Development 127, 177-186 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV2497

Signalling by FGF8 from the isthmus patterns anterior hindbrain and establishes the anterior limit of Hox gene expression

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Accepted 19 October; published on WWW 8 December 1999

SUMMARY

Current evidence suggests that the anterior segment of the vertebrate hindbrain, rhombomere 1, gives rise to the entire cerebellum. It is situated where two distinct developmental patterning mechanisms converge: graded signalling from an organising centre (the isthmus) located at the midbrain/hindbrain boundary confronts segmentation of the hindbrain. The unique developmental fate of rhombomere 1 is reflected by it being the only hindbrain segment in which no *Hox* genes are expressed. In this study we show that ectopic FGF8 protein, a candidate for the isthmic organising activity, is able to induce and repress gene expression within the hindbrain in a manner appropriate to rhombomere 1. Using a heterotopic, heterospecific grafting strategy we demonstrate that rhombomere 1 is able to express *Hox* genes but that both isthmic tissue and FGF8 inhibit their expression. Inhibition of FGF8 function in vivo shows that it is responsible for defining the anterior limit of *Hox* gene expression within the developing brain and thereby specifies the extent of the r1 territory. Previous studies have suggested that a retinoid morphogen gradient determines the axial limit of expression of individual *Hox* genes within the hindbrain. We propose a model whereby activation by retinoids is antagonised by inhibition by FGF8 in the anterior hindbrain to set aside the territory from which the cerebellum will develop.

Key words: Isthmus, Cerebellum, FGF8, Hindbrain, Midbrain, Rhombomere, Hox, Retinoid

INTRODUCTION

Distinct developmental strategies are deployed to impart regional identity and thereby pattern the vertebrate central nervous system along its anteroposterior axis. The hindbrain becomes organised into a series of repeated segments (rhombomeres) which exhibit both shared and unique developmental properties. Individual identity is imparted to each segment, at least in part, by expression of a unique combination of *Hox* genes. By contrast, immediately anterior to the hindbrain, the midbrain is patterned by a graded signal from an organiser tissue (the isthmus) located at the boundary between midbrain and hindbrain (reviewed by Lumsden and Krumlauf, 1996; Wassef and Joyner, 1997). Thus, the most anterior hindbrain segment, rhombomere 1 (r1), lies at the interface of these two different patterning mechanisms. r1 shares few developmental properties with posterior rhombomeres and is unique in its lack of *Hox* expression; *Hoxa2* is the most rostrally expressed *Hox* gene extending anteriorly to the r1/2 boundary (Prince and Lumsden, 1994). Furthermore, r1 displays a unique neuronal architecture reflected both in the presence of unique nuclei e.g. locus coeruleus and in the organisation of motor neuron cell bodies and their axonal trajectories. The trochlear motor nucleus arises in the anterior part of r1 leaving much of this rhombomere devoid of motor neuron cell bodies. Uniquely among the cranial somatic motor nuclei, its axons are repelled by netrin-1 and semaphorin D from the floor plate, to extend circumferentially and exit the neural tube in the dorsal midbrain (Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997).

Moreover, recent evidence indicates that the cerebellum, a structure unique to vertebrates, which is central to motor co-ordination and occupies almost a third of human cranial capacity, is entirely derived from r1 (Wingate and Hatten, 1999). The rostral limit of r1 is marked molecularly by the expression boundaries of the *Otx* genes (*Otx1* and *Otx2*) and *Gbx2*, which abut at the junction of the midbrain and hindbrain and define this boundary (Millet et al., 1996; Wassarman et al., 1997; Shamim and Mason, 1998; Hidalgo-Sanchez et al., 1999). Targeted mutations of these genes lead to either a rostral expansion ($Otx1^{-/-}$, $Otx2^{+/-}$) or reduction ($Gbx2^{-/-}$) of the cerebellum accordingly (Acampora et al., 1997; Wassarman et al., 1997). Absence of *Hox* expression appears to determine, at least in part, the caudal extent of the cerebellar anlage. Fate-mapping studies reveal that the caudal limit of the cerebellum anlage maps to the anterior limit of *Hoxa2* expression at the r1/2 boundary (Wingate and Hatten, 1999). Furthermore, a

targeted mutation of *Hoxa2* leads to a caudal expansion of the cerebellum (Gavalas et al., 1997), which is further extended into r2 and r3 territory when both paralogous genes *Hoxa2* and *Hoxb2* are absent (Davenne et al., 1999). Consequently, an understanding of the specification and patterning of r1, including its establishment as a '*Hox*-free' territory, will provide crucial insights into the evolutionary origins and the developmental initiation of this brain structure.

The isthmus (midbrain-hindbrain boundary) is an organising centre likely to play a role in patterning r1. Tissue grafting studies first identified the isthmus as a source of a signal(s) that specifies posterior midbrain and facilitates formation of the retinotectal map, and which can respecify posterior forebrain to develop as an ectopic midbrain (Wassef and Joyner, 1997 and references therein). The secreted signalling protein fibroblast growth factor 8 (FGF8) is the best candidate for this signal: ectopic application of FGF8 within the avian midbrain or posterior forebrain mimics the effects of isthmic tissue (Crossley et al., 1996; Sheikh and Mason, 1996; Lee et al., 1997; Shamim et al., 1999; Martinez et al., 1999). *Fgf8* is expressed in an appropriate temporal manner at the isthmus of all vertebrate classes (Crossley and Martin, 1995; Mahmood et al., 1995a; Christen and Slack, 1997), and mutational analyses in mice and zebrafish reveal that it is required for normal midbrain development (Meyers et al., 1998; Reifers et al., 1998; Picker et al., 1999).

There is also evidence that the isthmus can influence hindbrain development: isthmic tissue grafted within the anterior hindbrain causes the production of ectopic cerebellar structures and ectopic expression of *En2* normally expressed in posterior midbrain and anterior r1 (Martinez et al., 1995; Grapin-Botton et al., 1999). Furthermore, *Fgf8* has been implicated in patterning this region as the cerebellum is abnormal in mice hypomorphic for an *Fgf8* mutation, and absent in the *acerebellar* (*ace*) zebrafish mutant (Meyers et al., 1998; Reifers et al., 1998). However, while ectopic cerebellar derivatives and ectopic *En2* transcripts have been observed in the avian midbrain following implantation of FGF8-soaked beads (Martinez et al., 1999), surprisingly, a previous report has suggested that ectopic FGF8 protein does not mimic isthmic tissue in inducing *En2* in avian hindbrain (Crossley et al., 1996).

