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The homeoprotein *Xiro1* is required for midbrain-hindbrain boundary formation

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SUMMARY

The isthmic organizer, which patterns the anterior hindbrain and midbrain, is one of the most studied secondary organizers. In recent years, new insights have been reported on the molecular nature of its morphogenetic activity. Studies in chick, mouse and zebrafish have converged to show that mutually repressive interactions between the homeoproteins encoded by Otx and Gbx genes position this organizer in the neural primordia.

We present evidence that equivalent, in addition to novel, interactions between these and other genes operate in *Xenopus* embryos to position the isthmic organizer. We made use of fusion proteins in which we combined Otx2 or Gbx2 homeodomains with the E1A activation domain or the EnR repressor element which were then injected into embryos. Our results show that Otx2 and Gbx2 are likely to be transcriptional repressors, and that these two proteins repress each other transcription. Our experiments show that the interaction between these two proteins is required for the positioning of the isthmic organizer genes *Fgf8*, *Pax2* and *En2*. In this study we also developed a novel in vitro assay for the study of the formation of this organizer. We show that conjugating animal caps previously injected with *Otx2* and *Gbx2* mRNAs recreate the interactions required for the induction of the isthmic organizer. We have used this assay to determine which cells produce and which cells receive the Fgf signal.

Finally, we have added a novel genetic element to this process, *Xiro1*, which encode another homeoprotein. We show that the *Xiro1* expression domain overlaps with territories expressing *Otx2*, *Gbx2* and *Fgf8*. By expressing wild-type or dominant negative forms of *Xiro1*, we show that this gene activates the expression of *Gbx2* in the hindbrain. In addition, *Xiro1* is required in the *Otx2* territory to allow cells within this region to respond to the signals produced by adjacent *Gbx2* cells. Moreover, *Xiro1* is absolutely required for *Fgf8* expression at the isthmic organizer. We discuss a model where *Xiro1* plays different roles in regulating the genetic cascade of interactions between *Otx2* and *Gbx2* that are necessary for the specification of the isthmic organizer.

Key words: *Xenopus*, Iroquois, Midbrain, Hindbrain, Isthmus organizer

INTRODUCTION

The developing vertebrate brain is subdivided into three main territories: the forebrain, the midbrain and the hindbrain. The forebrain contains two vesicles, the telencephalon and diencephalon, while the midbrain forms one vesicle, the mesencephalon (mes). The hindbrain or rhombencephalon is further subdivided into transverse domains called rhombomeres. The isthmus between midbrain and hindbrain and the two most anterior rhombomeres are called the metencephalon (met), from which the pons and cerebellum develop. During the past decade, several studies have shown that the isthmus acts as an organizing center that patterns adjacent territories (reviewed by Alvarado-Mallart, 1993; Joyner et al., 2000; Liu and Joyner, 2001; Martínez, 2001; Rhinn and Brand, 2001). Chick-quail isthmic transplantation experiments have shown that the isthmus can induce ectopic midbrain structures when transplanted to the posterior diencephalon and cerebellum structures, when transplanted to the rhombencephalon (Gardner and Barald, 1991; Marin and Puelles, 1994; Martínez and Alvarado-Mallart, 1990; Martínez et al., 1995; Martínez et al., 1991). A key molecule in mediating the patterning effects of the isthmus is the diffusible molecule fibroblast growth factor 8 (Fgf8). In both chick and mouse, Fgf8 can activate the expression of many other mes-met genes, and directs the formation of ectopic midbrain and anterior hindbrain structures in the caudal diencephalon and mesencephalon (Crossley et al., 1996; Liu et al., 1999; Martínez et al., 1999; Shamim et al., 1999). Genetic studies in mouse and fish support the requirement of Fgf8 for the correct patterning of territories adjacent to the isthmus (Brand et al., 1996; Meyers et al., 1998; Reifers et al., 1998). *Fgf8* is

