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Fgf8 and *Gbx2* induction concomitant with *Otx2* repression is correlated with midbrain-hindbrain fate of caudal prosencephalon

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SUMMARY

Chick/quail transplantation experiments were performed to analyse possible factors involved in the regionalisation of the midbrain-hindbrain domain. The caudal prosomeres, expressing *Otx2*, were transplanted at stage HH10 into rostrocaudal levels of the midbrain-hindbrain domain, either straddling the intra-metencephalic constriction (type 1 grafts), or at rostral and medial levels of pro-rhombomere A1 (type 2 and 3 grafts, respectively); thus, in all situations, one border of the graft was in contact with the host *Gbx2*- and *Fgf8*-expressing domains. The area containing the graft, recognised by QCPN immunohistochemistry, was first analysed 48 hours after transplantation for *Otx2*, *Gbx2*, *En2* and *Fgf8*. Although in all three situations, a large part of the graft maintained *Otx2* expression, another part became *Otx2* negative and was induced to express *Gbx2* and *Fgf8*. These inductive events occurred exclusively at the interface between the *Otx2*-positive transplanted domain and the ipsilateral host *Gbx2*-positive rhombomere 1, creating a new *Otx2-Gbx2* boundary within the grafted territory. In type 1 and 2 grafts, the induced *Fgf8* domain is in continuity with the host *Fgf8* isthmic domain, whereas for type 3 grafts, these two domains are separate. High levels of *En2* expression were also induced in the area expressing *Gbx2* and *Fgf8*, and *Wnt1* and *Pax2* expressions, analysed in type 3 grafts, were induced at the intragraft *Otx2-Gbx2* new boundary. Moreover, at later embryonic stages, the graft developed meso-isthmo-cerebellar structures. Thus, gene expressions induced in the grafted prosencephalon not only mimicked the pattern observed in the normal midbrain-hindbrain domain, but is followed by midbrain-hindbrain cytodifferentiation, indicating that not only *Fgf8* but also confrontation of *Otx2* and *Gbx2* may play an essential role during midbrian-hindbrain regionalisation.

Key words: Isthmic organiser centre, *Wnt1*, *En2*, *Pax2*, Neural tube, Segmentation, Cerebellum, Mesencephalon, Chick/Quail chimera

INTRODUCTION

In the last decade, a number of studies using chick/quail transplantation experiments have analysed planar inductive processes in the avian neural tube. Nakamura et al. (1986, 1988) were the first to demonstrate that, after the closure of the neural tube, the fate of prosencephalon can still be modified by environmental factors. Later on, our group has shown that the plasticity of prosencephalon, mesencephalon and cerebellum can be related to induction or regulation of the expression of the transcription factor *En2* (Martínez and Alvarado-Mallart, 1990; Martínez et al., 1991). In the 10-somite avian embryo (stage HH10, see Hamburger and Hamilton, 1951), the cerebellar primordium, which expresses high levels of *En2*, can induce this gene in both p1 and p2 (Martínez et al., 1991) as well as in rhombomeres 2-5 (rh2-5; Martínez et al., 1995), a neuroepithelium that never expresses *En2* under normal conditions. In both cases, the induced primordium changes its original fate and develops, respectively, a mesencephalic and a cerebellar phenotype. The *En2*-expressing neuroepithelium appears as an organising centre (the 'isthmic organiser') implicated in the specification of the caudorostral sequence of mesencephalic structures (Marín and Puelles, 1994). The induced prosomeres can develop not only mesencephalon, but also cerebellum when integrated caudal to the midbrain-hindbrain (MH) boundary (Bloch-Gallego et al., 1996).

A number of transcription factors are expressed in the MH neuroepithelium. Some of them, such as *Otx1* and *Otx2*, are expressed rostral to the MH boundary (Simeone et al., 1992, 1993; Millet et al., 1996); some others, such as *Gbx2*, caudal to this boundary (Bulfone et al., 1993; von Bubnoff et al., 1995; Wassarman et al., 1997; Hidalgo-Sánchez et al., 1999), and others, such as *En1* and *En2* (Davis et al., 1988; Gardner et al., 1988) and three members of the *Pax* family, *Pax2*, *Pax5* and *Pax8* (Asano and Gruss, 1992), are expressed at both sides of the boundary, forming a decreasing gradient in rostral and caudal directions. In addition, two signalling

molecules: the oncogene *Wnt1* (McMahon et al., 1992; Bally-Cuif and Wassef, 1994) and the fibroblast growth factor 8 (*Fgf8*) (Crossley and Martin, 1995), are also expressed in the MH domain. The latter factor applied to prosencephalon can mimic the effect of the *En2*-positive grafts inducing the caudal prosomeres to express *En2* and to develop a mesencephalic phenotype (Crossley et al., 1996). Recent data from Martinez et al. (1999) have extended these observations demonstrating that FGF8 can also induce *Fgf8*, *Wnt1* and *En1* expressions and can repress *Otx2*, recreating an ectopic midbrain-hindbrain domain in both caudal prosencephalon and rostral midbrain. Surprisingly, this factor applied to rhombencephalon induces neither the expression of *En2* nor a cerebellar phenotype, as does the isthmic organiser.

Thus, in spite of the great number of studies concerning the isthmic organising centre (see the reviews of Hallonet and Alvarado-Mallart, 1997; Puelles et al., 1997), the molecular mechanisms involved in the observed *En2* inductive process, most probably reflecting those responsible for the specification of the various neural structures of the MH domain, are not totally understood. In particular, it is not yet known which signals are responsible for the choice made by an *En2*-induced primordium to acquire a mesencephalic or a cerebellar phenotype.

In the present paper, we report experiments using the chick/quail model, aimed at analysing presumptive phenotypic changes in the caudal two prosomeres after positioning them at three distinct levels of the MH domain, in contact to the host *Gbx2* and *Fgf8* domains. Our observations show that a midbrain-hindbrain molecular cascade, including *Fgf8*-, *Gbx2*-, *En2*-, *Pax2*- and *Wnt1*-induced expressions and *Otx2* repression, is selectively triggered all along the areas confronting the *Otx2*-positive grafted neuroepithelium and the host *Gbx2*-positive rhombencephalic domain. Moreover, the consecutive meso-isthmo-cerebellar cytodifferentiation of the grafted primordium strongly supports the view that, not only *Fgf8* expression, but also *Otx2* and *Gbx2* confrontation, are essential events during the regionalisation of the midbrain-hindbrain domain.

MATERIALS AND METHODS

Fertilised JA57 chick eggs (Morizeau, Eure et Loir, France) and Japanese quail eggs (La Caille de Chanteloup, France) were incubated in a humidified atmosphere at 38±1°C. They were used to obtain chick/quail chimeras by exchanging small portions of the neural tube at stage HH10.

Grafting experiments

Three types of transplantation have been carried out (Fig. 1). The surgical procedure has been described in detail elsewhere (Alvarado-Mallart and Sotelo, 1984). In all cases, the chick embryo was the host and the quail embryo the donor. The transplant was always confined to alar portions of p1-p2 (see Trujillo and Alvarado-Mallart, 1991) and was positioned in substitution of precise portions of the alar MH domain. After transplantation, the host chick eggs were closed with parafilm, sealed with paraffin and returned back to the incubator until fixation.

Processing of the tissue

For short-survival analysis, the embryos were fixed 48 hours after transplantation (stages HH19-21). Some embryos prepared for type 3 grafts were fixed at surgery or 3-4 hours after transplantation (stages HH10-12). The fixation was performed by overnight immersion in 4% paraformaldehyde solution, in 0.12 M phosphate buffer, pH 7.4, at 4°C. For long survival (stages HH36-42), we use the same fixative, intracardiac perfusion of the embryos and overnight immersion of the dissected brains. Fixed embryos and brains were rinsed twice in phosphate-buffered saline/Tween 0.1%, cryoprotected in 10% sucrose solution (in phosphate buffer), embedded in 7.5% gelatine (Sigma type A) 10% sucrose solution and sectioned in a cryostat. For the short-survival analysis, we mounted 12 μm serial sagittal sections in three or five series of parallel slides. For long-survival analysis, 20 μm serial sections were cut on the frontal or the sagittal plane and mounted in three series of parallel slides.

Chick Otx2, Gbx2, Pax2, Fgf8 and Wnt1 cDNA

The chick *Pax2* and *Gbx2* probes were PCR-amplified products from total chick embryo cDNA. The chick *Pax2* spans the region between aminoacids 83 and 365 (Adams et al., 1992), and the chick *Gbx2* fragment spans the region between aminoacids 5 and 333 (Kowenz-Leutz et al., 1997). The chick *Otx2* probe was the same as used previously (Millet et al., 1996), and the chick *Fgf8* and *Wnt1* probes were kind gifts of Drs Gail Martin and Marion Wassef, respectively.