Here we show that FGF8 protein is indeed able to mimic isthmic grafts into the hindbrain and can regulate gene expression in a manner appropriate to r1, revealing a difference in competence between the midbrain and hindbrain in response to the FGF8 signal. We have used a quail-chick heterotopic grafting strategy to investigate the role of the isthmus organiser in defining r1 as a '*Hox*-free' territory. We show by both ectopic expression and inhibition that FGF8 at the isthmus provides a repressive signal that establishes the anterior limit of *Hox* gene expression in the neural tube and positions the r1/2 boundary.

MATERIALS AND METHODS

Tissue transplantations

Donor chick or quail embryos were incubated to Hamburger and Hamilton stage 10-11 [HH 10-11; 10-13 somites; (Hamburger and Hamilton, 1951)], dissected in Howard's Ringer and pinned out on a Sylgard (Dow-Corning)-coated dish. To mark polarity, small focal injections of DiI C_{12} (Molecular Probes; 5 mg/ml in dimethyl formamide) were made into the anterior of the region to be grafted. The neural tube was excised and treated with Dispase I (Boehringer Mannheim) 1 mg/ml in L-15 medium (Life Technologies) containing 5 μg/ml DNAse I (Boehringer Mannheim) for 5 minutes to separate the neural tube from surrounding mesenchymal cells. The latter were then mechanically dissected away using a tungsten needle. The graft region was removed by further microdissection of either the left or right side of the neural tube and transplanted into stage-matched hosts in ovo.

Host chick embryos were 'windowed' and visualised by a sub-blastodermal injection of India ink. The vitelline membrane over the graft site was removed and tissue from the appropriate side of r4 for insertion of the graft was removed by microdissection using tungsten needles. The graft tissue was introduced with a serum-coated micropipette and manoeuvred into place. Eggs were sealed with tape and incubated for a further 24 hours prior to in situ hybridisation.

Implantation of FGF beads

Fragments of beads soaked in FGF8 (FGF8b isoform; R and D Systems), FGF4 (Sigma) and control beads (PBS-soaked) were prepared and implanted into HH 10-11 chick embryos as described by Shamim et al.(1999). For inhibition studies, 10 μl of 0.1 mg/ml anti-FGF8 or anti-FGF4 neutralising antisera were applied to 50 Affigel beads (BioRad) prior to implantantion. Embryos were incubated for 24 hours after implantation and processed for in situ hybridisation and immunohistochemistry.

Double whole-mount in situ hybridisation and immunohistochemistry

Whole-mount in situ hybridisation of embryos was performed as described by Shamim et al. (1999) using probes which have been previously reported (Guthrie et al., 1992; Prince and Lumsden, 1994; Hollyday et al., 1995; Shamim et al., 1999). For immunohistochemistry following in situ hybridisation, embryos were post-fixed in 4% w/v paraformaldehyde in PBS for 20 minutes and immunohistochemistry was performed using the quail-specific QCPN antibody (Hybridoma Bank, Iowa University, Iowa, USA) and a Cy-3-conjugated secondary antibody (Jackson Immunoresearch Laboratories) to visualise the grafted cells as described for whole vertebrate embryos (Mason, 1999).

BrdU labelling

Embryos in which a bead had been implanted for 24 hours were labelled with BrdU (Boehringer Mannheim) for 60 minutes, fixed, sectioned and immunohistochemistry performed as described by Shamim et al. (1999).

RESULTS

Neuromere boundaries form in a specific sequence between HH 9– and 12, and the morphological isthmic constriction is evident from HH 9– (Vaage, 1969). All experiments in this study were performed at HH 10, when the isthmus and rhombomeres are clearly identifiable and isthmic *Fgf8* expression is established (Shamim et al., 1999).

FGF8 induces ectopic expression of r1-associated genes in posterior hindbrain

We investigated whether or not FGF8 has a direct polarising and patterning influence on anterior hindbrain by introducing a local source of ectopic FGF protein into the hindbrain. FGF8 was delivered on heparin-coated acrylic beads (FGF beads),

and its ability to induce patterns of gene expression characteristic of r1 was examined. Beads were implanted unilaterally and spanning a rhombomere boundary i.e. contacting cells of two adjacent segments (e.g. r1 and r2). In all experiments identical results were also obtained using FGF4 soaked beads which shares FGF receptor specificity with FGF8 (Ornitz et al., 1996), and a homologue of which is also expressed at the isthmus in amphibians (Isaacs et al., 1992). Control beads soaked in PBS never produced a response (Table 1). We sought to resolve the question concerning the ability of FGF8 to induce, in hindbrain, genes expressed in both posterior midbrain and r1. These include *En1*, *En2* and *Pax2* that are inducible in midbrain by FGF8 (Crossley et al., 1996; Lee et al., 1997; Martinez et al., 1999; Shamim et al., 1999). *En2* is also inducible in hindbrain in response to an isthmic tissue graft (Martinez et al., 1995; Grapin-Botton et al., 1999).

24 hours after bead implantation (HH16-18) in situ hybridisation revealed ectopic expression of all three genes induced by an FGF bead. *En1* and *En2* transcripts were detected in a broad domain throughout those rhombomeres in contact with the bead (Fig. 1A-C; Table 1); by contrast, *Pax2* was induced only in a small number of cells close to the bead (Fig. 1D; Table 1). These results were reminiscent of our previous observations following FGF bead implants in midbrain (Shamim et al., 1999). As previously reported for *En2* induction following isthmus tissue grafts (Martinez et al., 1995), ectopic transcripts were never observed in rhombomeres that were not in direct contact with the bead, indicating that the FGF signal did not cross rhombomere boundaries. Moreover, in an identical manner to grafts of isthmic tissue, FGF8 efficiently induced *En2* in all rhombomeres examined (Table 1). However, FGF8 induced *En2* expression in both alar and basal plates (Fig. 1B), whereas isthmic tissue did so only in the alar plate (Martinez et al., 1995).

