E, the grafts extend in both the mesencephalic vesicle and rh1; in G-J, the grafts extend in both caudal rh1 and rostral rh2. The arrowheads in B,D and G-J point to the rh1/rh2 boundary. Scale bars, B-E and G,H, 250 μm; I,J, 500 μm.

1C), respectively. These transplants share therefore a common graft border: the so-called MHB constriction (arrows in Fig. 1B and C). Note that the other graft borders (rostral for type 1 and caudal for type 2 grafts) can vary from case to case (see dotted lines in Fig. 1B,C).

For type 3 grafts (Fig. 1D), we tried to transplant exclusively the *Otx2*-positive mesencephalic territory. However, since there is no landmark to recognise the caudal limit of *Otx2* expression in vivo, we intentionally moved the caudal border of these grafts to scan the area (in red in Fig. 1D) that should enclose the *Otx2* caudal limit, as observed in normal embryos stained by ISH with the *Otx2* chick probe (see below). Among the various caudal graft borders performed with this procedure, the most anterior defines type 3a grafts and the most posterior defines type 3b grafts (see dashed lines in Fig. 1D).

For type 4 grafts (Fig. 1E), we tried to transplant the *Otx2*-negative mesencephalic territory alone or with a portion of the rostral RhA1. In this case, the rostral graft border scans the area enclosing the *Otx2* caudal limit (in red in Fig. 1E) defining again two subtypes of grafts with different rostral graft borders (see dashed lines in Fig. 1E).

After transplantation, the host eggs were closed with parafilm sealed with paraffin and kept at 38±1°C for various survival times. The resulting chimeric embryos were analysed (i) 3 to 4 hours after transplantation, (ii) at embryonic days 3 to 4 (E3-E4) and (iii) at later embryonic stages (E11-E14).

Fixation

The developmental stage of normal chick and chimeric embryos were determined at the moment of fixation according to Hamburger and Hamilton (1951).

Embryos up to stage HH24 were fixed overnight by immersion in 4% paraformaldehyde solution (PF 4% in 0.12 M phosphate buffer, pH=7.4) at 4°C. The neural tubes were dissected, rinsed twice in phosphate-buffered saline/Tween 0.1% (PBT), dehydrated in increasing concentrations of methanol, and frozen and stored at –20°C in 100% methanol. Before treatment (ISH or immunocytochemistry), embryos were rehydrated in decreasing concentrations of methanol and washed twice in PBT.

Long-survival chimeras (stages HH37 to HH40) were perfused through the heart with PF 4% and postfixed overnight at 4°C in the same fixative. The dissected brains were then cryoprotected in 10% sucrose solution (in phosphate buffer, pH=7.4) for 24 hours, embedded in gelatine (7.5% solution of Sigma type A in the same sucrose solution) and frozen in isopentane cooled to –60°C with liquid nitrogen. The frozen brains were serially sectioned in a cryostat in the sagittal plan. The sections (20 μm thick) were mounted on two parallel sets of slides. One set was used for immunohistochemistry with the QCPN antibody (see below) to visualise the grafted cells, the other set was stained with cresyl violet/ thionine to study the cytodifferentiation.

Chick *Otx2* cDNA

A chicken embryonic cDNA library was screened using a murine *Otx2* cDNA probe. One of the positive clones was purified and a PCR-amplified fragment containing the most 3′ coding exon was used for in situ hybridisation experiments. Its sequence included the last 206 aa of the Otx2 protein and showed 98.5% identity to the murine *Otx2* coding sequence and 100% identity to the partial chick *Otx2* coding sequence previously reported (Bally-Cuif and Wassef, 1995).

Chick and mouse *Otx2* probes

The chick *Otx2* subclone was linearized with *Bam*HI or *Hin*dIII (Boehringer, Mannheim) and transcribed in the presence of Dig-UTP (Amersham) using T7 RNA polymerase or T3 RNA polymerase (Riboprobe Kit, Promega) to produce the antisense or sense probes, respectively. The mouse *Otx2* subclone (Simeone et al., 1992) was linearized with *Eco*RI or *Hin*dIII and transcribed in the presence of Dig-UTP using SP6 or T7 RNA polymerase to produce the antisense or sense probes, respectively. No signal was obtained when using the sense probes.

Whole-mount in situ hybridisation

ISH was carried out according to Wilkinson (1992). The probe was revealed using an anti-digoxigenin-alkaline-phosphatase Fab antibody (1/2000, Boehringer, Mannheim) and NBT-BCIP (Boehringer, Mannheim) as a substrate for the alkaline phosphatase. Embryos were then rinsed extensively in PBT and stored at 4°C in 80% glycerol.

Immunocytochemistry

Two primary antibodies were used in this study. The neural-specific anti-β-tubulin TuJ-1 antibody (a kind gift from Dr A. Frankfurter) was diluted 1/1500. A biotinylated horse anti-mouse antibody (1/200, Vector) was used as a secondary antibody and revealed with the ABC complex (1/400, Vector). The mouse monoclonal anti-quail antibody, QCPN (Developmental Studies Hybridoma Bank) was diluted 1/100 and revealed by the peroxidase/anti-peroxidase method of Sternberger (1970).

Whole-mount immunocytochemical reactions were preceded by treatment in a solution of lysine (0.1 M in PBS, gelatine 0.2%, triton 0.25%, azide 0.1%) for 1 hour at RT. For all the embryos stained with the TuJ-1 antibody and some of those stained by the QCPN antibody, immunohistochemistry was performed after *Otx2* ISH (following extensive washes in PBT). In all cases, after immunostaining, the neural tubes were rinsed in PBS-Triton X-100 (0.25%) and stored in glycerol 80% in PBT.

The QCPN immunocytochemistry on sections was preceded by an anti-endogenous peroxidase treatment (1 minute incubation in a 0.25% $KMNO_4$ solution in PBS, followed by a wash in 1% oxalic acid solution in PBS, until bleaching) and a 1 hour treatment in lysine solution as above. In this case, the DAB immunoreaction was intensified with a 0.6% solution of nickel ammonium sulphate in the DAB solution.

RESULTS

Changes of the caudal limit of *Otx2* expression from stage HH10 to HH24

During the first stages of neurulation, the expression of the *Otx2* gene extends through the anterior neural plate and tube and within the lateral borders of rhombencephalon (Simeone et al., 1992, 1993; Bally-Cuif and Wassef, 1994). Here, we will focus on the caudal limit of *Otx2* expression in the anterior neural tube and its relation with the so-called MHB constriction. This constriction can be identified at HH10, simultaneously with the individualisation of the mesencephalic and RhA1 vesicles. Before the closure of the neural tube (see Fig. 2A, illustrating stage HH6), the posterior limit of *Otx2* expression is not well defined. During the following stages, as the neural folds fuse at the level of the presumptive midbrain, the caudal *Otx2* limit becomes sharper (see Fig. 2B, illustrating stage HH8/9). At stage HH10, the *Otx2* expression extends through the prosencephalic and mesencephalic vesicles with a clear posterior limit somewhat rostral to the MHB constriction: the caudal fifth of the mesencephalic vesicle is *Otx2* negative (Fig. 2C). It is important to emphasise that the caudal limit of *Otx2* expression is not exactly transversal to the neural tube axis. This is better observed in flat mounted embryos (Fig. 3) where this limit forms a caudalward ‘beak’ at the level of the dorsal midline. During the following stages, the ‘mesencephalic’ *Otx2*-negative area progressively diminishes in size (see Fig. 2D and E illustrating stages HH12 and HH15). From stages HH17/18 onwards, this area disappears and the caudal

limit of *Otx2* expression coincides with the MHB constriction (see Fig. 2F and G illustrating stages HH20 and HH24). At these stages, the RhA1 splits into two vesicles, the first two rhombomeres (rh1 and rh2).