Chick probes

The chick *Otx2* subclone was linearised with *Bam*HI or *Hin*dIII (Pharmacia Biotech) and transcribed using T7 RNA polymerase (Pharmacia Biotech) or T3 RNA polymerase (Boehringer, Mannheinm) to produce the antisense and sense probes, respectively. For the chick *Gbx2* subclone, we used *Eco*RI and Sp6 RNA polymerase (Pharmacia Biotech) and *Hin*dIII and T7 RNA polymerase, respectively. The chick *Ffg8* subclone was linearised with *Eco*RI or *Xho*I (Pharmacia Biotech) and transcribed using T7 or T3 RNA polymerase, respectively. To obtain the antisense chick *Pax2* probe, we used *Xba*I (Pharmacia Biotech) and T7 RNA polymerase. For the chick *Wnt1* subclone, we used *Cla*I and T7 RNA polymerase and *Xba*I and T3 RNA polymerase to generate antisense and sense probes, respectively.

In situ hybridisation

In situ hybridisation was carried out as described by Schaeren-Wiemers and Gerfin-Moser (1993). For single labelling, we always used the digoxigenin-labelled probes, alkaline phosphatase-conjugated anti-digoxigenin antiserum (1:3500, Boehringer, Mannheinm) and NBT-BCIP (Boehringer, Mannheim) as substrate. For double labelling, fluorescein- and digoxigenin-labelled probes were hybridised together and detected one after another. The fluorescein probe was revealed first using an alkaline phosphatase-conjugated anti-fluorescein antiserum (Boehringer, Mannheim), diluted 1:2000, and Fast Red as the chromogene. The specificity of the probes was tested in normal embryos; no signal was obtained with the sense probes. All chick probes crossreact with quail tissue, but with lower intensity than for chick tissue.

Immunohistochemistry

The monoclonal antibody (mAb) 4D9 recognising specifically the *En2* protein (Patel et al., 1989), diluted 1/2, was used to visualise *En2* expression. To visualise the grafted cells, the mAb QCPN (Developmental Studies Hybridoma bank), diluted 1/100, was used. In some cases, one set of brain sections was also stained with the chicken anti-quail antibody of Lance-Jones and Lagenaur (1988),

diluted 1/500. The mAb QCPN and 4D9 were revealed by the peroxidase/antiperoxidase method of Sternberger et al. (1970) using a sheep anti-mouse secondary antibody, diluted 1/100 and DAB intensification with 0.6% nickel ammonium sulphate. For the chicken anti-quail antiserum, a biotinylated anti-chicken secondary antibody, diluted 1/10, and the ABC complex (Vector) were used.

RESULTS

The vesicles and constrictions observed in the MH domain during transplantation (stage HH10) and in short-survival chimeras do not correspond to the same entities (Millet et al., 1996; Hidalgo-Sánchez et al., 1999). To clarify the description, we use the recent nomenclature of Hidalgo-Sánchez et al. (1999) to define precisely MH domains receiving quail neuroepithelium. Thus, at HH10 (see Fig. 1), the so-called 'mesencephalic vesicle' is here named 'mes-metencephalic' vesicle and the constriction separating this vesicle from the pro-rhombomere A1 (RhA1) of Vaage (1969) is called 'intra-metencephalic', since it separate two portions of the cerebellar (metencephalic) neuroepithelium (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990).

Short-survival chimeric embryos

Type 1 grafts

Type 1 grafts correspond to those straddling the intra-metencephalic constriction (Fig. 1B), and were planned to substitute a large part of the cerebellar plate and also caudomedial portions of the alar mesencephalic vesicle (see Millet et al., 1996). In the six analysed embryos (Table 1), the bulk of the graft, recognised by its positive QCPN immunoreaction extended into caudal portions of the mesencephalic vesicle (Fig. 2A,C), only a very small portion was located in rh1 (* in Fig. 2C). In midsagittal sections, the host *Gbx2* rh1 territory was lacking; thus the caudal border of the graft contacted the host choroid tissue (Figs 2A, 7).

The transplanted primordium was induced to express *En2* (*n*=4, see Fig. 2E). However, in one case (not illustrated), a small portion of the graft was 4D9 negative. Interestingly, in this particular case, the grafted primordium was taken somewhat more rostral than in the other four cases. The sections hybridised with the *Otx2* probe (*n*=5) showed that most of the graft maintained its original *Otx2* expression (Fig. 2B-D), except in the area adapted to rh1, in which *Otx2* was repressed (* in Fig. 2D). The sections hybridised for *Gbx2* (*n*=3) showed that the *Otx2*-negative portion of the graft expressed *Gbx2* (* in Fig. 2F). These three cases and a fourth one were also hybridised with the *Fgf8* probe. In all of them, the *Otx2*-negative (*Gbx2*-positive) area of the graft was induced to express *Fgf8* (* in Fig. 2E), and was contiguous to the host *Fgf8*-positive neuroepithelium (see Fig. 7A summarising these results). Moreover, the induced *En2* expression was high in the *Gbx2/Fgf8*-induced area (Fig. 2E) and decreases as it extended within the *Otx2*-positive domain (not shown). We conclude that: (i) the caudal prosencephalic neuroepithelium, transplanted to the MH domain, can be induced to express not only *En2*, as previously reported (Bloch-Gallego et al., 1996), but also *Gbx2* and *Fgf8*; (ii) within the graft, the expression of these last two genes occurs concomitantly with *Otx2* repression, and (iii) within the grafted territory, *Otx2*, *Gbx2*, *Fgf8* and *En2* expression patterns reproduce those observed in the normal MH domain, the area in which the transplanted primordium becomes integrated.

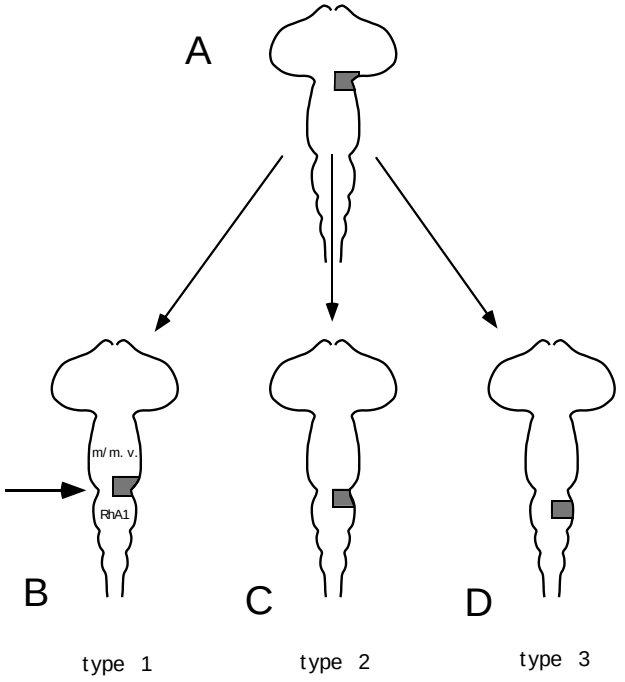

Fig. 1. Schematic representation of the three types of grafts performed in this study. (A) Schema of the donor quail embryo. The transplant, in grey, corresponds to alar portions of the two caudal prosomeres (p1-p2; see Puelles et al., 1997). This neuroepithelium was transplanted (long arrows) to three different levels of the MH domain, schematised in grey (B) for type 1 grafts, (C) for type 2 grafts, and (D) for type 3 grafts. The arrow in B points to the intrametencephalic vesicle, separating the mes-metencephalic vesicle (mes/met v.) and prorhombomere A1 (RhA1; see text).

Type 2 grafts

To determine if *Gbx2* expression can be induced in a larger portion of the *Otx2*-positive graft, the p1/p2 neuroepithelium was transplanted within pro-rhombomere A1 (RhA1, of Vaage, 1969), just caudal to the intra-metencephalic constriction (Fig. 1C).

The resulting chimeric embryos (*n*=7, see Table 1) were integrated within rh1. In midsagittal sections (Fig. 3A), its rostral border contacted the host mesencephalon and its caudal border the host choroid tissue (Fig. 3A,D), two *Otx2*-positive domains. In more lateral sections, however, the grafts were in contact to the host *Gbx2*-positive domain (Fig. 3E,G). These grafts showed a bulged shape (Fig. 3A,E), with the exception of the area contacting the host ipsilateral rh1, which adopted a flat shape, similar to the surrounding host tissue (Fig. 3E,H).