Ectopic FGF8 protein also induces *Fgf8* gene expression in the midbrain (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999); however we were never able to induce ectopic *Fgf8* in anterior hindbrain (Fig. 1E; Table 1). In the midbrain FGF8 plays a key role in stimulating cell proliferation, as demonstrated by both BrdU and DiI cell labeling following an FGF bead implantation (Martinez et al., 1999; Shamim et al., 1999). Furthermore transgenic analysis of *Fgf8* under the control of the *Wnt1* regulatory elements results in a massive proliferation of neural precursors in the midbrain but not in the dorsal hindbrain (Lee et al., 1997). We were therefore interested in the effects of FGF8 delivered locally on a bead into the hindbrain. 24 hours after the introduction of an FGF bead into r1 or r2, dividing cells were labelled with a short pulse of BrdU introduced directly into the lumen of the neural tube and incubated for a further 60 minutes before fixation and immunohistochemical detection of incorporated BrdU. In agreement with the work of Lee et al. (1997), the number of dividing cells was not markedly increased on the side of the neural tube that received the bead. However, a small local increase in the number of dividing cells was seen tightly associated with the bead (data not shown), but this mitogenic effect was small in comparison to the massive proliferative effect of ectopic FGF8 in midbrain (Shamim et al., 1999),

Thus, in contrast to a previous report (Crossley et al., 1996), these data indicate that FGF8 can induce ectopic expression of genes that are normally expressed in anterior r1, suggesting a role in establishing polarity within r1. Moreover, FGF8 protein alone is sufficient to mimic isthmus tissue grafts into hindbrain.

FGF8 represses expression of *Wnt1* and *Wnt3a* in the hindbrain

While r1 shares some patterns of gene expression with posterior midbrain, it differs in others. For example, it is distinguished from both midbrain and the rest of the hindbrain by transiently lacking expression of both *Wnt1* and *Wnt3a* in dorsal midline cells (Fig. 2A; Hollyday et al., 1995; L. Tumiotto, A. Lumsden and A. Graham, personal communication). In the midbrain, *Wnt1* is also expressed in a dorsoventral ring immediately anterior to the isthmus and abutting the *Fgf8* expression domain there (Fig. 2A; Hollyday

Table 1 Summary of FGF bead implants into the hindbrain

Ggene	Implant site	Bead	Ectopic induction	Total embryos	Gene	Implant site	Bead	Repression	Total embryos
En1	r1/2	FGF	3	8	*Hoxa2*	r1/2	FGF4	7	11
		PBS	0	7			FGF8	7	13
							PBS	0	7
En2	r1/2	FGF4	5	15		r3/4	FGF4	0	5
		FGF8	25	40			FGF8	0	4
	r2/3	FGF4	5	15					
		FGF8	22	42	*Hoxb1*	r3/4	FGF4	0	3
	r3/4	FGF4	2	9			FGF8	0	1
		FGF8	2	5					
					Wnt1	r1/2	FGF4	7	11
Pax2	r1/2	FGF4	3	8			FGF8	10	19
		FGF8	3	20		r2/3	FGF4	9	10
		PBS	0	5		r3/4	FGF4	6	6
							FGF8	3	12
Fgf8	r1/2	FGF4	0	12		r2/3	PBS	0	6
		FGF8	0	30					
					Wnt3a	r2/3	FGF4	3	8
Hoxa2	r1	α-FGF8	5	13			FGF8	1	4
		α-FGF4	0	6					
		PBS	0	6					

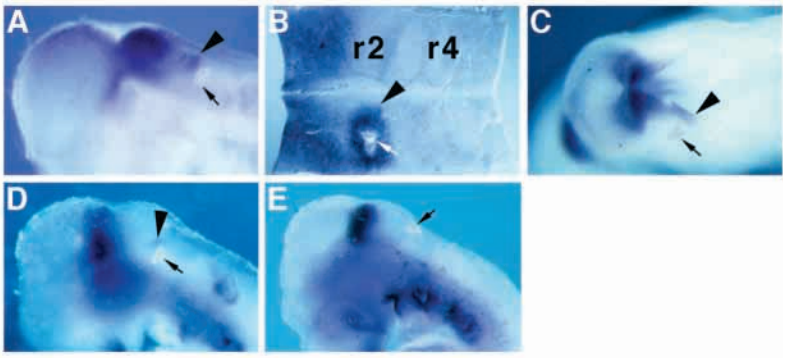

Fig. 1. Analysis of ectopic gene induction following implantation of FGF-soaked beads into anterior hindbrain. In all plates anterior is to the left, ectopic expression is indicated by an arrowhead and position of bead is indicated by an arrow. B shows a flat-mounted hindbrain opened along the dorsal midline such that the floorplate is medial in the preparation and dorsal regions are lateral. (A) In situ hybridisation detects *En2* in its normal expression domain in the posterior midbrain and anterior r1, and ectopic transcripts adjacent to the FGF bead at the r2/3 boundary. (B) Following insertion of an FGF bead across the r1/2 boundary, ectopic *En2* transcripts extend along the entire dorsoventral axis in r1 and r2 but do not extend across the r2/3 boundary into r3. (C) Dorsal view of an embryo following insertion of an FGF bead at the r1/2 boundary shows ectopic *En1* transcripts induced in r1 and r2, posterior to its normal domain of expression. (D) Lateral view of an embryo showing *Pax2* expression both in its normal domain and in a small group of cells adjacent to an FGF bead implanted at the r1/2 boundary. (E) FGF protein does not induce *Fgf8* gene expression within the hindbrain.

et al., 1995 and references therein). Both FGF8 and isthmic tissue induce ectopic expression of *Wnt1* in midbrain and posterior diencephalon (Bally-Cuif and Wassef, 1994; Crossley et al., 1996; Sheikh and Mason, 1996; Martinez et al., 1999; Shamim et al., 1999). However, when FGF beads were introduced into the hindbrain, expression of both *Wnt1* and *Wnt3a* was lost from the roof plate on the side of the neural tube that received the bead graft. Furthermore, repression spanned several rhombomeres indicating that it was not restricted by boundary structures in roof-plate tissue (Fig. 2B-D; Table 1). Again, all rhombomeres were sensitive to the FGF signal (Table 1). Taken together, these data indicated a differential competence between anterior hindbrain and midbrain with respect to their response to FGF8. A differential competence to propogate the FGF signal was also revealed between the main body of the rhombomere and the roof plate region, consistent with the lack of morphological boundary structures in the latter.