A similar analysis in mouse embryos shows that the presence of a transient *Otx2*-negative portion within the caudal mesencephalic vesicle is not peculiar to the avian species: the caudal limit of *Otx2* expression lies rostral to the MHB constriction at embryonic day 9.5 (E9.5), while coinciding with the constriction 24 hours later, at E10.5 (see Fig. 2H and I).

These observations raise questions concerning the mechanisms of this shift in the posterior limit of *Otx2* expression from the caudal mesencephalon to the MHB constriction. It could result from either an up-regulation of the *Otx2* gene in previously *Otx2*-negative cells, or from the translocation of the 'mesencephalic' *Otx2*-positive cells towards the rhombencephalon. It is likely that the HH10 *Otx2*-negative mesencephalic territory corresponds to the 'mesencephalic' cerebellar primordia depicted by fate map experiments using the chick-quail model (see Introduction). However, the analysis of chick/quail chimeras revealing that the cerebellar primordium extends, at stage HH10, on both sides of the MHB constriction, was performed only at stages older than E5. Therefore, these studies did not determine at which moment during development the 'mesencephalic' cells shift towards the rhombencephalon. We have thus performed similar experiments but analysed the chimeras after shorter survival times to determine whether this shift follows a time course that is compatible with the one just described for the posterior limit of *Otx2* expression.

Cells from the HH10 caudal mesencephalic vesicle are found within rhombomere 1 as soon as stage HH14

Two main types of homotopic transplantations were performed at HH10 to study the fate of both the caudal mesencephalic vesicle and RhA1. As illustrated in Figs 1B,C and 4A,F, type 1 transplantations concern the caudal mesencephalic vesicle, while type 2 concerns RhA1. These two types of transplants share a common border: the caudal border in type 1 and the rostral border in type 2, coinciding with the MHB constriction (arrows in Figs 1B,C and 4A,F). All chimeric embryos (n=16 for type 1 and n=18 for type 2 grafts) were analysed by means of the anti-quail, QCPN, antibody. From stage HH14 onwards, the type 1 grafts extend to both sides of the MHB constriction (see stages HH15, HH16, and HH17 in Fig. 4B,C/D and E, respectively), whereas type 2 grafts become located solely in the caudal portion of rh1 and has shifted caudally with respect to the MHB constriction (see stages HH14, HH16 and HH19 in Fig. 4G/H,I and J respectively). These latter grafts can invade parts of rh2 (Fig. 4G-J) when the graft involves the entire HH10 RhA1 (Fig. 4F). Thereby, cells derived from the HH10 MHB constriction (caudal border for type 1 and rostral border for type 2 grafts) become located within rh1 starting at stage HH14/15. It can be concluded that the constriction separating the mesencephalic vesicle from rh1 at stage HH14 and onwards is not formed by cells derived from what appears to be the 'MHB' boundary at HH10. As discussed below, the term MHB constriction should not be used to designate the constriction observed at stage HH10. The results also show that the shift of constriction, resulting in a relocation of the caudal 'mesencephalic' cells within the rhombencephalon, is a very early event that takes place between stages HH10 and HH17.

The *Otx2*-positive neuroepithelium evolves in parallel with the first β-tubulin-positive cells of the dorsal mesencephalon

As previously described in the mouse and chick (Moody et al., 1989; Easter et al., 1993, Chédotal et al., 1995), the TuJ-1 antibody, recognising a class III neuron-specific β-tubulin isotype, can be used as a marker for the first postmitotic neurons. Based on their location, birthdate and axonal projection, the first-labelled cells include the mesencephalic sensory neurons of the fifth nerve (MesV), lying all along the mesencephalic dorsal midline, and the tecto-bulbar neurons spreading over the dorsal tectum (Easter et al., 1993, Chédotal et al., 1995; Sheperd and Taylor, 1995). In the chick, these neurons are first observed at stage HH14 and can be followed at least to stage HH20 (Chédotal et al., 1995). Therefore, it is interesting to compare the localisation of these mesencephalic cells with respect to the neuroepithelial area expressing the *Otx2* gene. At all stages between HH14 and HH20, in chick embryos double stained with the *Otx2* chick probe and with the TuJ-1 antibody, dorsal mesencephalic β-tubulin-positive cells just overlie the *Otx2*-positive neuroepithelium (Fig. 5A-C). Furthermore, between stages HH14 and HH20, the caudalmost of these neurons undergo the same apparent displacement towards the constriction as does the posterior limit of *Otx2* expression. These results together with those obtained with type 1 and 2 grafts provide evidence that, from HH10, the caudal limit of *Otx2* expression represents the caudal limit of the mesencephalon. In other words, the *Otx2*-negative portion of the HH10 mesencephalic vesicle may represent the 'mesencephalic' isthmocerebellar neuroepithelium depicted by previous experiments using the chick/quail model (Martinez and Alvarado-Mallart, 1989; see also Hallonet et al., 1990; Alvarez Otero et al., 1993; Hallonet and Le Douarin, 1993).

From stage HH10, the *Otx2* caudal limit of expression may represent the caudal end of the mesencephalic neuroepithelium

To verify this hypothesis, two other types of homotopic transplants were carried out aimed at transplanting the mesencephalic *Otx2*-positive territory or the 'mesencephalic' *Otx2*-negative territory (types 3 and 4 respectively, Fig. 1D and E). Here, the important graft border was the one corresponding to the caudal *Otx2* arrest (the caudal graft border of type 3 grafts and the rostral border of type 4 grafts). As illustrated in Figs 3 and 5A, this caudal *Otx2* limit is not totally transversal to the neural tube. Its oblique border line cannot be visualised in vivo at the moment of transplantation, and thus was difficult to follow during surgery, particularly its characteristic caudalward 'beak' at the level of the dorsal midline. Therefore, we decided to keep all borders of the grafts transversal to the tube. Furthermore, we intentionally varied the caudal limit of type 3 grafts and the rostral border of type 4 grafts, within an area that should enclose the *Otx2* posterior limit as observed by ISH in the normal chick and quail embryos (area schematised in red between broken lines in Fig. 1D and E). For type 3 grafts (the extreme caudal borders corresponding to types 3a and 3b, see Fig. 1D), we tried to exchange the caudal *Otx2*-positive neuroepithelium between chick and quail embryos, free from most