After mAb 4D9 (*n*=4) staining, the grafts exhibited a characteristic decreasing gradient of *En2* (Fig. 3D,H); high expression was always observed in the area adapted to the host

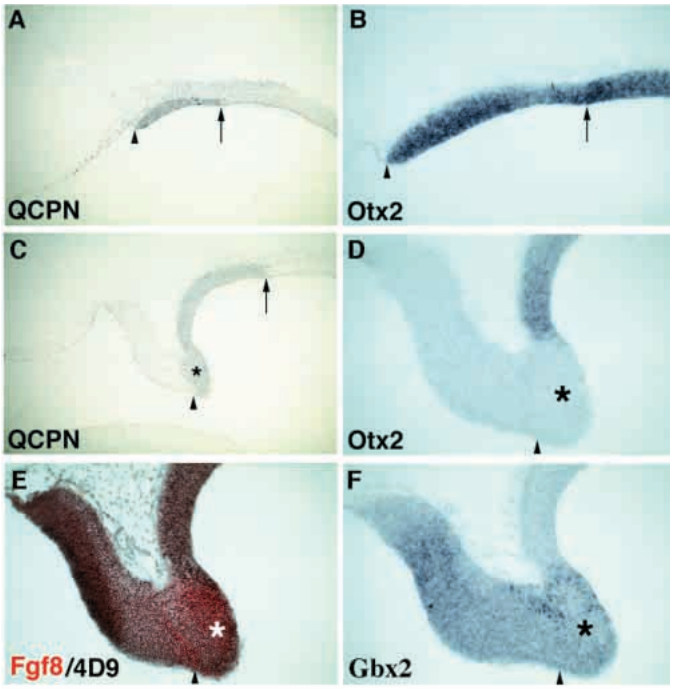

Fig. 2. Short-survival type 1 grafts. Serial sagittal sections of case MH 174, rostral to the right. (A,B) Medial sections; (C-F) lateral sections. The antibodies and probes used are noted in each figure; the *Fgf8* probe was revealed by the *fast red* chromogene (red letters). The extent of the graft, evident in A and C, is delimited in all figures by the arrow (rostral border) and the arrowhead (caudal border). Note in medial sections that the graft maintains *Otx2* expression (B). In lateral sections, a portion of the graft (* in C,D,E,F) is adapted to the host *Gbx2*-positive neuroepithelium. This area has lost *Otx2* expression (D) and has been induced to express *Gbx2* (F) *Fgf8* (H) and high levels of *En2* and thus is 4D9 immunopositive (E). A, ×25; C, ×13; B,D,F, ×50.

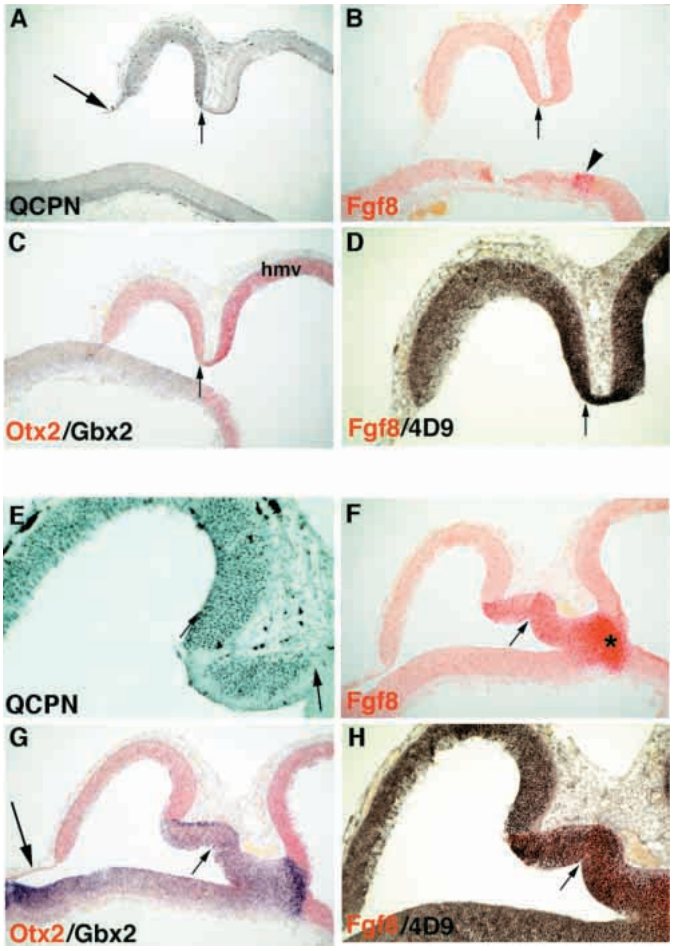

Fig. 3. Short-survival type 2 grafts. Serial sagital sections of case MH167 (rostral to the right). The antibodies and probes used are indicated in each figure; red letters indicate the probe revealed by the *fast red* chromogene. (A,E) The extent of the graft is obvious in the QCPN-stained sections (the small arrow points to the graft rostral border). (E) The section passes tangentially to the graft border thus, some host, QCPN-negative, cells are intermingled with grafted, QCPN-positive, cells; but, this area contains exclusively grafted cells in sections labelled by HIS (F,G), data confirmed by cresyl violet counterstaining. (A-D) At medial level, the graft contacts the *Otx2* host mesencephalic vesicle (hmv, in C) and maintains *Otx2* expression (C). Note the absence of *Fgf8* (B) and *Gbx2* (C) expressions in this portion of the graft. The arrowhead in B points to the *Fgf8*-positive host basal plate. (E-H) Laterally, the rostral border of the graft contacts the *Gbx2*-positive host neuroepithelium and the contiguous portion has adopted a flat shape, has been induced to express *Gbx2* and has lost *Otx2* (G); this flat territory is also *Fgf8* positive (F). Note, in this picture, that *Fgf8*-induced domain concerns also the contiguous host *Gbx2* neuroepithelium, as an extension of the *Fgf8*-positive isthmic ring (*). Note in H that the induced *En2* expression is high in both the *Gbx2/Fgf8*-expressing domain and the surrounding *Otx2*-positive grafted domain. Neither *Fgf8*- nor *Gbx2*-induced expressions are observed at the caudal border of the graft, contiguous to the host choroid tissue (large arrow in A, G). A, ×24; B,C,F,G, ×32; D,H, ×48; E, ×70.

cerebellar plate (Fig. 3H) and decreased as it invaded the bulged shaped area (Fig. 3D,H). However, in two cases, a small portion of the graft remained *En2*-negative (* in Fig. 4B,D). This *En2*-negative area maintained its original *Otx2* expression (* in Fig. 4F) and was *Gbx2*-negative (* in Fig. 4E) and *Fgf8*-negative (expression tested in case MH176, see Table 1). In these two cases, as in type 1 grafts with partial induction of *En2*, the transplants were obtained from quail neuroepithelium taken somewhat more rostral than in the other six cases.

Single-labelled sections with the *Otx2* probe (n=6) or double *Otx2/Gbx2* hybridisation (n=4) showed that type 2 grafts remained *Otx2* positive (Fig. 3C,G), with the exception of the area adapted to the host rh1. This latter region became *Gbx2*-positive (Fig. 3G), recreating inside the graft a new, and ectopic, *Otx2-Gbx2* interface. The induced *Gbx2* territory also expressed *Fgf8* (Fig. 3F,H). Moreover, *Fgf8*-induced expression, although in continuity to the host *Fgf8*-positive isthmic ring (Fig. 3F), selectively followed the interface confronting the grafted territory to the host *Gbx2*-expressing domain (Fig. 8B). Importantly, in the area contacting the host *Otx2*-positive mesencephalon, the graft maintained its original *Otx2* expression and was *Gbx2* and *Fgf8* negative, although this interface is always in close apposition to the host *Fgf8*-positive isthmic domain (Fig. 8B). Also of interest was the constant absence of *Gbx2* and *Fgf8* induction at the interface between the graft and host choroid tissue, another *Otx2*-positive domain (Fig. 3B,C,F,G). Moreover, neither *Gbx2* nor