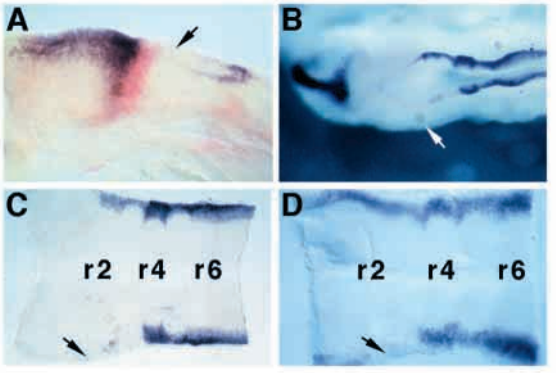

Fig. 2. Repression of *Wnt* expression following FGF bead implantation into anterior hindbrain. In all plates anterior is to the left and position of bead is indicated by an arrow. (A) Lateral view of a HH 12 embryo showing normal *Wnt1* (blue) and *Fgf8* (red) expression in adjacent domains at the isthmus, and *Wnt1* expression in the dorsal midline of the midbrain and dorsal roof plate of the hindbrain. The arrow indicates the lack of *Wnt1* expression in r1. (B) Dorsal view of an embryo showing normal *Wnt1* expression on the contralateral side of the brain, and inhibition of expression in r2 and r3 following an FGF bead implant at the r1/2 boundary. (C) Flat-mount preparation of the embryo in B. (D) *Wnt3a* expression repressed by an FGF bead inserted at the r2/3 boundary. Expression is not detected in r2 and r3 and is reduced in r4.

r1 is competent to express *Hox* genes when grafted posteriorly within the hindbrain

The most striking feature of r1 when compared with the other segments of the hindbrain is its lack of *Hox* gene expression; *Hoxa2*, the most anterior *Hox* transcript, has an anterior limit of expression at the r1/2 boundary (Prince and Lumsden, 1994). Current models suggest that axial limits of *Hox* gene expression within the hindbrain are established by their differential responsiveness to a gradient of a retinoid morphogen, probably acting in concert with a signal(s) from post-otic paraxial mesoderm. In this model, *Hoxa2* is activated by the lowest morphogen concentrations and its anterior limit of expression reflects the position at which morphogen concentrations fall below this threshold (see e.g. Itasaki et al., 1996; Muhr et al., 1997; Godsave et al., 1998; Grapin-Botton et al., 1998; Maden et al., 1998; Woo et al., 1998; Muhr et al., 1999). The ability of FGF8 to repress *Wnt* expression within the hindbrain prompted us to investigate an alternative possibility: that the anterior limit of *Hox* expression within the developing brain might be established by a repressive influence from the isthmus, possibly mediated by FGF8.

We first determined whether or not r1 was competent to express *Hox* genes when grafted ectopically to a caudal location within the hindbrain. Previous studies involving transplantation of hindbrain tissue reveal that rhombomeres generally display a plasticity of cell fate when grafted posteriorly but maintain characteristics of their axial level when grafted anteriorly. (Itasaki et al., 1996; Grapin-Botton et al., 1997; Gould et al., 1998 and references therein). We examined the ability of r1 to express *Hox* genes when transplanted to r4, within the otic region of the hindbrain where the isthmus is reported to maintain its organiser abilty when grafted heterotopically (Martinez et al., 1995; Grapin-Botton, 1999). Quail donor r1 (excluding any isthmic cells) was grafted unilaterally into a chick host in place of host r4 (Fig. 3A). Donor r1 tissue was distinguished from host tissue with a quail-specific antibody. To ensure that no isthmic tissue was transferred with the r1 graft, donor embryos were selected at random and assayed for *Fgf8* expression as a marker of isthmic tissue following removal of the r1 graft as previously described (Irving and Mason, 1999). In all cases, a region of *Fgf8*-

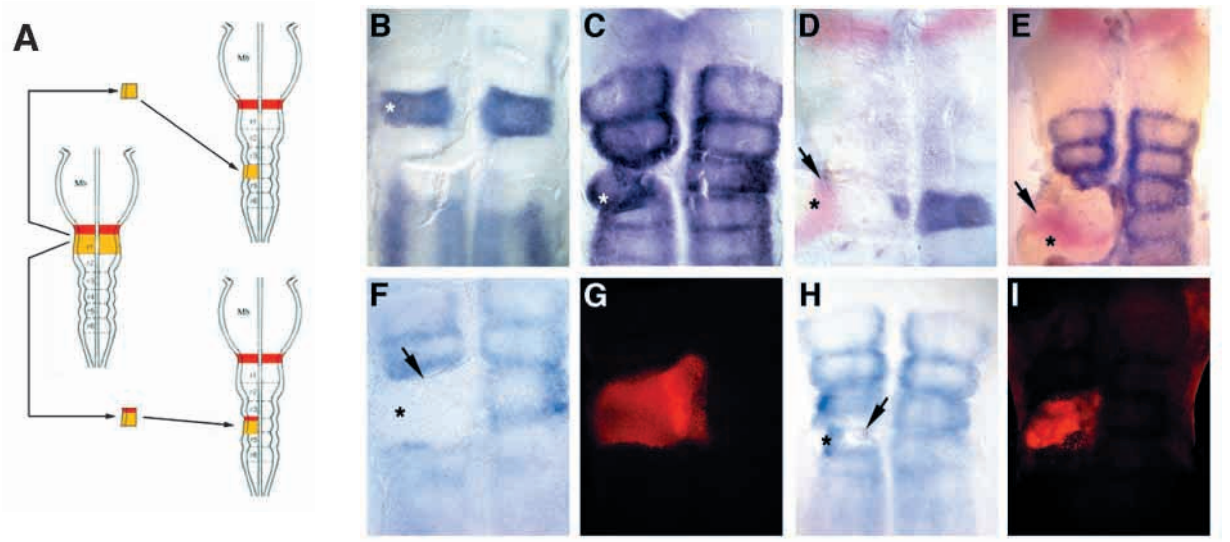

Fig. 3. Expression of *Hox* genes in r1 following grafting posteriorly within the hindbrain is regulated by the presence of isthmic tissue or FGF8. In all cases the tissue graft was inserted into r4 on the left side of the embryo at HH10. All preparations are flat-mounted and oriented with anterior to the top of the plate and the grafted tissue is indicated by an asterisk. (A) Schematic representation of the grafting strategy. (B,C) r1 expresses *Hoxb1* (B) and *Hoxa2* (C) when transplanted ectopically to r4 in the absence of isthmic tissue. (D,E) When r1 is grafted to the position of r4 together with isthmic tissue, *Hoxb1* (D) and *Hoxa2* (E; blue colour) are never induced within the graft. Presence of isthmic tissue is confirmed by hybridisation for *Fgf8* transcripts (red colour; arrow). (F) An FGF8-soaked bead (arrow) grafted into r4 together with quail r1 lacking isthmic tissue, prevents expression of *Hoxa2* within the graft. (G) Detection of grafted quail donor r1 tissue by indirect immunofluorescence using the QCPN antibody on the specimen shown in F shows that no grafted quail cells express *Hoxa2*. (H) Control experiment showing that insertion of a PBS bead (arrow) together with a quail r1 graft does not inhibit *Hoxa2* expression within the graft. (I) QCPN immunohistochemistry shows the extent of the r1 graft in H.