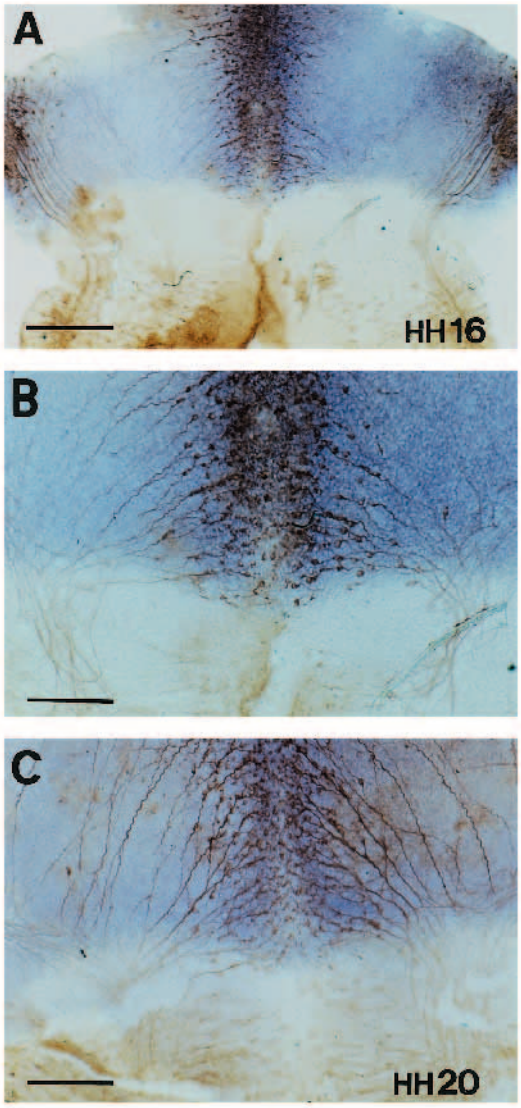

Fig. 5. Flat-mounted neural tubes of normal chick embryos stained by ISH with the chick *Otx2* probe (in violet) and by immunohistochemistry with the TuJ-1 anti-β-tubulin antibody (in brown). (A) Stage HH16; (B) high magnification of A; (C) stage HH20. Note that in each case the β-tubulin-positive neurons overlie precisely the *Otx2*-positive neuroepithelium, including the caudalwards 'beak'. Scale bars, A,C, 20 μm; B, 10 μm.

of the *Otx2*-negative cells. For type 4 grafts (the extreme rostral borders corresponding to types 4a and 4b, see Fig. 1E), we tried to exchange the *Otx2*-negative portion of the HH10 mesencephalic vesicle (with or without portions of RhA1), free from most of the *Otx2*-positive cells. The resulting chimeric embryos were analysed at three different stages. We first wanted to verify, by analysing the chimeras 3 to 4 hours after transplantation, that the scanned region (in red in Fig. 1D and E) contained the *Otx2* caudal limit, in both donor and host. Then, we analysed the chimeric embryos at stages HH16 to HH20 (20 to 40 hours after transplantation), in order to determine whether the whole *Otx2*-negative territory of the HH10 mesencephalic vesicle became located within rh1. Finally, an analysis performed at later embryonic stages (long-survival chimeric embryos, HH37 to HH40) allowed the study of the cytodifferentiation of the grafted areas.

Chimeric embryos analysed 3-4 hours after transplantation

4 hours after transplantation, the grafts are already integrated into the host neural tubes, but the chimeric embryos have changed very little in shape since they are still at late stage HH10 or at stage HH11. Thus, these experiments gave a good idea of the area removed in the host at the moment of surgery and the one taken from the donor for transplantation. Such chimeric embryos (n=10 for type 3 grafts and n=10 for type 4 grafts) were treated by in situ hybridisation with the chick *Otx2* probe and analysed in flat mounted preparations, to determine how the graft borders (caudal in type 3 and rostral in type 4)

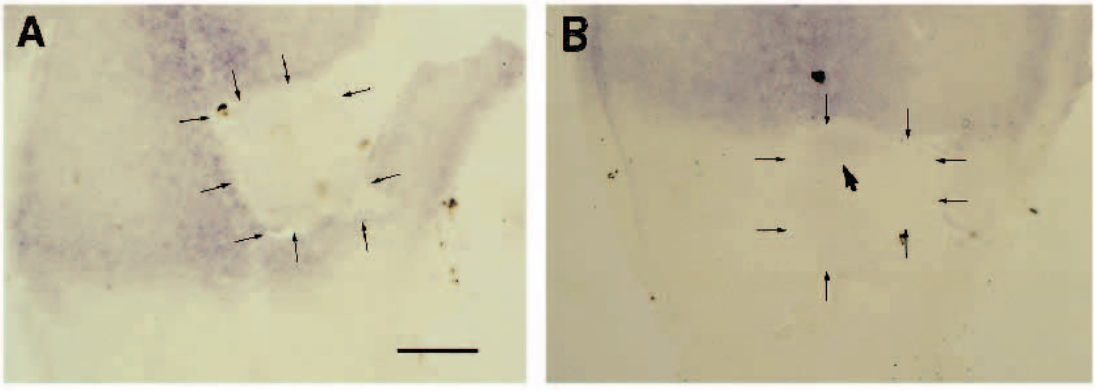

Fig. 6. Flat-mounted neural tube bearing a type 3 (A) or type 4 (B) graft, fixed 3-4 hours after transplantation and treated by ISH with the chick *Otx2* chick probe. The neural tube has been opened along the ventral midline (in A, the lateral host tube has been broken during this process). The grafts are encircled by thin arrows. (A) Note that the graft lies entirely in the *Otx2*-positive territory. This graft is also *Otx2* positive. However, due to the low affinity of the chick *Otx2* probe for quail tissue, the *Otx2* labelling in the graft is very pale and not as obvious in this picture. This case corresponds to a type 3a graft. (B) The graft lies mainly within the *Otx2*-negative territory but also extends within the *Otx2*-positive territory. It contains some *Otx2*-positive cells mediorostrally (see large arrow). This case corresponds to a type 4 a/b graft. Scale bar, 135 μm.