expressed in the metencephalon that abuts the domain of expression of another diffusible molecule *Wnt1* in the mesencephalon. In addition, engrailed 1/engrailed 2 (*En1/En2*) and the paired homeobox genes *Pax2/Pax5* are expressed both in the midbrain and hindbrain territories and, as well as *Wnt1*, are required for the correct midbrain and cerebellum development (reviewed by Joyner et al., 2000; Liu and Joyner, 2001; Martínez, 2001). *Otx1/2* and *Gbx2*, genes that encode homeoproteins, are essential for the positioning and maintenance of the isthmus organizer as well as for midbrain and cerebellum development. These are the earliest expressed genes in the prospective midbrain-hindbrain organizer territory with restricted expression domains. At early gastrula the *Otx1/Otx2* genes are expressed in the anterior neuroectoderm abutting the *Gbx2* expression domain at the prospective midbrain-hindbrain boundary (Simeone et al., 1992a; Simeone et al., 1992b; Wassarman et al., 1997). Their complementary expression domains suggest mutual repression. Gain- and loss-of-function mutations have confirmed this hypothesis and shows their requirement for midbrain and cerebellum development (Acampora et al., 1998; Broccoli et al., 1999; Katahira et al., 2000; Millet et al., 1999; Rhinn et al., 1998; Wassarman et al., 1997). However, a recent study by Garda et al. (Garda et al., 2001), has shown that the initial expression domains of *Otx2* and *Gbx2* do not come into contact but are instead separated by a gap of *Otx2*- and *Gbx2*-negative cells. Soon after, the expression domains of these two genes overlap, and *Fgf8* is first detected within this overlapping territory. Fgf8 then overactivates *Gbx2*, causing *Otx2* repression and the generation of a sharp boundary between *Otx2* and *Gbx2*. This sharp boundary maintains *Fgf8* expression that continues to act positively on *Gbx2* and negatively on *Otx2*. Fgf8 also activates other midbrain-hindbrain genes whose domains of expression are later refined by a complex crossregulation mechanism (Garda et al., 2001; Wurst and Bally-Cuif, 2001). In addition, other factors such as the *Hes1*, *Hes3* and *Her5* also participate in the establishment of this border (Müller et al., 1996; Hirata et al., 2001).

The iroquois (Iro) genes belong to the TALE class of homeobox-encoding proteins (Bürglin, 1997). As their discovery as prepattern factors required for proneural and provein gene activation (Gómez-Skarmeta and Modolell, 1996; Leyns et al., 1996), they have been shown to participate in many developmental processes (reviewed by Cavodeassi et al., 2001). Both *Drosophila* and vertebrates Iro genes, have an early functional requirement for the specification of large territories, and a late function necessary for the subdivision of these territories into more restricted domains (reviewed by Cavodeassi et al., 2001). Thus, in *Drosophila* the Iro genes are required for the formation of the dorsal eye, head and mesothorax (Cavodeassi et al., 2000; Diez del Corral et al., 1999). In *Xenopus laevis* they participate in the specification of the Spemann organizer (Glavic et al., 2001) and the neuroectoderm (Gómez-Skarmeta et al., 2001). Later during development, the Iro genes help pattern the *Drosophila* imaginal discs and vertebrate neuroectoderm and heart (Bao et al., 1999; Bellefroid et al., 1998; Bruneau et al., 2001; Cavodeassi et al., 1999; Christoffels et al., 2000a; Gómez-Skarmeta et al., 1998; Gómez-Skarmeta and Modolell, 1996; Kehl et al., 1998; Leyns et al., 1996). In *Drosophila*, the Iro genes have been shown to be essential for the formation of several organizer centers in both the eye and wing imaginal discs (Cavodeassi et al., 1999; Diez del Corral, 1999; Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998). Although most of the vertebrate Iro genes have restricted patterns of expression in the midbrain-hindbrain boundary, their functions in the formation of this organizer center have not been explored (Bellefroid et al., 1998; Bosse et al., 2000; Bosse et al., 1997; Bruneau et al., 2001; Christoffels et al., 2000b; Cohen et al., 2000; Gómez-Skarmeta et al., 1998; Goriely et al., 1999; Peters et al., 2000; Tan et al., 1999).

In this work, we have examined whether *Gbx2* and *Otx2* function as activators or repressors in midbrain-hindbrain boundary formation in *Xenopus*. In addition, we have used conjugates of injected animal caps to recreate the isthmus organizer in vitro. This and other assays allowed us to explore how the *Xenopus* Iro gene, *Xiro1*, participates in the formation of this organizer.