negative cells was clearly visible between the isthmus and the anterior excision point of the graft, indicating that no isthmic cells had been transferred (data not shown and see also Irving and Mason, 1999). Furthermore, 24 hours after grafting, no *Fgf8* expression was detected associated with the graft (data not shown). In situ hybridisation with both *Hoxb1*, which specifically marks r4 in the anterior hindbrain at this stage (Guthrie et al., 1992), and *Hoxa2* revealed that grafted r1 expressed both *Hox* genes in accordance with its new axial level (Fig. 3B,C; Table 2). Therefore, r1 is competent to express *Hox* genes and respond to positional information from the host environment at HH 10. It is noteworthy that other rhombomeres (r3 and r5) fail to induce *Hoxb1* when transplanted to r4 at this or earlier stages (Guthrie et al., 1992; Kuratani and Eichele, 1993). Rather than reflecting an early specification of r4 followed by loss of any local inducing signal for *Hoxb1* (Guthrie et al., 1992; Kuratani and Eichele, 1993), our study indicates that the inducing signal is still present in the host environment at HH 10 and that r1 is competent to respond to it.

Presence of isthmic tissue prevents *Hox* gene expression in grafted r1

Previous studies involving transplantation of r1 produced conflicting results concerning its ability to express *Hox* genes (Grapin-Botton et al., 1995; Itasaki et al., 1996; Grapin-Botton et al., 1997; Hunt et al., 1998) but importantly, these studies did not address the presence or absence of isthmic tissue

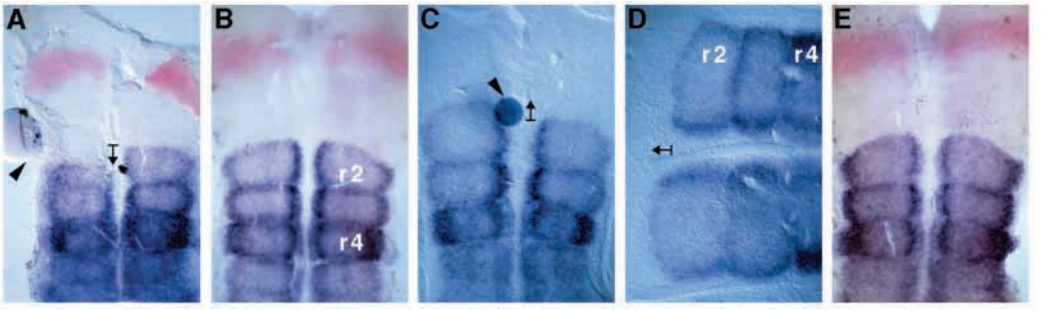

Fig. 4. Analysis of Hoxa2 expression following implantation of either FGF-beads (arrowheads) or anti-FGF antibodies into r1. Hindbrains are flatmounted and anterior is to the left in D and to the top in A-C and E. Double in situ hybridisation reveals *Hoxa2* transcripts in blue and *Fgf8* transcripts in red. (A) *Hoxa2* expression is repressed in r2 following an FGF bead implant at the r1/2 boundary. Compare the extent of expression on the implant and contralateral sides of the hindbrain. (B) Control experiment showing no shift in the limits of *Hoxa2* expression when a PBS bead is implanted into r1. (C,D) *Hoxa2* expression is expanded into r1 after implanting a bead soaked in an anti-FGF8 blocking antibody within in r1. Compare the distance between *Fgf8* at the isthmus and the rostral *Hoxa2* boundary on the implant and contralateral sides. (E) No shift in Hoxa2 expression is seen when an anti-FGF4 blocking antibody is introduced into r1. Arrows show shift in axial limit of gene expression.

Table 2. Summary of grafts

Type of graft into r4	Donor r1 species	Total number of grafts	Number of integrated grafts	Ectopic *Hox* expression in r1 graft	% of embryos with ectopic *Hox* expression	Significance relative relative to appropriate control*
r1	quail	12	8	6	75	
	chick	21	18	15	83	
r1+ isthmus	quail	12	12	0	0	*P*=<0.001
	chick	4	2	0	0	*P*=<0.001
r1+FGF8 bead	quail	13	11	2	18	*P*=<0.05
	chick	23	10	4	40	*P*=<0.002
r1+FGF4 bead	quail	9	6	0	0	*P*=<0.001
r1+PBS bead	quail	20	14	8	57	
	chick	13	9	7	78	

*Statistical probabilities were calculated using Student's *t*-test.

following grafting. We investigated whether the isthmus might repress *Hox* expression in r1 tissue if the two were transplanted posteriorly together. We performed grafts of r1 including adjacent isthmic tissue. Due to the larger size of this piece of tissue, the graft was made unilaterally into both r4 and anterior r5 but the majority of the grafted tissue was located in r4. After 24 hours, in cases where *Fgf8*-positive (isthmic) tissue was detected within the graft, neither *Hoxb1* nor *Hoxa2* expression was observed within the grafted tissue (Fig. 3D,E; Table 2). Thus, the presence of the isthmus within the graft confers a dominant specification upon r1 such that it is now unresponsive to positional cues associated with r4.

To test whether FGF8 could mimic this isthmic activity and repress *Hox* genes in r1, we first implanted an FGF bead together with an r1 graft (minus isthmic tissue) into r4. FGF8 was sufficient to prevent *Hox* expression within the graft. In most cases, expression of *Hoxa2* or *Hoxb1* was completely absent from the r1 graft (Fig. 3F,G and data not shown; Table 2). However, in a few instances *Hox* expression was detected within the graft but distal to the bead, suggesting that a critical concentration of FGF8 is required to maintain r1 identity (data not shown). When r1 was grafted to the position of r4 with control beads soaked in PBS both *Hoxb1* and *Hoxa2* were efficiently induced in the graft (Fig. 3H,I and data not shown). Therefore, FGF8 alone is sufficient to prevent induction of *Hox* gene expression in r1 tissue grafted into posterior hindbrain.