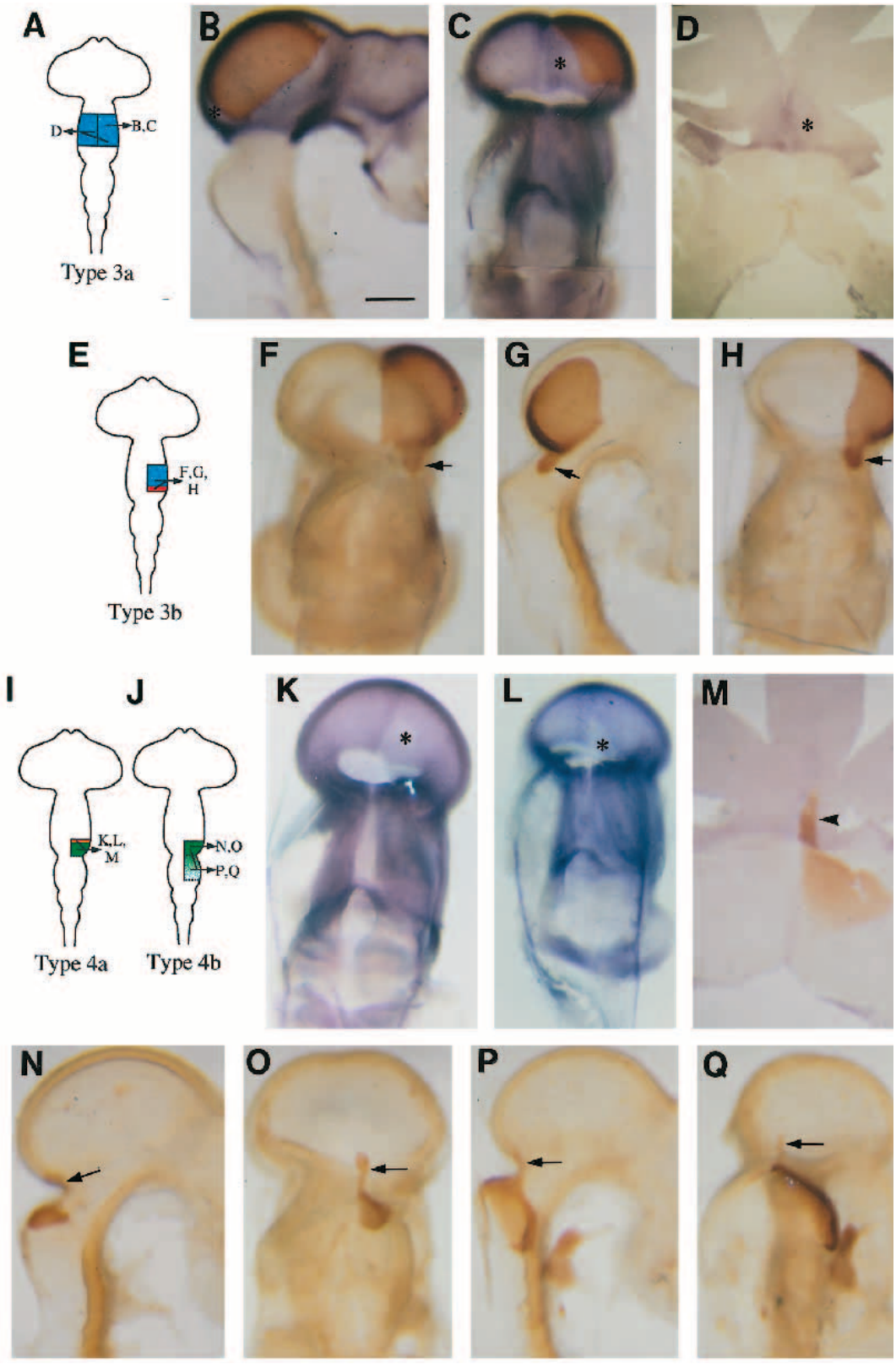

Fig. 7. Chimeric neural tubes bearing types 3 and 4 grafts, treated by ISH with the chick *Otx2* probe (in violet in D and K-L), or by immunohistochemistry with QCPN anti-quail antibody (in brown in F-H and N-Q) or by both techniques (B, C and M). (B-D) Type 3a transplantations as in A. Lateral (B) and dorsocaudal (C) views of the same embryo bearing an unilateral graft. (D) Flat-mounted neural tube with a bilateral graft. (F-H) Type 3b transplantations as in E; (F,H) a dorsocaudal view of two different chimeras; (G) lateral view of the chimera illustrated in H. (K-L) Dorsocaudal views of two different chimeras with type 4a transplantations as in I. (M) Flat-mounted neural tube of a third case bearing a more posterior graft border (type a/b transplant). (N-Q) Type 4b transplantations as in J. (N-O) and (P-Q) are lateral and dorsocaudal views of two different chimeras, respectively. The low affinity of the chick *Otx2* probe for quail tissue allows the localisation of the quail graft when located in the *Otx2*-positive territory because it is paler than the *Otx2*-positive chick tissue (* in K and L). The reverse is also true, a host *Otx2*-positive territory can be recognized clearly when surrounded by an *Otx2*-positive paler labelled graft (* in B-D). The * in B-D and K-L and arrowhead in M indicate the triangular-shaped area in the dorsocaudal *Otx2*-positive territory which has the opposite (chick or quail) phenotype to the rest of the mesencephalon. This area is reduced to a thin vertical line of cells along the dorsal midline in type 3b and 4b grafts. This is more conspicuous for type 4b (arrows in N-Q) since this area can be depicted by its immunoreaction to the anti-quail antibody in these cases. Note also that, in type 3b, the graft extends caudally to the *Otx2* limit within the lateral *Otx2*-negative rhombencephalon (arrows in F-H). The corollary of this result is obtained with types 4a/b and 4b grafts: the graft extends medially but not laterally in the rostralmost rhombencephalon and the most caudal *Otx2*-positive territory (M-Q). Scale bar, 500 μm for all photos.

are located with respect to the *Otx2* arrest in these 20 cases. The graft, recently integrated into the host neural tube, can easily be distinguished without treatment with the anti-quail antibody. In general, there was a good correspondence between the hole made in the host and the portion taken from the donor. With the exception of two cases in which these two parameters were too rostral with respect to the *Otx2* caudal limit, the graft borders common to the two types of grafts are in the close vicinity of the caudal *Otx2* limit. The most anterior of these graft borders (corresponding to type 3a and 4a grafts) were entirely located in the *Otx2*-positive region (see Fig. 6A illustrating a type 3a graft and schema in Fig. 8A). The most posterior graft borders always cross the *Otx2* caudal limit, isolating either a little *Otx2*-positive region, dorsomedially located (see arrow in Fig. 6B), or even just the *Otx2*-positive caudalward 'beak' on the dorsal midline. The latter case corresponds to types 3b and 4b grafts while the former can be considered as intermediate between subtypes a and b (types 3a/b and 4a/b, see Fig. 8A).

These observations show that the scanned area (in red in Fig. 1D and E) indeed contains the oblique *Otx2* caudal limit and that the transversal graft borders are either totally located inside the *Otx2*-positive territory or cross the *Otx2* caudal limit (see Fig. 8A).

Chimeric embryos analysed 20-40 hours after transplantation

A conspicuous result of type 3 and 4 grafts is that their common border, transversal at the moment of surgery, becomes oblique, or even almost vertical, in its medial part. The precise location of this curved line with respect to the *Otx2*-positive territory will be described in detail for each type of graft; it is illustrated in Fig. 7 and schematised in Fig. 8B.