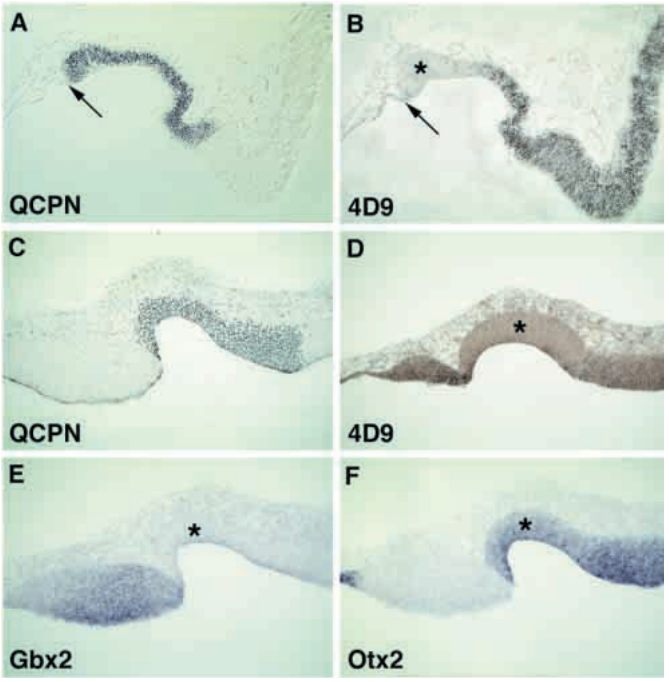

Fig. 4. Two cases of type 2 grafts with partial induction of *En2*. Serial sagittal sections, rostral to the right. (A,B) Case J32. (A) The extent of the graft is evident, the arrow points to its caudal border contiguous to the host choroid tissue. (B) The * labels the 4D9-negative portion of the graft, contiguous to the one expressing high levels of *En2*. (C-F) Case MH176. (C) The extent of the graft is evident. (D-F) The * labels the *En2*-negative portion of the graft. This area is contiguous to the *Gbx2*-positive neuroepithelium (E) and has maintained *Otx2* expression (F). A-F, ×50.

Fgf8 expressions were induced at the interface between the graft and the contralateral host rh1 (Fig. 8B), a region expressing *En2* and *Gbx2*, which is bordered in its rostral portion by the *Fgf8*-positive isthmic domain.

Type 3 grafts

Some chimeric embryos were transplanted in the middle of RhA1. In this situation (*n*=15, see Table 1), the transplanted tissue was found: (i) within the *Otx2*-positive choroid tissue (*n*=2), (ii) between the caudal *Gbx2*-positive cerebellar plate and the *Otx2*-positive choroid tissue (*n*=12), or (iii) totally surrounded by the *Gbx2*-positive cerebellar plate (*n*=1). The two grafts surrounded by host choroid tissue (J36, J141, not illustrated) were very small, maintained their original *Otx2* expression and were *En2* negative. Moreover, case MH141, also tested for *Fgf8* and *Gbx2*, showed the absence of induction of these two other genes.

When the grafts were located within the cerebellar plate or between the cerebellar plate and the choroid tissue (Figs 5A,D,G, 6A), the bulk of the transplant exhibited a bulged shape with only a small portion adapted to the host rh1. Gene expression in these grafts was similar to that described for lateral levels of type 2 grafts. Thus, when in contact with the ipsilateral host rh1, the graft was induced to express *En2* (*n*=6; Figs 5F, 6D), *Gbx2* (*n*=10; Fig. 5B,H) and *Fgf8* (*n*=11; Figs 5C,I, 6D,E) with a concomitant loss of *Otx2* (*n*=9; Figs 5B,E,H, 6B; see Fig. 8C summarising these data). Here again, no induction of *En2*, *Gbx2* or *Fgf8* was ever observed at the interface between the graft and the host choroid tissue (Figs 5B,C,H,I, 6D,E). It is important to emphasise that, although in 3 out the 11 cases, the *Fgf8*-positive ectopic domain appeared

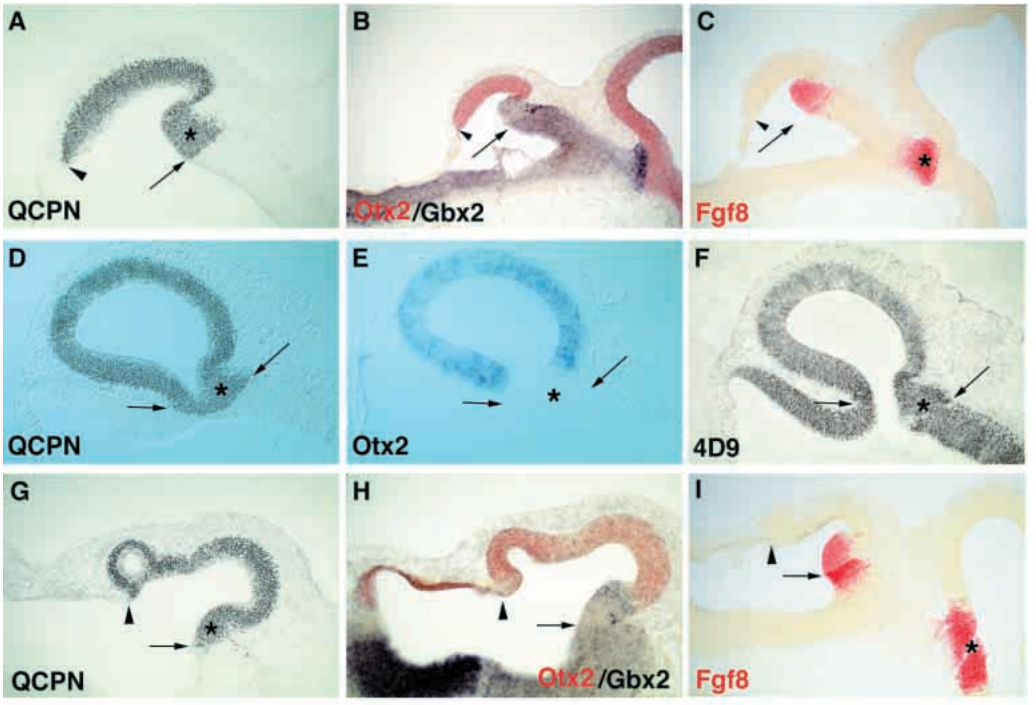

Fig. 5. Short-survival type 3 grafts. Serial sagittal sections (rostral to the right) of cases MH144 (A-C), J30 (D-F), MH163 (G-I). The antibodies or probes used for each section are noted; for double labelling, the red letters indicate the probe revealed by the *fast red* chromogene. Note in A-C and G-I that the rostral border of the graft is contiguous to caudal portions of the host cerebellar plate (arrows), and its caudal border contiguous to the host choroid tissue (arrowheads). Conversely, in J30 (D-F), the graft, delimited by the arrows, has been integrated into medial portions of the cerebellar plate. In the three cases, the area of the graft adapted to the cerebellar plate (* in A,D-G) has lost *Otx2* expression (B,E,H). This area expresses *Gbx2* (B,H) and *Fgf8* (C,I). Note that the induced *Fgf8* domain is clearly apart from the host *Fgf8* isthmic ring (* in C,I). Interestingly, in J30 (D-F), all borders of the graft, contact to the *Otx2*-negative (presumably *Gbx2*-positive) territory (E) and express high levels of *En2* (F). Note also, that neither *Fgf8* nor *Gbx2* expressions are induced in the interface graft/choroid tissue (arrowheads in B,C,H,I). A,D,H, ×50; B,C,I, ×25.

Table 1. Short-survival chimeric embryos and the various tests applied at each case

	Cases	QCPN	4D9	*Otx2*	*Gbx2*	*Fgf8*	*Pax2*	*Wnt1*
Type 1	J22	+	+	+				
	J27	+	+	+				
	MH137	+	+			+		
	MH174	+		+	+	+		
	MH177	+	+	+	+	+		
	MH185	+		+	+	+		
Type 2	J32	+	+	+				
	J89	+	+			+		
	MH142	+		+	+	+		
	MH147	+		+	+	+		
	MH167	+		+	+	+	+	+
	MH176	+	+	+	+	+		
	MH180	+	+	+		+		
Type 3	J36	+	+	+				
	J28	+	+	+				
	J30	+	+	+				
	J88	+		+	+	+		
	J162	+	+	+	+	+		
	MH130	+		+	+	+		
	MH132	+	+			+		
	MH141	+	+	+	+	+		
	MH144	+		+	+	+		
	MH145	+		+	+	+		
	MH149	+		+	+	+	+	+
	MH151	+		+	+	+		
	MH153	+	+	+	+	+	+	+
	MH163	+	+	+	+	+		
	MH168	+		+	+	+	+	+

as an expansion of the host isthmic *Fgf8*-expressing domain (as was observed for type 2 grafts), in the other 8 cases, the in situ and ectopic *Fgf8*-positive domains were clearly separated (Figs 5C,I, 6E).