FGF8 defines the anterior limit of *Hox* gene expression in the neural tube

As FGF8 was able to prevent *Hox* gene expression in the r1 grafted ectopically into posterior hindbrain, we investigated whether FGF8 at the isthmus normally functions to actively repress *Hox* genes in r1 and thus define the anterior limit of their expression within the hindbrain. FGF beads were placed in r1 at HH 9 and 24 hours later in situ hybridisation to detect *Hoxa2* transcripts revealed that the anterior limit of *Hoxa2* expression was displaced caudally on the side of the embryo that received the bead graft. Notably, rather than observing a local loss of gene expression around the bead, the entire boundary of *Hoxa2* expression shifted posteriorly and remained perpendicular to the floorplate; even beads integrated in an extreme dorsal position evoked this response (Fig. 4A; Table 1). This was not due to mechanical disturbance, as PBS beads had no effect (Fig. 4B; Table 1). By contrast, FGF beads placed at the r3/4 boundary had no effect on *Hoxa2* or *Hoxb1* expression in these segments (Table 1 and data not shown). It is also unlikely that the caudal shift in *Hoxa2* expression was due to increased proliferation in r1. It has been previously reported that ectopic FGF8 acts as a mitogen only in midbrain and not in hindbrain (Lee et al., 1997), and we find that FGF beads inserted into r1 or r2 caused only a slight local increase in proliferation around the bead in these rhombomeres (see above). Moreover, the relative size of the metencephalic territory (r1 plus r2) on the implanted side compared with the unoperated sides of embryos (see e.g. Fig. 4A) remained unaltered.

To confirm the role of FGF8 in establishing the anterior limit of *Hoxa2* expression within the brain we sought to inhibit its activity. We therefore applied a specific anti-FGF8 blocking antiserum to inhibit the endogenous signal. Anti-FGF8 antibody was introduced on Affigel beads unilaterally into anterior r1 and 24 hours after bead implantation we found that *Hoxa2* expression was shifted rostrally. Again, the entire boundary of expression shifted such that the r2 territory (as defined by *Hoxa2*) was expanded at the expense of r1 tissue on the side of the embryo that received the bead graft (Fig. 4C,D; Table 1). It is noteworthy that the r3 territory is also slightly enlarged suggesting that the normal influence of FGF8 extends beyond the metencephalic (r1/r2) territory. A control anti-FGF4 blocking antibody, previously shown to be active in vivo (Shamim et al., 1999), had no effect on *Hoxa2* expression (Fig. 4E; Table 1).

DISCUSSION

We have investigated the role of FGF8 from the isthmus in patterning r1 by using a combination of ectopic protein expression, inhibition-of-function and grafting strategies.

FGF8 regulates gene expression in the anterior hindbrain

We found that ectopic FGF8 protein introduced into the hindbrain is sufficient to regulate gene expression in a manner characteristic of r1. The transcription factors, *En1*, *En2* and *Pax2*, normally expressed in r1, are induced in all posterior rhombomeres by FGF8. These data suggest that FGF8 mediates the *En2*-inducing activity demonstrated for isthmic tissue grafted into hindbrain (Martinez et al., 1995), although

preliminary data reported by others had previously suggested that FGF8 was unable to induce *En2* in hindbrain tissue (Crossley et al., 1996). Moreover, the inductive response to both FGF8 and isthmic tissue signals does not cross rhombomere boundaries. However, the response to FGF8 differs from that to isthmic grafts in one respect: the former induces *En2* in both alar and basal plates whereas the latter does so only in the alar plate (Martinez et al., 1995).

FGF8 also induces ectopic *En1*, *En2* and *Pax2* expression within the avian midbrain (Sheikh and Mason, 1996; Martinez et al., 1999; Shamim et al., 1999). However temporal studies showed that both *En1* and *Pax2* transcripts are normally detected in the mid-hindbrain region prior to *Fgf8* mRNA suggesting that FGF8 at the isthmus functions to maintain rather than induce their expression in posterior midbrain and anterior r1 (Shamim et al., 1999). The same conclusion has been drawn by others from studies of gene expression and the *acerebellar* (*ace*) *Fgf8* and *no ishmus* (*noi*) *Pax2.1*mutants in zebrafish (Lun and Brand, 1998; Reifers et al., 1998).

However, the response to ectopic FGF8 in hindbrain differs from that of midbrain in a number of respects. While *Wnt1* is induced in both midbrain and diencephalon (Crossley et al., 1996; Sheikh and Mason, 1996; Martinez et al., 1999; Shamim et al., 1999), together with *Wnt3a*, it is repressed by FGF8 in hindbrain. This reflects the normal transient lack of transcripts for these genes in r1 (Fig. 2; Hollyday et al., 1995; L. Tumiotto, A. Lumsden and A. Graham, personal communication). In addition, FGF8 is unable to induce its own expression in hindbrain tissue whereas it does so efficiently in midbrain (Martinez et al., 1999; Shamim et al., 1999), although conflicting results have been reported concerning *Fgf8* induction in posterior diencephalon (Crossley et al., 1996; Shamim et al., 1999). The inability of FGF8 protein to induce *Fgf8* transcripts within the hindbrain is consistent with a recent study which showed that, within the hindbrain, only r1 is competent to express *Fgf8* and that expression requires a diffusible activity from the midbrain (Irving and Mason, 1999).

The differing responses of midbrain and hindbrain to ectopic FGF8 identifies a difference in competence between these tissues and is consistent with the different developmental fates of midbrain and r1 either side of the isthmic organiser (e.g. tectum anteriorly and cerebellum posteriorly). The isthmic organiser is established at the site of juxtaposition of *Otx2* and *Gbx2* expression within the neuroepithelium, and their expression precedes that of all genes associated with the mid-hindbrain territory including *Fgf8* (Bally-Cuif et al., 1995; Niss and Leutz, 1998; Shamim and Mason, 1998). Indeed, studies of mice in which *Otx* or *Gbx2* function has been perturbed suggest that they participate in formation of the isthmic organiser (Wassarman et al., 1997; Acampora et al., 1998). Hence, they may also underly the differences in midbrain and hindbrain response to FGF8.