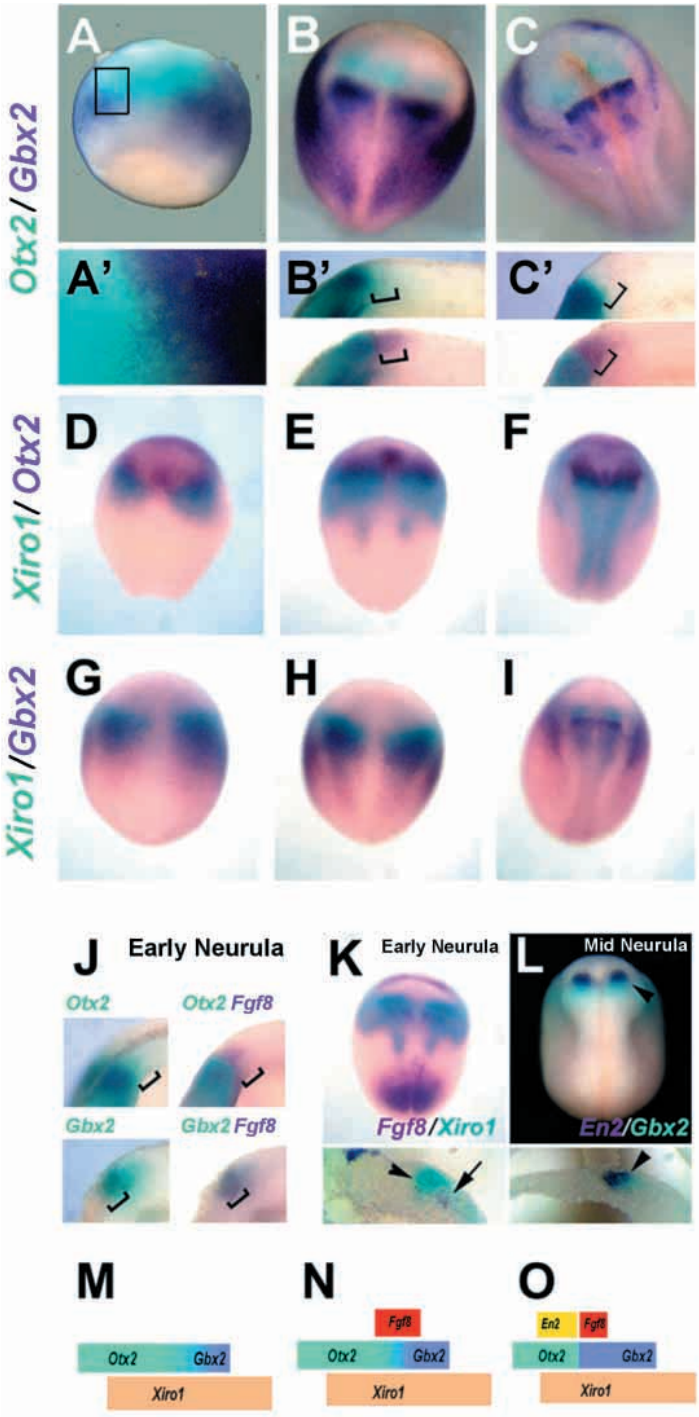
MATERIALS AND METHODS

Plasmid constructions, *in vitro* RNA synthesis and microinjection of mRNAs

The *Otx2* and *Gbx2* homeodomain coding regions were amplified using the following primers 5′-ATGCCGTGAATTCGCT-CAGCC-3′/5′-CACTCTCGAGGCTCACTTCCC-3′ and 5′-ACCTG-GACTAGAATTCAGATGAC-3′/5′-TTGCTTGCTCGAGCTGCTGG-3′ respectively. *Eco*RI and *Xho*I sites (underlined) were used to fuse them to the *engrailed* repressor domain (EnR) or the E1A transactivator domain in the pCS2-MT-NLS-EnR and pCS2-MT-NLS-E1A plasmids (donated by N. Papalopulu). The fragments generated were digested with *Eco*RI and *Xho*I restriction enzymes and cloned in pBS SKII and were subsequently sequenced. To obtain the E1A fusion proteins the pCS2-MT-NLS-E1A vector and the homeodomain fragments were double digested with *Eco*RI and *Xho*I restriction enzymes and then ligated together. The EnR fusion constructs were generated by exchanging the E1A domain, excised with *Xho*I and *Kpn*I, from the pCS2-MT-NLS-*Otx*-E1A or pCS2-MT-NLS-*Gbx2*-E1A with the EnR-coding sequence, excised with the same enzymes, from the pCS2-MT-NLS-EnR vector. *Xiro1* constructs are described elsewhere (Gómez-Skarmeta et al., 2001). All cDNAs were linearized and transcribed, as described by Harland and Weintraub (Harland and Weintraub, 1985) with GTP cap analog (New England Biolabs). SP6, T3 or T7 RNA polymerases were used. After DNAse treatment, RNA was extracted using phenol-chloroform, column purified and precipitated with ethanol. For injections, mRNAs were resuspended in DEPC-water and injected using 8-12 nl needles in two-cell stage embryos.

Whole-mount in situ hybridization, X-Gal, Myc staining and histology

Antisense RNA probes for *Xiro-1* (Gómez-Skarmeta et al., 1998), *Gbx2* (von Bubnoff et al., 1995), *Otx2* (Blitz and Cho, 1995), *Pax2* (Heller and Brändli, 1997), *En2* (Hemmati-Brivanlou et al., 1991), *Fgf8* (Christen and Slack, 1997), *Wnt1* (Wolda et al., 1993), were synthesized from cDNAs using digoxigenin or fluorescein (Boehringer Mannheim) as a label. Specimens were prepared, hybridized and stained using the method of Harland (Harland, 1991). NBT/BCIP or BCIP alone were used as substrate for alkaline phosphatase. X-Gal staining was performed according to Coffman et al. (Coffman et al., 1993). Antibody staining was performed after in situ hybridization of the embryos using anti Myc mouse monoclonal antibodies from BabCo, and according to the method described by Turner and Weintraub (Turner and Weintraub, 1994). Histology was performed as described by Mayor et al. (Mayor et al., 2000).