Type 3 grafts were also analysed for *Pax2* and *Wnt1* expression (*n*=3). Both genes were observed within the host tissue mimicking the characteristic MH pattern. *Pax2* expression was induced at both sides of the intragraft *Otx2-Gbx2* boundary (Fig. 6C) and *Wnt1* expression was restricted to the grafted *Otx2*-positive territory, with an abrupt stop at the intragraft *Otx2-Gbx2* boundary (Fig. 5F).

These observations suggest that apposition of the *Otx2* grafted territory and the *Gbx2* host neuroepithelium triggers a molecular cascade including the induction of *Gbx2*, *Fgf8*, *Pax2*, *En2* and *Wnt1* expression as well as the repression of *Otx2*. However, it is also possible, in accordance with Crossley et al. (1996) and Martinez et al. (1999), that the FGF8 protein emanating from the host isthmic domain could be implicated in the observed inductive process. Indeed, although induced and host *Fgf8* expression are segregated at stage HH20, analysis performed at surgery (either of host embryos before the positioning of the graft, *n*=6, or of chimeric embryos three-four hours after grafting, *n*=8) showed that the hole performed in the host embryo always removed the caudalmost *Fgf8*-expressing cells (Fig. 7A), and that the graft contacted, at least during a short period, the host *Fgf8*-expressing domain (Fig. 7B). Nevertheless, it has to be emphasised, that for both type 2 and 3 grafts, *Fgf8*- and *Gbx2*-induced expressions selectively followed the *Gbx2-Otx2* host-graft interface.

Long-survival analysis

Twelve chimeric embryos, bearing type 1-3 grafts, were fixed between stages HH36 and 42 (E10-17; Table 2). As will be detailed below, the localisation and the meso-isthmo-cerebellar cytodifferentiation of the transplanted cells are in accordance with the genetic expression observed in short-survival embryos. Moreover, in each group, at least one case has an ectopic pineal gland formed by quail cells (Table 2, see Figs 9G,H, 10A); interestingly, these grafts were dissected from slightly more rostral portions of prosencephalon than in the other cases, as was reported for short-survival embryos with partial induction of *En2*.

Type 1 grafts

The two analysed embryos (Table 2) presented an unexpectedly normal brain, although the histological analysis detected that quail grafted cells contributed to the main alar structures of the in situ MH domain, the mesencephalon, isthmus and cerebellum, and that, in one case, they also formed an ectopic pineal gland. Within the mesencephalon, the grafted cells formed a dorsorostral band in the optic tectum (Fig. 9A), which is known to originate from caudal mesencephalic neuroepithelium (Goldberg, 1974), an area that at stage HH20 was formed by quail cells. The caudalmost mesencephalic trigeminal cells, also originating from caudal portions of the *Otx2*-positive mesencephalic neuroepithelium (Millet et al., 1996), were of quail phenotype (not illustrated). The nucleus isthmic principalis, in its pars magnocellularis and parvocellularis (Fig. 9A,B), contained numerous quail neurones. Within the cerebellum, numerous quail cells were found in lobules I-V (one case), and in lobules I-VI (the other case; Fig. 9A). Quail cells also contributed to the medial cerebellar nucleus (not shown). These grafted portions exhibited a qualitatively normal cytoarchitecture (Fig. 9C). No quail cells were observed in the external granular layer. These observations indicate that part of the grafted primordium has substituted for the rostromedial portions of the cerebellar neuroepithelium (Alvarez-Otero et al., 1993; Millet et al., 1996). This portion most probably corresponds to the area that at early stages was adapted to the host rh1, and which was induced to express *Gbx2*, *En2* and *Fgf8* and where *Otx2* was repressed (see Fig. 8A).

Type 2 and 3 grafts

These two groups will be described together since they exhibit a similar cytodifferentiation (Table 2). In all cases, the graft contributed to the in situ cerebellum and developed mesencephalic ectopic structures. In most cases, some of the in situ isthmic nuclei contained quail cells.

In case G960 (type 2 graft), the ectopic mesencephalon was represented by an ectopic tectal-like structure (Fig. 9E,F) containing quail mesencephalic trigeminal neurones (Fig. 9F). This group also differs from the others because numerous quail cells were found in the external granular layer of the cerebellar lobules VI and VII (Fig. 9D). In this case, the quail cells also contributed to the other types of cerebellar cortical neurones and to Bergmann glia (Fig. 9D), as well as to the isthmic neurones (not shown).

Table 2. Long-survival chimeric embryos: structures containing and/or formed by the transplanted cells

	Cases	In situ optic tectum	In situ mes. Vth N	In situ isthmus nuclei	In situ cerebellum	Ectopic optic tectum	Ectopic mes. Vth N	Ectopic pineal gland
Type 1	MH90 (E12)	+++	+	+	+++			++
	MH207 (E15)	+++	+	+	+++			
Type 2	G960 (E17)			++	+++		++	++
	G958 (E17)			++	+	+++		++
	J18 (E12)			++	+++	+++		
	J19 (E12)			+	++	+++		
	J175 (E15)			++	++	+++		
	J180 (E15)			+	++	++		
Type 3	J13 (E12)			+	+	+++		
	G1015 (E10)			++	+++	+++		++

In all other cases (n=5, for type 2 and n=2 for type 3 grafts, see Table 2), the ectopic mesencephalon consisted of a supernumerary optic tectum with characteristic lamination (Fig. 10A,B,D). However, variations in the number and location of the quail cells were observed within the chimeric cerebellum and isthmic region. Two chimeric brains showed a very small number of cells dispersed within the cerebellum (probably representing glial cells), while in the six others, the quail cells have clearly differentiated as cortical cerebellar neurones, including Purkinje cells (Fig. 10E). Only in three cases, a very small number of quail cells within the external granular layer was found (not shown). As illustrated in Fig. 9A-C, the two cases bearing type 3 grafts are particularly different. Case G1015 (Fig. 10A) may be compared to case J30 (Fig. 4D-F), since the graft is integrated in medial portions of cerebellum: the quail cells form an ectopic optic tectum and contribute to both isthmic region and cerebellum. In case J13 (Fig. 10B,C), the graft has been more caudally integrated, as in case MH163 (Fig. 4G-L) and thus, in addition to the supernumerary tectum and some dispersed cells in the cerebellar white matter, the quail cells contributed to form the most caudal cerebellar lobule, but not isthmic nuclei.

DISCUSSION

The present observations show that, with the exception of the area developing the ectopic pineal gland, the transplanted primordium (both the area that loses and the one that maintains *Otx2* expression) always changes its original prosencephalic fate and develops a meso-isthmo-cerebellar phenotype. These phenotypic changes are preceded by a molecular cascade including induction of *Fgf8* and *Gbx2* gene expression and *Otx2* repression throughout the grafted areas contacting the host *Gbx2*-positive domain, as well as induction of *En2*, *Pax2* and *Wnt1* gene expression within the graft. In the three tested situations, the isthmic- and cerebellar-induced structures appear integrated within the in situ host isthmus and cerebellum, as is also the case for the induced mesencephalon in type 1 grafts. Conversely, for type 2 and 3 grafts, the induced mesencephalon always develops in an ectopic position. Considering that the *Otx2-Gbx2* common boundary marks the frontier between mesencephalon and cerebellum (Millet et al., 1996; Hidalgo-Sánchez et al., 1999) and that, within the transplant, an induced *Otx2-Gbx2* boundary was always detected, we conclude that grafted mesencephalic cells originate from the area maintaining *Otx2* expression and induced to express a gradient of *En2* (mimiking an ectopic mesencephalic vesicle), while grafted cerebellar and isthmic cells originate from the area induced to express *Fgf8* and *Gbx2* and high levels of *En2*. Only the quail cells observed within the pars magnocellularis of nucleus isthmic principalis may arise from the *Otx2*-expressing mesencephalic domain (see Vaage, 1973; Puelles and Martínez de la Torre, 1987; Millet et al., 1996).