FGF8 regulates the anterior limit of *Hox* gene expression in the neural tube

(i) r1 is competent to express *Hox* genes

Unilateral, heterotopic grafts of r1, lacking isthmic tissue as defined by *Fgf8* expression, into the position of r4 in the hindbrain revealed that r1 is competent to express *Hox* genes characteristic of the axial level of r4 (*Hoxa2* and *Hoxb1*). Others have reported that rhombomere grafts (r3, r5) into r4 were unable to express *Hoxb1*. This led to the suggestion that specification of this rhombomere occurred at a stage in development earlier than that used for grafting (HH 10; 10 somites), and that signal(s) specifying r4 identity were subsequently lost (Guthrie et al., 1992; Kuratani and Eichele, 1993). However, grafts of r1, performed at identical or later stages, showed that the patterning signal is still present in the local environment. Thus, the inability of r3 and r5 to express *Hoxb1* would seem to reflect a difference in competence between them and r1 to respond to the patterning cues. The former are distinguished from other rhombomeres by expression of *Krox20* at stages prior to those used for grafting (Nieto et al., 1991) and this specification may render them unresponsive to a *Hoxb1*-inducing signal. Furthermore, the *Hoxb1* 5′ transcriptional regulatory region that restricts expression to r4 contains a repressor element that specifically blocks expression in r3 and r5 (Studer et al., 1994).

(ii) Isthmic tissue or FGF8 prevents *Hox* gene expression in r1 grafts

Grafts of r1 together with isthmic tissue showed that the isthmus is a source of an inhibitory signal that prevents *Hox* gene expression in tissue grafted at the level of r4. Moreover, recombinant FGF8 protein was sufficient to mimic this isthmic property. Previous studies of r1 tissue grafted posteriorly within the hindbrain have produced conflicting results concerning its ability to express *Hox* genes associated with its new axial level (Grapin-Botton et al., 1995; Itasaki et al., 1996; Grapin-Botton et al., 1997; Hunt et al., 1998). However, the presence or absence of isthmic tissue was not examined, although one report showed induction of ectopic cerebellar-like structures indicating its presence in the grafts (Hunt et al., 1998). Consistent with our own data, the latter study also failed to detect ectopic *Hox* expression in r1.

Fgf8 expression was maintained within the graft when it was placed at the level of r4. This was consistent with previous work which had shown that isthmic tissue retained its organiser ability when grafted into midbrain, diencephalon and into otic or pre-otic hindbrain (Alvarado-Mallart et al., 1990; Martinez et al., 1991; Bally-Cuif and Wassef, 1994; Martinez et al., 1995). When grafted into spinal cord, however, the isthmus loses its organiser ability concommitent with loss of *Fgf8* expression and induction of *Hox* gene expression in the grafted tissue (Grapin-Botton et al., 1999). The latter data lend further indirect support to our findings that *Fgf8* is responsible for repressing *Hox* genes in r1.

While either isthmic tissue or FGF8 were able to prevent *Hox* expression in r1 grafted into posterior hindbrain, it was notable that neither were able to repress endogenous *Hox* expression in rhombomeres (r3 and r5) adjacent to r1 grafts. A number of explanations are possible. The first is that the presence of rhombomere boundary cells prevented r3 or 5 being exposed to the isthmic or FGF8 signal. However, we consider this unlikely as FGF8-coated beads implanted into r2/3 or 3/4 failed to repress either *Hoxa2* or *Hoxb1* expression. Alternative explanations are that (i) an ‘activating’ signal was able to antagonise isthmic or FGF8 effects, or (ii) that r3 and r5 have already been specified such that they are no longer responsive to these signals.

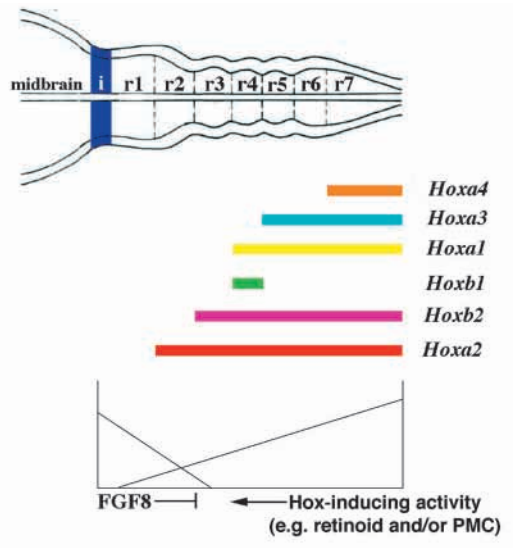

Fig. 5. A model for the regulation of *Hox* expression in the anterior hindbrain. The anterior limit of each member of the Hox gene family is set by a decreasing morphogen gradient established from the spinal cord and/or post-otic paraxial mesoderm. More 3′ genes on the Hox cluster are activated by increasingly lower morphogen concentrations. The anterior limit of this activation is restricted however, by an opposing gradient of FGF8 signalling from the isthmus. This FGF signal is dominant over the retinoid signal at anterior hindbrain levels, providing an anteriorising cue that defines the r1/2 boundary and maintains a territory free of *Hox* gene expression that will give rise to the cerebellum. Abreviations: i, isthmus; PMC, paraxial mesoderm-derived caudalising activity.

(iii) Ectopic FGF8 or anti-FGF8 neutralising antiserum regulate the anterior limit of *Hoxa2* expression within the developing brain

We confirmed the role of FGF8 in regulating *Hox* gene expression in the anterior hindbrain by manipulating its expression/function in r1 in situ. We showed that ectopic FGF8, introduced into r1 on acrylic beads, could repress *Hoxa2* expression within a territory which was normally fated to become r2 and to express *Hoxa2*. Application of a neutralising antibody against FGF8 had the opposite effect, extending the *Hoxa2*-positive domain anteriorly into prospective r1. A neutralising antiserum against FGF4 had no effect.

Taken together, our data show that FGF8 signalling from the isthmus is responsible for establishing the anterior limit of *Hox* gene expression in the neural tube in vivo by repressing *Hoxa2* in r1. This effect clearly influences the position of the morphological r1/2 segmental boundary and, interestingly, it may also influence the positioning of the r2/3 boundary. In a number of experiments involving application of the anti-FGF8 serum, the size of r3 was also apparently increased (see e.g. Fig. 4c). This suggests that the influence of FGF8 extends as far as the prospective r3 territory and that it may participate in positioning both the r1/2 and r2/3 boundaries.