Chimeric embryos with type 3 grafts (n=20) show that, at all stages analysed (HH16-HH20), most of the grafted cells lie within the mesencephalic vesicle and are therefore *Otx2* positive (Fig. 7B-D and F-H). However, there are important differences in the caudal extent of the grafted cells, related to the small variations in the area dissected at stage HH10. In type 3a grafts in which, at surgery, the caudal graft border was at its most anterior position (see schemes in Figs 7A and 8A), the graft remains located entirely in the *Otx2*-positive region. Laterally, its caudal border is very close to the *Otx2* caudal limit but dorsomedially, it delimits a large triangular shaped area in the mesencephalic vesicle (expressing *Otx2*) which is formed by host cells (negative for the anti-quail antibody) (Figs 7B,C, 8B). This triangular region does not result from a grafting artefact concerning the dorsal midline since it is also observed in chimeric embryos with bilateral grafts (Fig. 7D). When the caudal graft border is moved posteriorly, as observed in chimeras of type 3b (see scheme in Figs 7E, 8A), laterally the graft concerns not only mesencephalic *Otx2*-positive cells but also the rostral part of the *Otx2*-negative rhombencephalon (Figs 7F-H, 8B). Dorsomedially, the graft still excludes few *Otx2*-positive cells of the mesencephalon (negative for the anti-quail antibody) located along the dorsal midline (see Figs 7F-H, 8B).

Type 4 grafts provide complementary results. Most of the grafted epithelium (whether it includes RhA1 or not) becomes located within the *Otx2*-negative rhombencephalon (Fig. 7M-Q). However, in all cases, we also observed some *Otx2*-positive grafted cells located within the *Otx2*-positive dorsomedial portions of mesencephalon (Fig. 7K-Q). The rostral extent of the grafted area in the mesencephalon and rostral rhombencephalon varies depending on the extent of the area used for the graft at stage HH10. In grafts of type 4a, with the most anterior rostral graft border (see schemes in Figs 7I, 8A), the *Otx2*-positive part of the graft forms a dorsomedial triangular-shaped area (Figs 7K-L, 8B) very similar to that formed by host chick cells in type 3a grafts. This mediorostral *Otx2*-positive part of the graft diminishes in size when the caudal graft border is moved posteriorly, as observed in the case illustrated in Fig. 7M (corresponding to a type 4a/b transplantation). However, in this case, laterally the graft no longer concerns the rostralmost part of the *Otx2*-negative rhombencephalon. Finally, when the caudal graft border reaches its most posterior position (types 4b grafts, see schemes in Figs 7J, 8B), the mediorostral part of the graft extends into the *Otx2*-positive mesencephalon as a thin line of cells along the dorsal midline (analogous to the few chick cells excluded from the graft along the dorsal midline in type 3b) while laterally the graft excludes the rostralmost rhombencephalon (Figs 7N-Q, 8B).

In conclusion, our analysis has shown that, at stage HH20, type 3 grafts mainly remain located within mesencephalon while type 4 grafts are mainly relocated within rhombencephalon. The unexpected morphogenetic movements depicted by these grafts deserve discussion (see below). Nevertheless, taking into account that the scanned area (between graft borders 'a' and 'b', see Fig. 8A) has a composite phenotype, *Otx2*-positive and *Otx2*-negative, our results strongly support the view that all *Otx2*-negative cells of the HH10 mesencephalic vesicle, and only them, switch caudally towards the rhombencephalon (see Discussion). It was therefore interesting to examine the cytodifferentiation of these types of grafts at later embryonic stages.

Long-survival chimeras

The cytodifferentiation of chimeric embryos with type 3 and 4 grafts have been analysed at embryonic stages HH37-HH40. The grafted cells were recognised by their imunoreactivity to the anti-quail antibody. The chimeras presenting severe malformations were discarded. Those selected for analysis are completely in agreement with the data obtained by the short-survival analysis: type 3 grafts develop mainly mesencephalic grisea and type 4 grafts develop mainly cerebellar and isthmic structures. Furthermore, depending on the precise location of the common graft borders, 'a', 'a/b' or 'b', some mesencephalic and isthmo/cerebellar structures are present or excluded from the grafted area.

In the following description, we will focus on three cases fixed at stage HH40, representative of these graft borders: case S419 with an 'a' graft border (type 3a graft), case G835 with an 'a/b' graft border (type 4a/b graft) and case G860 with a 'b' graft border (type 4b graft).

Case S 419 (type 3a graft)

In this case, the graft provides a large portion of the tectum, with the exclusion of its most rostromedial portion (not illustrated). As a result of the rotation of the tectal anlage (see Goldberg, 1974), this rostromedial part of the tectum actually arise from the mediocaudal part of the mesencephalic neu-

roepithelium which, as observed in our short-survival analysis (Fig. 8), was excluded from this type of grafts. In addition, grafted cells are found in other mesencephalic alar grisea, such as the nucleus mesencephalicus lateralis, pars dorsalis (Mld) and the nucleus tegmenti pedunculo-pontinus, pars compacta (Tpc, the avian substantia nigra). No quail cells have been observed either in the cerebellum or in the isthmic nuclei (even in the nucleus isthmi, pars magnocellularis, described by Vaage, 1973 and Puelles and Martinez de la Torre, 1987) to have a mesencephalic origin.

In the other two cases, G835 and G860, bearing type 4 transplantations, the HH10 graft contains a few portion of RhA1 territory in addition to the caudal mesencephalic vesicle (Fig. 9A).In both cases, the grafted cells were mostly located in cerebellum and isthmus but were also found in a portion of the mesencephalon (Fig. 9B-I). Due to the complex migratory pathways of the postmitotic cerebellar cells (see in particular Alvarez Otero et al., 1993), the detailed analysis of the chimeric cerebella is beyond the scope of this paper. Here, we will focus mainly on the extent of the grafted cerebellar ependyme which is reminiscent of the grafted cerebellar neuroepithelium.

Case G835 (type 4a/b graft)

In this chimeric brain, the grafted cells spread widely in medial but not lateral cerebellum. In the medial sections, the caudal portion of the cerebellar ependyme is formed by quail grafted cells, while its rostral portion is formed by chick host cells (Fig. 9D). In no sections are quail cells observed to reach the rostral end of the cerebellar ependyme and, consequently, quail Purkinje cells are almost completely absent from rostral lobules I-III. Granule cells are numerous throughout the medial sections because the grafted epithelium extends up to the choroidal tissue (see Alvarez Otero, 1993). Since previous studies have demonstrated that the HH10 mesencephalic vesicle gives rise to the mediorostral part of the cerebellum (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990; Alvarez Otero et al., 1993; Hallonet and Le Douarin, 1993), these observations indicate that a part of the 'mesencephalic' cerebellar primordium was excluded from this graft. This excluded portion of the cerebellar primordium may correspond to the laterorostral portion of the HH10 *Otx2*-negative region which is excluded from this type of graft, as shown in our short-survival analysis (Fig. 8).