Mutual regulation of *Otx2*, *Gbx2*, *Pax2*, *En2*, *Fgf8* and *Wnt1* gene expressions

One important result is that, in all situations, the *Gbx2-Otx2* boundary, created at surgery between graft and host, has disappeared at stage HH20. At this stage, a newly formed *Fgf8-Gbx2-Otx2* interface, mimiking the MH boundary, is observed within the graft. *En2*-induced expression crosses this newly formed boundary, extending with a decreasing gradient throughout the grafted domain, while the induced, but more restricted, *Pax2* and *Wnt1* expressions, are also related to this newly formed boundary. In normal chick embryos, *Gbx2* and *Otx2* genes are expressed before gastrulation (see Bally-Cuif et al., 1995, for the chick *Otx2* gene; Niss and Leutz, 1998, and Shamin and Mason, 1998, for the chick *Gbx2* gene) that is, more precociously than *Fgf8*, *En2*, *Pax2* and *Wnt1* genes, which start to be expressed almost simultaneously during neurulation (see Ohuchi et al, 1994 and Crossley and Martin, 1995, for *Fgf8*; Asano and Gruss, 1992; Stoykova and Gruss, 1994, for *Pax2*; Gardner et al., 1988, for *En2*, and Bally-Cuif and Wassef, 1994, for *Wnt1*, our unpublished observation for all these genes in the chick embryo). Moreover, *Otx2*- and *Gbx2*-expressing domains are always confronted and seem exclusive of each other (Hidalgo-Sánchez et al., 1999). Several data of the literature provide evidence of a mutual regulation between all these analysed MH genes. Retinoic acid-mediated repression of *Otx2* is accompanied by an anteroposterior repatterning of the neural tube involving the anteriorisation of midbrain and hindbrain genes such as *Wnt1*, *En2* and *Hoxb1* (Simeone et al., 1995; Avantaggiato et al., 1996; Ang et al., 1994). In mice lacking *Gbx2*, there is a caudal shift of

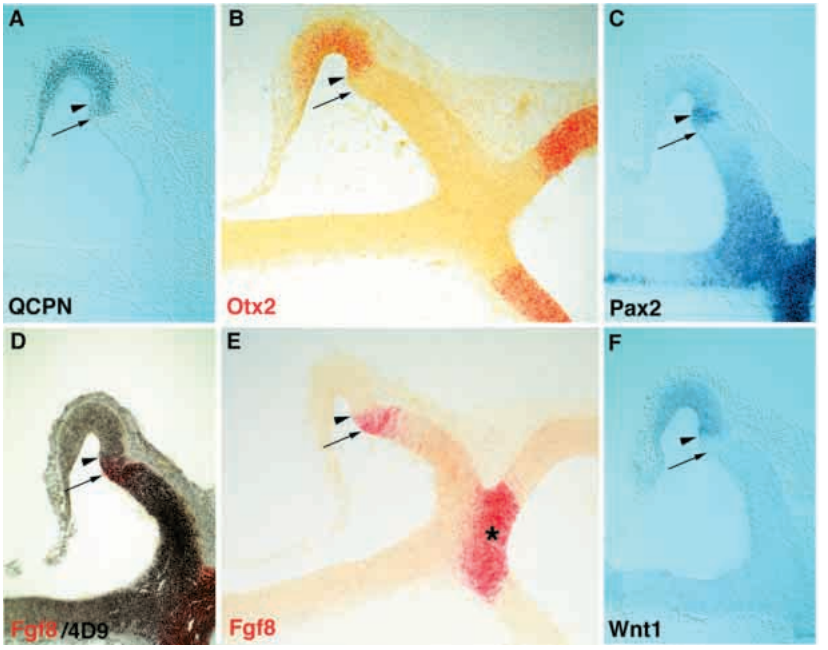

Fig. 6. Short-survival type 3 grafts. Serial sagittal sections of case MH168, rostral to the right. The graft is integrated to the caudal extremity of the cerebellar plate. The probes and antibodies used are noted in each figure; the probes revealed by the *fast red* chromogene are noted by red letters. The arrows point to the rostral border of the graft, in contact to the host cerebellar plate, in this area, *Otx2* has been repressed (B). The intragraft *Otx2+/Otx2–* boundary is indicated in all figures by an arrowhead, note that *Pax2* (C), *En2* (D), *Fgf8* (D, E) and *Wnt1* (F) are all present at this interface. Note also that the induced *Fgf8* expression is clearly separated from the isthmic *Fgf8*-positive ring (* in E). A-F, ×45.

the *Otx2* expression boundary together with a mis-expression of *Wnt1* and *Fgf8* (Wassarmann et al., 1997). In transgenic mice, in which the *Fgf8* gene was ectopically expressed, a regulation of *En2* expression by this signalling factor was also reported (Lee et al., 1997). Kelly and Moon (1995) have shown that *Pax2* overexpression in zebrafish embryos results in expansion of the *Wnt1*-expressing domain. Molecular evidence for *Pax* regulating the *En* gene family has been obtained by Joyner's group (Song et al., 1996), and was also confirmed by Funahshi et al. (1999). Lun and Brand (1998) also reported that, in the *Pax2.1* zebrafish mutant, *no isthmus* (*noi*), *Wnt1* and *Fgf8* gene expressions are initiated but, later on, eliminated. *Wnt-1* expression was induced in caudal diencephalon separating this *Otx2*-positive domain from an ectopic cerebellar graft (Bally-Cuif and Wassef, 1994). Recently, Shamin et al. (1999) have shown that ectopic induction of *Fgf8* expression can be obtained by retroviral surexpression of *En1*. The reverse is also true since Martinez et al. (1999) have demonstrated that ectopic application of FGF8 within caudal diencephalon and rostral midbrain, induces the expression of *Fgf8*, *En1* and *Wnt1* and represses *Otx2*. Moreover, mutant mice in which *Otx2* was replaced with *Otx1* showed at early midgastrula stage a visceral endoderm-restricted translation of *Otx1* RNA that was sufficient to rescue the specification of the anterior neural plate even though no OTX2 gene products were identified within the neuroectoderm. Subsequently, the anterior patterning was lost and the MH markers, such as *Gbx2*, *Fgf8*, *En2*, *Wnt1* and *Pax2*, were rostrally shifted and altogether co-expressed at the tip of the mutant embryos. These mutants will result in a sharp headless phenotype (Acampora et al., 1998). Therefore, OTX gene products are required, at early gastrula, to specify an early neural plate and, at late gastrula, for maintenance of anterior patterning and positioning of the MH boundary.

Fgf8, *Otx2* and *Gbx2* together contribute to the newly formed MH boundary

So, although we do not yet know which is the chronological sequence of the genetic induction observed in the present work, we propose that, from all observed expressions, *Fgf8* may be induced first when the transplanted primordium comes in contact with the *Fgf8*-positive isthmic domain and may selectively regulate its own expression throughout the original *Otx2-Gbx2* graft-host interface. This *Fgf8* expression, invading the grafted territory, may repress *Otx2* allowing the induction of *Gbx2* within the repressed area and translating the *Otx2-Gbx2* interface within the grafted area. It is most

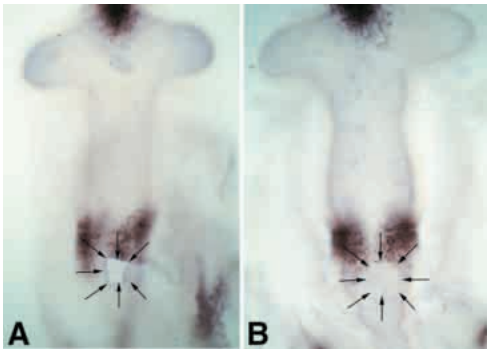

Fig. 7. Embryos prepared for type 3 grafts. (A) A stage HH10 chick embryo prepared to receive a type 3 graft and stained by HIS with the *Fgf8* probe. Note that the hole in the right side of the neural tube (surrounding by arrows) has removed some the most caudal portion of the *Fgf8*-positive isthmic domain (compare with the non operated left side). (B) A chimeric embryo with a type 3 graft (surrounding by arrows) and fixed 4 hours after grafting (stage HH11) and stained by HIS with the *Fgf8* probe. Note that the rostral border of the graft is still in contact with the host *Fgf8*-positive isthmic domain. A-B, ×35.