Our conclusions are consistent with the temporal expression of both *Fgf8* and *Hoxa2*: *Hoxa2* transcripts are first observed at HH 8 in the neural tube, extending progressively more rostral during its early development. However, the anterior limit of *Hoxa2* expression is defined and formation of the morphological r1/2 boundary occurs at HH 11 (Prince and Lumsden, 1994). By contrast, *Fgf8* expression is first detected in the propsective isthmic territory at HH 8+ (Shamim et al., 1999), before the *Hoxa2* expression domain has extended to its anterior limit and prior to the establishment of rhombomere boundaries (Vaage 1969; Lumsden, 1990). The apparent regulation by FGF8 of the position of both the r1/2 and r2/3 rhombomere boundaries suggests that FGF8 may initially act as a diffusible signal influencing hindbrain patterning over multiple segments prior to the appearance of inter-rhombomeric boundaries. However, signals from FGF8 (this study) and isthmic tissue (Martinez et al., 1995) are not able to traverse boundaries. In this respect, it is noteworthy that boundary cells express a repertoire of genes that distinguish them from the main body of rhombomeres (Heyman et al., 1995; Mahmood et al., 1995b, 1996) and gap-junctional communication is lost across them (Martinez et al., 1992). In this way they may aid the isolation of each segment and the establishment or maintenance of individual rhombomere identities. By contrast, there is no morphological or molecular evidence evidence that boundaries exist within the roof plate. Consistent with this, we found that the FGF signal is propogated over a number of rhombomere lengths in that structure, as evinced by the down-regulation of *Wnt1* and *Wnt3a* in the roof plate across a number of adjacent segments.

Activation of *Hox* expression in the developing hindbrain

Inhibition of FGF8 showed that signals for activation of *Hoxa2* expression extend into the prospective r1 territory but are normally antagonised by a dominant and repressive effect of FGF8. Current evidence concerning activation and differential axial expression of *Hox* genes within the hindbrain suggest that these processes are mediated by multiple signalling molecules (see above), including a retinoid, originating from post-otic paraxial mesoderm and/or spinal cord. The retinoid morphogen is believed to form a gradient within the hindbrain with individual *Hox* genes being activated at different concentrations (Itasaki et al., 1996; Grapin-Botton et al., 1997; Maden et al., 1998; Berggren et al., 1999). Other activities, such as those derived from paraxial mesoderm, may contribute to the establishment or function of this gradient. In support of this hypothesis, there are a number of studies which show that *Hox* genes are induced at defined retinoid concentrations and in accord with both their postition within the chromosomal gene cluster and their relative axial limits of expression (Simeone et al., 1990, 1991; Godsave et al., 1998). Ectopic application of retinoic acid is sufficient to change *Hox* expression patterns within the hindbrain, leading to a posterior transformation of rhombomere identity (Conlon and Rossant, 1992; Marshall et al., 1992; Gale et al., 1996). The activation of *Hox* genes by retinoic acid may be mediated either directly via interaction of retinoid receptors with retinoic acid response elements (RAREs) within the *Hox* cluster (Dupe et al., 1997), or indirectly via the Cdx family of transcription factors, which themselves are regulated by retinoic acid (Subramanian et al., 1995; Taneja et al., 1995).

Our evidence that FGF8 represses *Hox* expression in anterior hindbrain was unexpected given the increasing body of evidence which indicates that FGF signalling induces *Hox* expression at spinal cord levels via activation of members of the *Cdx* family (Pownall et al., 1998 and references therein).

However, it is noteworthy that others have previously reported that FGFs are unable to activate *Hox* genes at hindbrain levels. (see e.g. Godsave et al., 1998; Grapin-Botton et al., 1998; Pownall et al., 1998).

It is becoming clear that FGF8 is involved in multiple patterning events along the anteroposterior axis of the developing neural tube and that these occur at approximately the same developmental stages. FGF8 from the neuropore region patterns the forebrain (Shimamura and Rubenstein, 1997), isthmic FGF8 is involved in establishment of both midbrain and r1 identity/polarity (Crossley et al., 1996; Sheikh and Mason, 1996; Lee et al., 1997; Meyers et al., 1998; Reifers et al., 1998; Picker et al., 1999; Shamim et al., 1999; Martinez et al., 1999 and this study) while at spinal cord levels it is one of several FGFs implcated in patterning that tissue (Godsave et al., 1998; Pownall et al., 1998 and references therein). Differential competence must therefore underlie these divergent responses to the same protein and this is consistent with recent evidence that the neural plate is already regionalised along its anteroposterior axis at the time of its induction (see e.g. Shamim and Mason, 1998).

Conclusion: a model for the regulation of *Hox* gene expression in the anterior hindbrain

Our data shows that FGF8 establishes the anterior limit of *Hox* expression within the developing brain, thereby establishing the r1 territory. The results of inhibition of FGF8 function show that the activating influence on *Hoxa2* expression normally extends within the prospective r1 territory but is antagonised by FGF8. Increasing levels of FGF8 by application of protein on beads causes the anterior limit of *Hoxa2* expression to shift posteriorly.

We propose that axial patterns of *Hox* expression in the anterior hindbrain are determined by opposing gradients of activating (retinoid and paraxial mesoderm-derived caudalising activity) and inhibiting (FGF8) morphogens (Fig. 5). The latter predominates in r1 and maintains it as a '*Hox*-free' hindbrain territory. Current evidence suggests that the cerebellum is very likely derived entirely from r1 and the isthmus (Wingate and Hatten, 1999). Loss of *Hoxa2* expression causes expansion of the cerebellar primordium into r2 in mutant mice (Gavalas et al., 1997) and zebrafish lacking FGF8 also lack cerebellae (Reifers et al., 1998). Moreover, FGF8 locally induces characteristics of r1 when expressed ectopically in midbrain (Martinez et al., 1999; Irving and Mason, 1999). Taken together with this study, these data suggest that a major role of FGF8 signalling from the isthmus is to establish that territory of the brain that will ultimately give rise to the cerebellum.

We are grateful to Andy McMahon (*Wnt1*), Anthony Graham (*Wnt3a*), Esther Bell and Andrew Lumsden (*Hoxa2* and *Hoxb1*), Cairinne Logan (*En1* and *En2*) and Peter Gruss (*Pax2*) for providing clones used in this study. We thank Richard Wingate and Anthony Graham for allowing us to cite unpublished data and Richard Wingate, Huma Shamim, Esther Bell and Leah Toole for technical advice. Members of the lab are thanked for critical reading of the manuscript. This work was funded by the MRC, Human Frontier Science Program and The Wellcome Trust.

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