The grafted cells also give rise to isthmic nuclei, including the isthmo-optic nucleus (Fig. 9C), a large ventral part of the nucleus isthmi, pars parvocellularis (Fig. 9B) and to a lesser extent the nucleus isthmi, pars magnocellularis (not illustrated).

Finally, this graft also concerns a large mediorostral portion of the tectum (mediocaudal in origin, see Fig. 9B,C) as well as the tectal comissure and several cells of the nucleus mesencephalic of the fifth nerve (see Fig. 9E). These mesencephalic structures may correspond to the mediorostral *Otx2*-positive portion contained in this type of graft (Fig. 8A) forming at stage HH20 a triangular-shaped region in the *Otx2*-positive mesencephalon (Fig. 8B).

Case G860 (type 4b graft)

In this case, the quail cells spread both in medial (Fig. 9F) and lateral cerebellum. Again, a rostromedial portion of the cerebellar ependyme is formed by chick cells (Fig. 9F). Accordingly, almost no grafted Purkinje cells are observed within the rostral lobules I-III.

Outside of the cerebellum, grafted cells are found in isthmic nuclei located in a dorsoventral band (not shown), and also in a ventral part (more reduced than in case G835) of the nucleus isthmi, pars magnocellularis and pars parvocellularis (Fig. 9H). Concerning the mesencephalic grisea, only a few grafted cells are found in the tectal comissure (Fig. 9G) and in the mesencephalic nucleus of the fifth nerve (not illustrated). Also, the number of grafted cells in the mediorostral tectum is dramatically reduced (Fig. 9I).

DISCUSSION

The observations presented above show (i) the existence of a transient *Otx2*-negative portion in the caudal mesencephalic vesicle between stages HH10 and HH17/18, (ii) this transient *Otx2*-negative 'mesencephalic' neuroepithelium is not peculiar to the avian embryo but is also observed in the mouse embryo at E9.5 and (iii) the first mesencephalic postmitotic neurons overlie the mesencephalic *Otx2* neuroepithelium from stage HH14 to HH20, and indeed the caudalmost postmitotic neurons evolve in parallel with the *Otx2* caudal limit. Although it has not been possible to separate *Otx2*-positive from *Otx2*-negative territories in our chick/quail chimeras, our analysis performed after three different postoperative periods strongly indicates that (iv) the HH10 'mesencephalic' *Otx2*-negative neuroepithelium ends with a rh1 relocation at stage HH18/20 and differentiates isthmic nuclei and mediorostral cerebellum. Finally, (v) we have depicted local caudorostral morphogenetic movements in the mediodorsal part of the caudal *Otx2*-positive neuroepithelium.

The constriction observed at stage HH10 between the mesencephalic vesicle and the prorhombomere A1 is not the MHB constriction

The results of type 1 and 2 grafts clearly show that the cells derived from the constriction observed at stage HH10 between the mesencephalic vesicle and RhA1 are located within the rhombencephalon as early as stage HH14. Between stages HH14 and HH20, a new constriction is formed that derives from more rostrally located cells, most probably those located around the HH10 *Otx2* caudal border. In other words, the term 'MHB constriction' designates two distinct entities of the neural tube at stages HH10 and HH20. Puelles et al. (1995), by inserting a black nylon thread into the locus of the midbrain/hindbrain fold, have already proposed that this constriction is not yet fixed at stages HH10-15.

In types 1 and 2 grafts, all grafted cells remain grouped together without any mixing with surrounding host cells. Therefore the 'shift' of *Otx2*-negative cells, from the HH10 caudal mesencephalic vesicle towards the HH20 rh1, is not the result of a real movement of neuroepithelial cells but the consequence of the change of position of the constriction.

Therefore, the use of the term 'MHB constriction' should be restricted to designate only that constriction observed from stage HH18 onwards. This term should not be used to designate the constriction observed at stage HH10/12 since it is located within the presumptive cerebellar neuroepithelium (and disap-

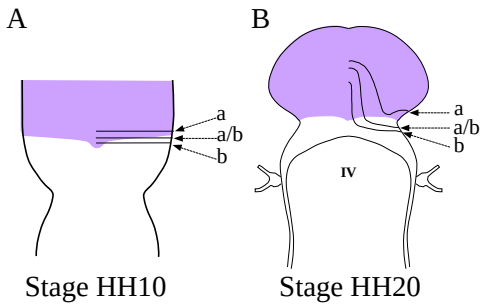

Fig. 8. Dorsal views of the MHB territory at stages HH10 and HH20, schematising the results obtained with types 3 and 4 grafts. The *Otx2* expression is in violet. Three different graft borders (a, a/b and b) are schematised at transplantation (A) and at fixation (B). Note the dramatic change of orientation of these three lines (see text). IV, IVth ventricle.

pears during the following stages). The possibility that this constriction represents an intracerebellar neuromeric boundary can not be excluded by the present data (see below).

The caudal limit of *Otx2* expression as an early marker for the caudal border of the mesencephalon

We have seen that the first mesencephalic postmitotic neurons, identified by their TuJ-1 immunoreactivity, evolve in parallel with the caudal limit of *Otx2* expression, and that these two markers simultaneously reach the MHB constriction at stage HH18. Also, both the progressive disappearance of the *Otx2*-negative neuroepithelium from the mesencephalic vesicle and the translocation of cells from the caudal mesencephalic vesicle towards rh1 have similar kinetics. The difficulty of transplanting exclusively *Otx2*-positive or exclusively *Otx2*-negative territories has been compensated for by scanning the area containing the oblique *Otx2* caudal limit. This procedure allows us to analyse the fate of three distinct HH10 regions (schematised at HH10 in Fig. 8A): (i) a strictly *Otx2*-positive part of the mesencephalic vesicle ending caudally at the level of the 'a' graft border, (ii) the *Otx2*-negative part of the mesencephalic vesicle (with or without RhA1) ending rostrally at the graft border 'b' (which also contains a very small number of *Otx2*-positive cells located in the caudalwards 'beak' close to the dorsal midline) and (iii) a tiny region lying between the graft borders 'a' and 'b' which contains almost the entire oblique *Otx2* caudal limit, and which has a composite *Otx2* phenotype: *Otx2*-positive, rostromedially and *Otx2*-negative, caudolaterally.

The first region, containing exclusively *Otx2*-positive cells, stays in the *Otx2*-positive HH20 mesencephalon (Fig. 8B), and develops only mesencephalic grisea. The second region, containing almost exclusively *Otx2*-negative cells, is almost totally relocated within the HH20 *Otx2*-negative rhombencephalon (Fig. 8B), and develops almost exclusively isthmic and cerebellar structures. The few *Otx2*-positive cells contained in this region (forming the caudalwards 'beak') are probably those remaining in the HH20 mesencephalon, forming a thin band of cells along the dorsal midline (Fig. 8B), and giving rise to a few tectal and comissural cells. The third region, containing both *Otx2*-positive and -negative cells, spreads on both sides of the caudal *Otx2* limit at stage HH20 and develops isthmo/cerebellar structures but also mesencephalic grisea.