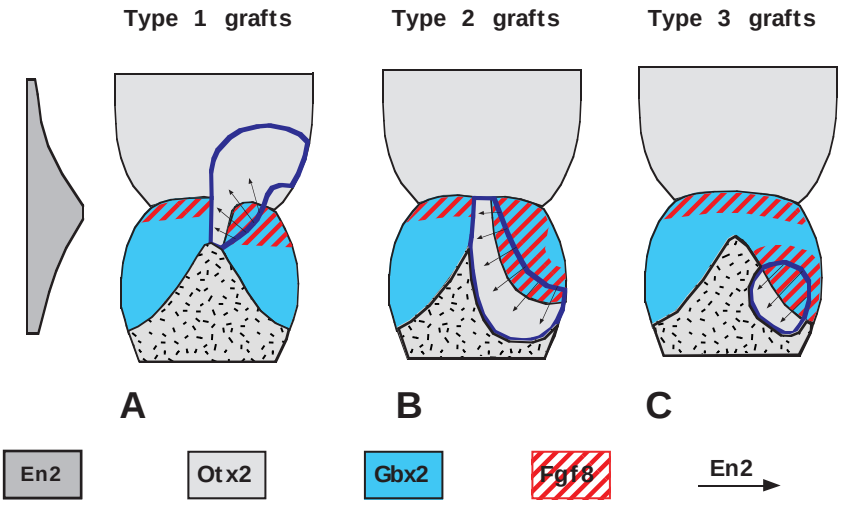

Fig. 8. Schematic representation of short-survival chimeric embryos with type 1 (A), type 2 (B) and type 3 (C) grafts. Dorsal view of the chimeric neural tubes illustrating the localisation of the graft (delimited by the blue line). The light-grey colour labels *Otx2*-positive territories in both the host and the graft: the weave pattern differentiates the choroid tissue from mesencephalon (plain gray). In blue, *Gbx2* territories from both host and graft. Normal and induced *Fgf8*-positive territories are labelled by red stripes. The dark-grey area, to the left of A, schematises the normal gradient of *En2* expression. The arrows in A-C, indicate the decreasing gradient of the induced *En2* expression within the graft. Note that, in the three cases: (i) an *Otx2-Gbx2* boundary is formed within the graft, which is ectopic with respect to the host one for types 2 and 3 grafts, (ii) *Gbx2*- and *Fgf8*-induced expressions are in contact to the host *Gbx2*-positive territory, (iii) this area is negative for *Otx2*, (iv) the induced gradient of *En2* starts in the *Gbx2/Fgf8*-induced area, and (v) in type 3 grafts, the *Fgf8*-induced territory is clearly separated from the host *Fgf8*-positive isthmic ring.

possible that all other inductive events may be triggered at this newly formed boundary. The fact that neither *Fgf8*- nor *Gbx2*-induced expressions have ever been observed at the interface between the graft and the contralateral *Gbx2*-positive neuroepithelium does not contradict our view. On the contrary, it indicates that the isthmic inductive factors are arrested at the roof plate, as was the case in other studies using unilateral *En2*-positive grafts (Martínez et al., 1991; Gardner and Barald, 1991; Bloch-Gallego et al., 1996). Moreover, the results obtained through type 2 grafts strongly support our view that *Fgf8*, *Otx2* and *Gbx2* together contribute to the induction of the new MH boundary. *Fgf8* expression is induced all along the interface between the graft and the host *Gbx2*-expressing territory, which extends to the caudal pole of rh1 far away from the *Fgf8*-positive isthmic ring. Conversely, the area of the graft confronting the *Otx2*-positive mesencephalon, which lies in close continuity to the host *Fgf8*-positive isthmus (see fig 8B), is not induced to express this gene.

The *Gbx2-Otx2* common boundary as an essential cue for MH regionalisation

Our data showing that all inductive events take place exclusively in areas where *Otx2*- and *Gbx2*-expressing territories confront recall the situation observed in the normal MH domain. Since, as already discussed, these two genes are the first to be expressed, it is likely that *Otx2* and *Gbx2* confrontation could play an essential role during the regionalisation of the in situ MH boundary. The fact that, in *Gbx2* null mutants (Wassarman et al., 1997) and in mice replacing *Otx2* with *Otx1* (Accampora et al., 1998), both *Fgf8* and *Wnt1* transcripts are found to be expressed, although abnormally, does not totally contradict this hypothesis. First, as discussed by Wassarmann et al. (1997) and Accampora et al. (1998), it is possible that, although *Gbx2* and *Otx2* seem not indispensable to initiate *Fgf8* and *Wnt1* expressions, their presence may be necessary for the normal inductive interactions needed to establish the isthmic organiser centre. Second, other not yet known transcription factors could be redundant with *Gbx2*, and could initiate *Fgf8* and *Wnt1* expression in the *Gbx2* null mutants. A redundancy of *En1* and *En2* (Hanks et al., 1995), *Otx1* and *Otx2* (Acampora et al., 1997) and *Pax2* and *Pax5* have already been reported (Lun and Brand, 1998). In all previous analysis with heterotopic grafts, cerebellum is formed from areas supposed to express *Gbx2* and to be negative for *Otx2*: for instance, (i) the in situ rh1, contacting either a rostrocaudally inverted mesencephalon (Martinez and Alvarado-Mallart, 1991; Marín and Puelles, 1994) or a diencephalic *Otx2*-positive graft (Bloch-Gallego et al., 1996), and (ii) the isthmic neuroepithelium ectopically transplanted both to the mesencephalic-p1 boundary or to the p2 domain (see Martinez et al., 1991; Marín and Puelles, 1994; Bloch-Gallego et al., 1996). In all these cases, the areas juxtaposed to the *Gbx2*-positive cerebellar territory and which will develop a mesencephalic phenotype, are supposed to express *Otx2* (rostral mesencephalon, p1, p2). Moreover, a cerebellar phenotype is exclusively induced from the *Gbx2*-positive caudal rhombomeres confronted to the isthmo-cerebellar (*Gbx2*-positive) domain (Martinez et al., 1994). Accordingly, in the recent experiments of Martinez et al. (1999), using FGF8-impregnated beads, the *Otx2*-expressing prosencephalon is induced to express *En2* and becomes mesencephalon, while the *Fgf8*-induced area, not only represses *Otx2*, but develops a cerebellum. It remains to determine whether this *Otx2* repressed area is also induced to express *Gbx2*.

That *Otx2-Gbx2* confrontation plays an essential role in MH domain regionalisation fits with the interpretation of Meinhardt (1983) which proposed that interaction of two differently ‘pre-specified’ zones could generate an organiser centre characterised by the induction of a graded morphogen at the interface. In the in situ MH domain, the graded morphogen would be the product of the *Fgf8* gene, expressed

in normal conditions just caudal to the *Otx2* domain. The possibility that the WNT1 protein would contribute, as a graded morphogen, to regionalised the MH domain is sustained by the fact that, in *Wnt1* null mutants, the MH domain is deleted (McMahon and Bradley, 1990; Thomas and Capecchi et al., 1990). However, as reported by McMahon et al. (1992), Serbedzija et al. (1996) and Lee et al. (1997), *Wnt1* seems not implicated in initiating *Fgf8* and *En2* but in maintaining their expression.

As a modulation of our hypothesis, it is necessary to recall that all *Otx2*-expressing territories are not competent to express *En2* and to change their cytodifferentiation when confronted to the *En2*- (and *Gbx2*)-expressing domain. This is the case for telencephalon and p3 neuroepithelium (Martínez et al., 1991; Alvarado-Mallart, 1993; Bloch-Gallego et al., 1996; Crossley et al., 1996) and could be also the case for the pineal gland primordium (this paper). The possibility that the *En2*-induced arrest observed within some of our grafts could take place at the p1-p2 interprosomeric boundary, included within the graft, is difficult to accept. Indeed, we have previously shown that arrest of *En2*-inductive factors can occur at the p1-p2 and p2-p3 interprosomeric boundaries (Bloch-Gallego et al., 1996) data which has been recently confirmed by Martinez et al. (1999). However, if this was the case in our present experiments, it would be necessary to accept that the non-induced p1 or p2 neuroepithelium, isolated from its normal environment,