Altogether, these results strongly suggest that, as early as stage HH10, the caudal *Otx2* limit represent the boundary between mesencephalic and isthmo-cerebellar presumptive territories.

With this idea in mind, it is interesting to recall the suggestion that the *Wnt-1* gene, which encodes a signalling molecule, is implicated in the formation of the 'mesencephalic/metencephalic' boundary (Bally-Cuif, 1994, 1995; Bally-Cuif and Wassef, 1995). These authors have analysed the embryonic development of the 'swaying' mutant mouse in which the Wnt-1 protein is not functional, and reported that there is a perturbation of the *Otx2* caudal limit, with patches of *Otx2*-positive cells intermixed within *Otx2*-negative territories and vice versa. In addition, Bally-Cuif (1994) has shown some mixing of cerebellar and tectal (mesencephalic) neurons in the adult. However, at stage HH10, the expression of the *Wnt-1* gene does not yet coincide with the caudal *Otx2* limit. *Wnt-1* expression spreads dorsally over the entire mesencephalic vesicle up to the constriction, and it is not until stage HH14-15 that it forms the characteristic ring overlying the *Otx2* caudal boundary (Bally-Cuif and Wassef, 1994). Therefore, it is possible that the boundary between cerebellar and mesencephalic territories could be established without the direct involvement of the *Wnt-1* gene but that this gene is necessary for its stabilisation.

The experiments of Avantaggiato and collaborators (1996), showing co-ordinate anteriorisation in the expression pattern of *Otx2*, *Pax-2*, *Wnt-1*, *En-1* and *En-2* genes in RA-induced anteroposterior repatterning of rostral CNS, supports the possibility that *Otx2* is directly involved in defining MHB regional identity, possibly interfering in the transcriptional regulation of genes functionally defined as essential to the establishment of this region (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Wurst et al., 1994). Recently, the *Drosophila otd* gene product has been demonstrated to be involved in the regulation of the *en* gene (Royet and Finkelstein, 1995). Both *en* and *wg* gene expressions are lost in *otd* mutant, leading to the hypothesis that they are direct targets of the head gap genes orthodenticle (*otd*), empty spiracles (*ems*) and button-head (*btd*) (Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990). Furthermore, it has been shown (Hirth et al., 1995) that the mutation of the *otd* gene results in the elimination of the first anterior brain neuromere, suggesting that this homeobox gene is required for the development of a specific brain segment in *Drosophila*. Preliminary results from mice carrying different functional copies of *Otx1* and *Otx2* indicate that these two genes are required to specify regional identity in a dosage-dependent mechanism (Tuorto et al., unpublished data).

Altogether these previous findings and those reported here support the possibility that *Otx2* plays an important role in the MHB region, such a role could be part of a complex mechanism involving evolutionarily conserved gene combinations, defining morphogenetic fields that distinguish the midbrain from the rostralmost hindbrain.

Single or dual neuromeric origin of cerebellum

The fate map experiments of Martinez and Alvarado-Mallart (1989) and Hallonet et al. (1990), demonstrating that at HH10 the cerebellar primordium spreads to both the mesencephalic and the first rhombencephalic vesicles, have opened up the possibility that the cerebellum has a dual neuromeric origin