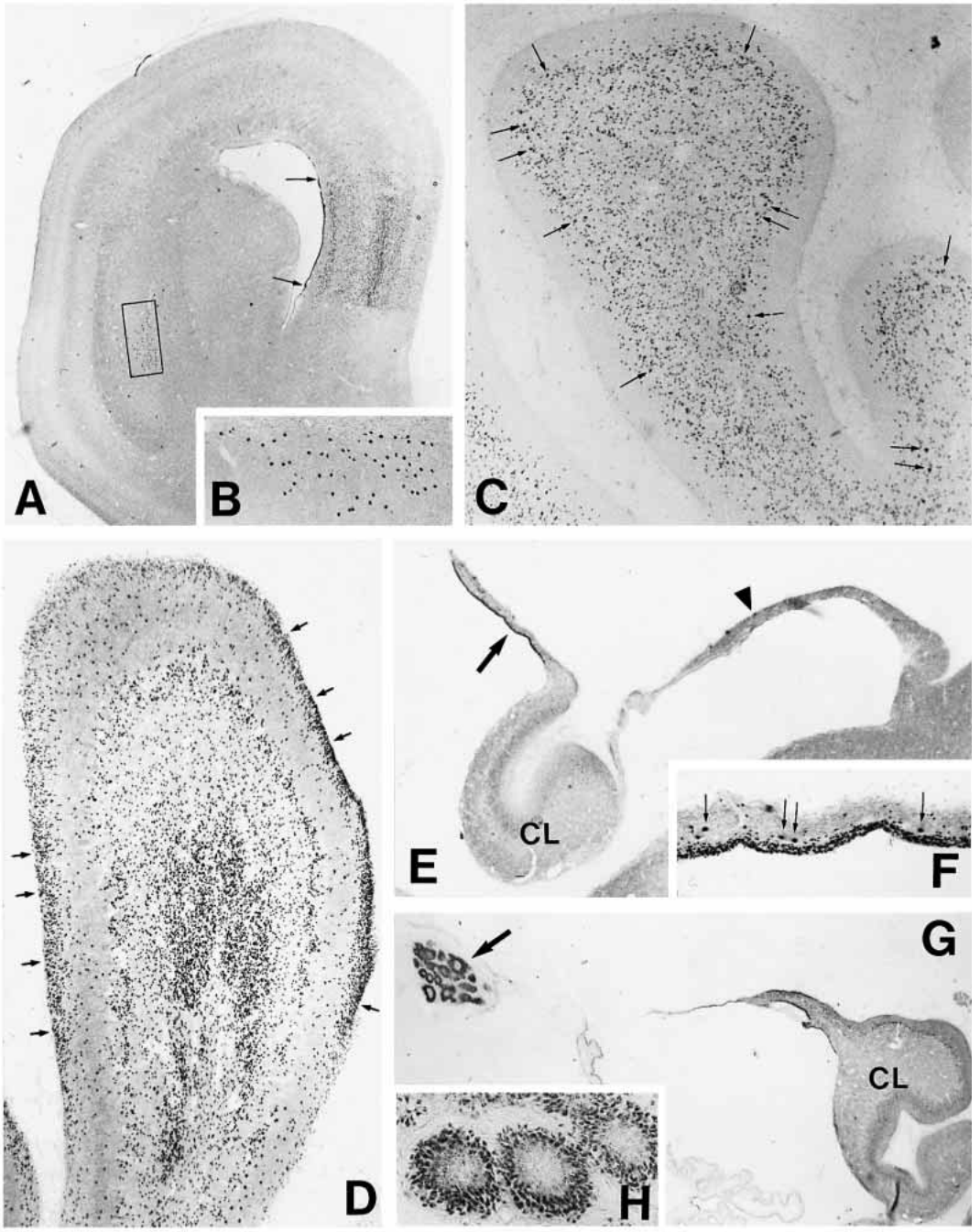

Fig. 9. Long-survival type 1 and 2 grafts. Sagittal sections stained with the QCPN antibody, rostral to the right. (A-C) Case MH207 (type1). Note in A that quail cells have contributed to a small segment of the optic tectum (between arrows), and to the nucleus isthmi, pars parvocellularis (framed area) illustrated at higher magnification in B. (C) Detail of the cerebellar lobule 6, the arrows point to several Purkinje cells. Note the normal cytoarchitecture of this chimeric cerebellum. (D-H) Case G960 (type 2). (D) Cerebellar lobule 7, which contains numerous quail cells, even within the external granular layer (arrows); note its normal cytoarchitecture. (E) The ectopic tectal-like commissure (arrow) attached to a cerebellar lobule (CL); the arrowhead points to the in situ host tectal commissure. (F) Higher magnification of the ectopic commissure; arrows point to quail mesencephalic trigeminal neurones. (G) The ectopic pineal gland (arrow) formed by the grafted cells, evident in the area magnified in H. A, ×15; B, ×70; C, ×67; D, ×120; E, ×15; F, ×76; G, ×14; H, ×180.

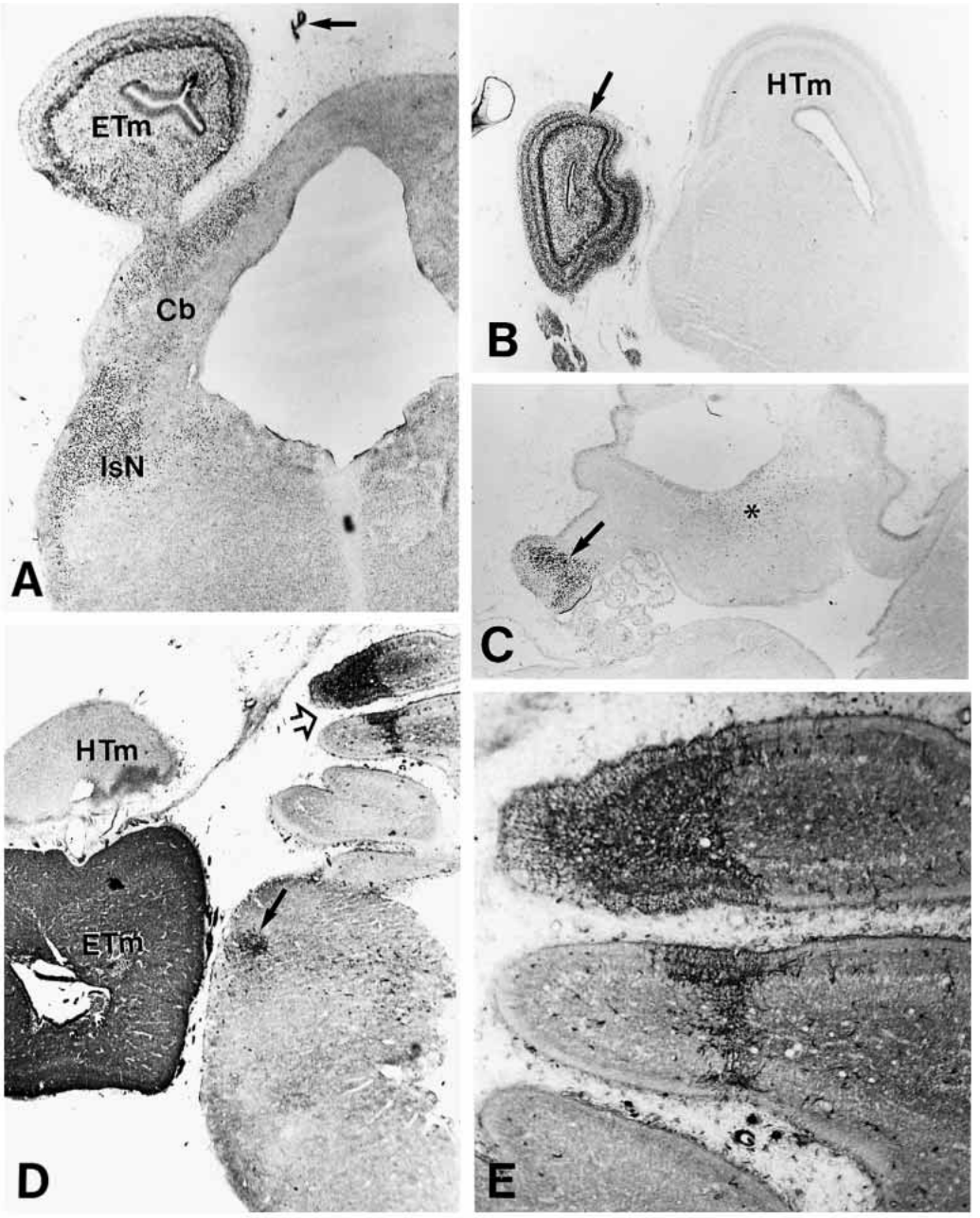

Fig. 10. Long-survival type 3 grafts. (A) Frontal section of case G1015 stained with the QCPN monoclonal antibody. The graft has given rise to an ectopic optic tectum (ETm) and an ectopic pineal gland (arrow). The quail cells have also contributed to the in situ cerebellum (Cb) and isthmic nuclei (IsN). (B,C) Sagittal sections of case J13 stained by the QCPN anti-quail antibody, rostral to the right. (B) The grafted ectopic tectum (arrow); (C) quail cells that contributed to the in situ cerebellum. Some of these cells are observed in the cerebellar white matter (*); other quail cells form the most caudal cerebellar lobule (arrow). (D) A frontal section of case J175 stained with the anti-quail antibody of Lance-Jones and Lagenaur (1988). Note that the grafted cells have developed an ectopic optic tectum (ETm), but also they have contributed to the in situ cerebellum (open arrow), illustrated at higher magnification in E. The arrow in D points to quail cells within the isthmic region. HTm, host optic tectum. A, ×30; B, ×15; C, ×23; D, ×15; E, ×60.

would always differentiate as a pineal gland. More likely would be that, in the cases with partially induced grafts, the transplanted primordium would be restricted to p2 neuroepithelium but, within this prosomere, a boundary, separating the pineal gland neuroepithelium, would arrest the *En2*-inductive factors. Further analysis, using prosencephalic molecular markers would be necessary to confirm this hypothesis.

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