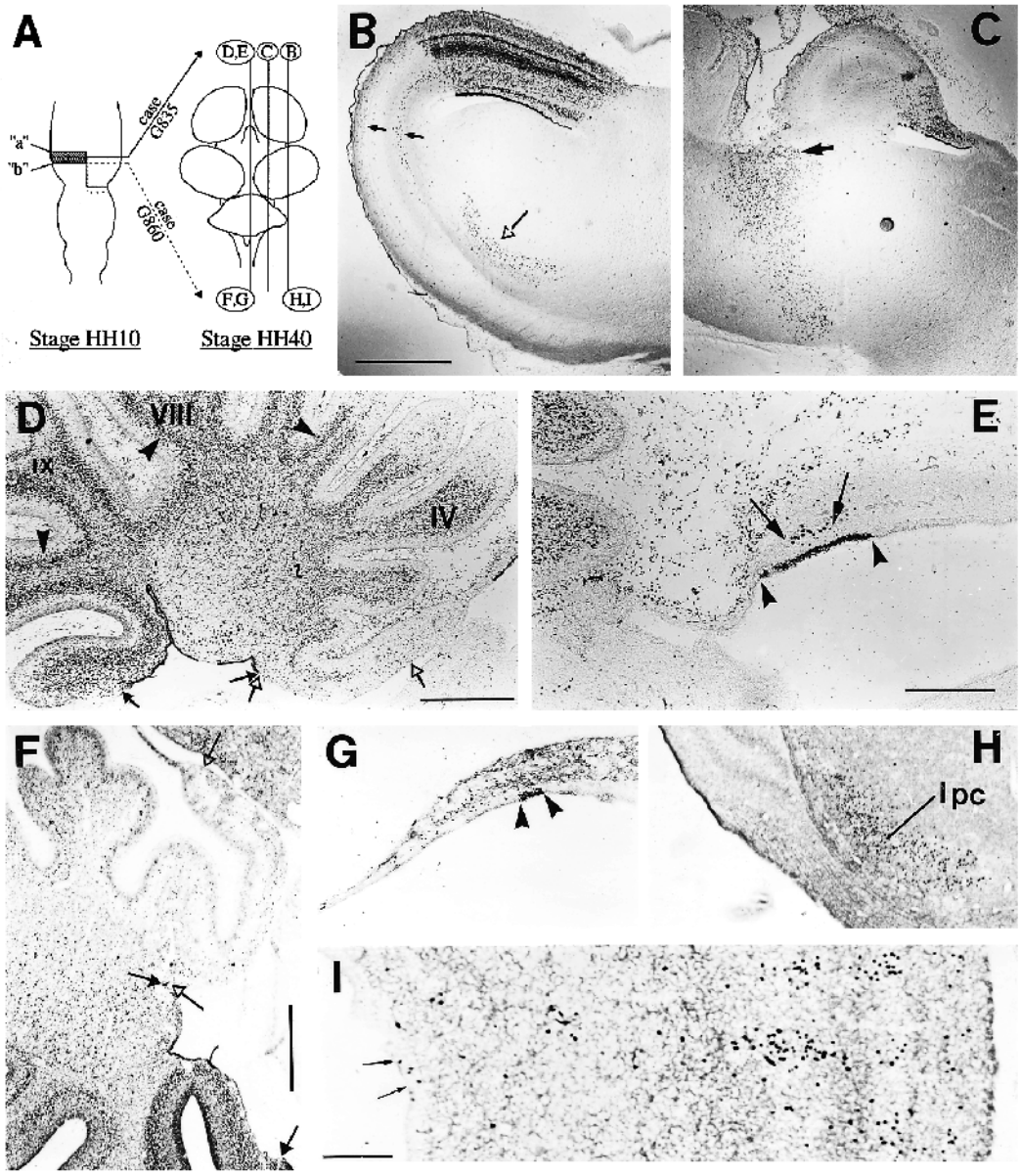

Fig. 9. Sagittal sections of HH40 chimeric brains. The grafted cells are revealed by the QCPN anti-quail antibody. Sections in B-E, G-H are oriented with the rostral end on the right and the caudal on the left. F is oriented with the rostral end up and the caudal down. I is a detail of the optic tectum, dorsal surface on the right. (A) A scheme of the areas used as the graft at stage HH10 and the levels of the illustrated HH40 sections. (B-E) Sections from case G835 bearing a type 4 a/b graft. (B) Lateral section passing through the chimeric mesencephalon illustrating that the graft concerns a large rostrodorsal segment of the tectum. The small arrows point to the tangentially migrating cells in the upper layer of the Stratum Griseum et Fibrosum Superficiale and in the Stratum Griseum Centrale (see Senut and Alvarado-Mallart, 1987). Note the presence of grafted cells in a large ventral portion of the nucleus isthmi, pars parvocellularis (open arrow). (C) A more medial section passing through mesencephalon, isthmus and lateral cerebellum. The grafted cells are located in the rostrodorsal tectum and tectal comissure. They spread also in a dorsoventral band of cells in the isthmus. The arrow points to the isthmo-optic nucleus. (D) Medial cerebellar section. The extent of the graft in the cerebellar ependyme is delimited by plain arrows. Note that the rostralmost portion of this ependyme is formed by chick cells (negative for the antibody, between open arrows). Note also numerous quail granule cells throughout cerebellum. Grafted Purkinje cells (arrowheads) are present in caudal lobules but absent in lobules I-III. IV, VIII and IX = lobules IV, VIII and IX. (E) Medial section illustrating the grafted cells in the ependyme of the tectal comissure (delimited by arrowheads) and in the mesencephalic nucleus of the Vth nerve (between arrows). (F-I) Sections from case G860 bearing a type 4b graft. (F) Detail of a medial section of the chimeric cerebellum. Note that, again, the quail cells are found in the caudal portion of the ependyme (between plain arrows) while the rostralmost portion is formed by chick host cells (between open arrows). Consequently, few postmitotic cells are observed in the rostral lobules I and II. (G) Detail of the tectal comissure. Note the small portion of the ependyme formed by quail cells (arrowheads). (H) Detail of a lateral section passing through the nucleus isthmi, pars parvocellularis (Ipc) containing numerous quail cells in its ventral part. (I) Detail of a more dorsal portion of the same section as illustrated in H. Note the small number of quail cells present in the rostral optic tectum. These grafted tectal cells form three thin bands extending from the ventricle to the surface. Note the few quail cells within the tectal ependyme (between arrows). Scale bars, B,C, 1 mm; D and F,H, 500 μm; E,G, 250 μm; I, 100 μm.

(see Introduction). Marin and Puelles (1995), using rhombencephalic transplants between chick and quail embryos, have reported that, in addition to rh1, rh2 also contributes to the cerebellar primordium, giving rise to the most lateral (auricular) portion of the adult avian cerebellum. A multi-neuromeric origin of the cerebellum cannot be excluded by our results. However, as we have just discussed, the constriction observed at stage HH10 does not represent the MHB boundary, and it is not yet clear that it represents an interneuromeric boundary. Further analysis of regulatory genes expressed at the level of the MHB domain would perhaps reveal unknown cerebellar interneuromeric boundary(ies).

It is generally accepted that, within the MHB domain, the isthmic territory is interposed between the cerebellar and mesencephalic domains. However, experiments to precisely determine the localisation of the isthmic primordium in the

early neural tube are lacking. In particular, it is not known whether this territory is located within the *Otx2*-positive or -negative neuroepithelium. It is interesting to note that, according to Vaage (1973), Puelles and Martinez de la Torre (1987) and Marin and Puelles (1994), one of the classically defined isthmic nucleus, the nucleus isthmi, pars magnocellularis, has its origin within the mesencephalic neuroepithelium. Based on our analysis performed at stage HH40, the nucleus isthmi, pars parvocellularis and pars magnocellularis, seems to arise from a territory located at stage HH10 between graft borders 'a' and 'b'. However, since this region has a heterogeneous *Otx2* phenotype, it is difficult to determine whether the nuclei isthmi, pars magnocellularis arises from the *Otx2*-positive or -negative territory. More work is needed to determine definitively the precise localisation of the isthmic primordia.

Despite the difficulties pertaining to this localisation, our results have led us to consider the caudal *Otx2* limit as the mesencephalic boundary. At stage HH18, this boundary also coincides with the MHB constriction and with the ring of *Wnt-1*-expressing cells. As suggested by the experiments of Bally-Cuif et al. (1995), this *Wnt-1*-positive ring may represent a clonal restriction boundary. Therefore at this stage, the caudal *Otx2* limit appears as a real interneuromeric boundary. Conversely, since at stage HH10 the *Otx2* caudal limit coincides neither with a limit of *Wnt-1* expression nor with a constriction, it is not possible to ascertain whether, at this stage, the *Otx2* caudal limit has already acquired all the characteristics of an interneuromeric boundary.

Caudorostral morphogenetic movements in the caudal mesencephalon

We have reported that the graft borders common to types 3 and 4 grafts (graft borders 'a', 'a/b' and 'b' in Fig. 8) undergo a change in the orientation of their medial part, from initially transversal to an oblique or even rostrocaudal orientation. All the other graft borders: the rostral one in type 3, the caudal one in type 4 and all graft borders in types 1 and 2 transplantations, keep their transversal orientation. Furthermore, it has to be noticed that the portion of the graft borders that is reoriented at stage HH20 concerns only the portion located in the *Otx2*-positive territory (see Fig. 8B). These observations indicate that only the *Otx2*-positive cells located in the dorsomedial mesencephalic vesicle, close to the *Otx2* caudal limit, undergo caudorostral morphogenetic movements between stages HH10 and HH20.

The significance of these morphogenetic movements is not yet understood. However, our observations can be related to those of Shimamura and Takeichi (1992) who reported the existence of a local and transient patch of expression of E-cadherin in the embryonic mouse caudal mesencephalon on both sides of the dorsal midline, that is in the area where we have observed morphogenetic movements. Shimamura et al. (1994) have suggested that the *Wnt-1* signalling molecule is involved, directly or indirectly, in the regulation of this patch of E-cadherin expression. Furthermore, Shimamura and Takeichi (1992) have described an alteration of *en* gene expression pattern together with a slight reduction of the lateral expansion of the mesencephalon on flat-mounted cultured explant, when blocking E-cadherin activity with a specific antibody. It is tempting to suggest that the E-cadherin plays a role in the caudorostral morphogenetic movements described here.

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