Embryos, micromanipulation and dexamethasone treatments

Xenopus embryos were obtained as described previously (Gómez-Skarmeta et al., 1998) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Dissections and conjugates were performed as described by Mancilla and Mayor (Mancilla and Mayor, 1996). Dexamethasone treatment was performed as described by Kolm and Sive (Kolm and Sive, 1995). Dexamethasone was included in the culture medium at stage 9.5-10 or 12-12.5 and maintained until the embryos were fixed.

RESULTS

Xiro1 is co-expressed with *Otx2* and *Gbx2* in *Xenopus* embryos

The expression patterns of *Otx2*, *Gbx2* and *Xiro1* were examined by whole-mount double in situ hybridization to address the possible role of each gene in isthmus development. As described previously, *Otx2* expression is restricted during gastrulation to the anterior region of the embryo (Blitz and Cho, 1995). By the end of gastrulation, *Otx2* is located in the anterior neural plate including the presumptive forebrain and midbrain territories. At this time, *Gbx2* begins to be expressed (von Bubnoff et al., 1995) in two patches within the neural tissue, which overlap in the most anterior region with the *Otx2*-expressing cells (Fig. 1A,A'). At mid neurula stage, *Otx2* and *Gbx2* expression domains begin to separate (Fig. 1B). Still, a faint graded *Otx2* expression is detected in sections which overlap with the *Gbx2* expression domain (Fig. 1B'). Finally, the faint graded *Otx2* expression becomes narrower by the late neurula stage and the boundary between *Gbx2* and *Otx2* expression domains becomes sharp (Fig. 1C,C'). *Xiro1* is co-expressed with both *Otx2* and *Gbx2* during the earliest stages analyzed (Fig. 1D,G). The co-expression territory of *Xiro1* and *Otx2* corresponds to the presumptive midbrain territory. This overlap between the anterior region of *Xiro1* expression and the caudal expression of *Otx2* is maintained and refined

Fig. 1. Comparison between *Otx2*, *Gbx2* and *Xiro1* expression. Embryos were fixed at late gastrula (stage 12-12.5) (A,A',D,G), early neurula (stage 13-14) (B,B',E,H,J,K) and mid neurula (stage 17-18) (C,C',F,I,L), and double in situ hybridization and sectioning were carried out for each pair of genes. The whole mounts are dorsal views oriented with anterior to the top and the sections and inset are oriented with anterior to the left. (A-C) *Otx2* (green) and *Gbx2* (purple) are expressed in complementary domains that overlap in the isthmus region. (A') Higher magnification of the square shown in A. Notice the overlapping expression of both genes. (B',C') Upper panels show a sagittal section of an embryo after the first chromogenic reaction for *Otx2* detection (green). Lower panels show the same embryo after the second chromogenic reaction for *Gbx2* detection (purple). Notice the overlap in the expression of both genes at the early neurula stage (bracket in B'), which disappears at the mid neurula stage (bracket in C'), to generate a sharp boundary of *Otx2*/*Gbx2* expression. (D-F) *Otx2* (purple) and *Xiro1* (light blue) overlap at the presumptive midbrain domain. (G-I) *Gbx2* expression (purple) is almost completely included in *Xiro1* (light blue)-expressing territory. (J) Position of *Fgf8* expression. The initial isthmus expression of *Fgf8* appears at early neurula stage in the region where *Otx2* and *Gbx2* are co-expressed (brackets). This early expression precedes the establishment of the sharp border described for *Otx2* and *Gbx2*. Images were taken from the same embryos after the first gene detection (right panels, green for *Otx2* and *Gbx2*) and at after the second chromogenic reaction (left panels, purple for *Fgf8*). (K) Double in situ hybridization for *Fgf8* (purple) and *Xiro1* (green) mRNAs. The *Fgf8* isthmus expression is included in the *Xiro1*-positive cells at this stage (arrow). Arrowhead points the anterior limit of *Xiro1*. (L) Double staining for *En2* (purple) and *Gbx2* (green). *En2* is expressed mainly in the *Otx2* domain with a faint graded co-expression with *Gbx2* at stage 17 (arrowhead). (M-O) The expression patterns observed by whole-mount in situ hybridization during the three stages described above. The positions of *Fgf8* and *En2* expression are also shown. Note the refinement in the *Otx2*-*Gbx2* overlapping region and the co-expression domains of *Xiro1*, *Otx2* and *Gbx2*.

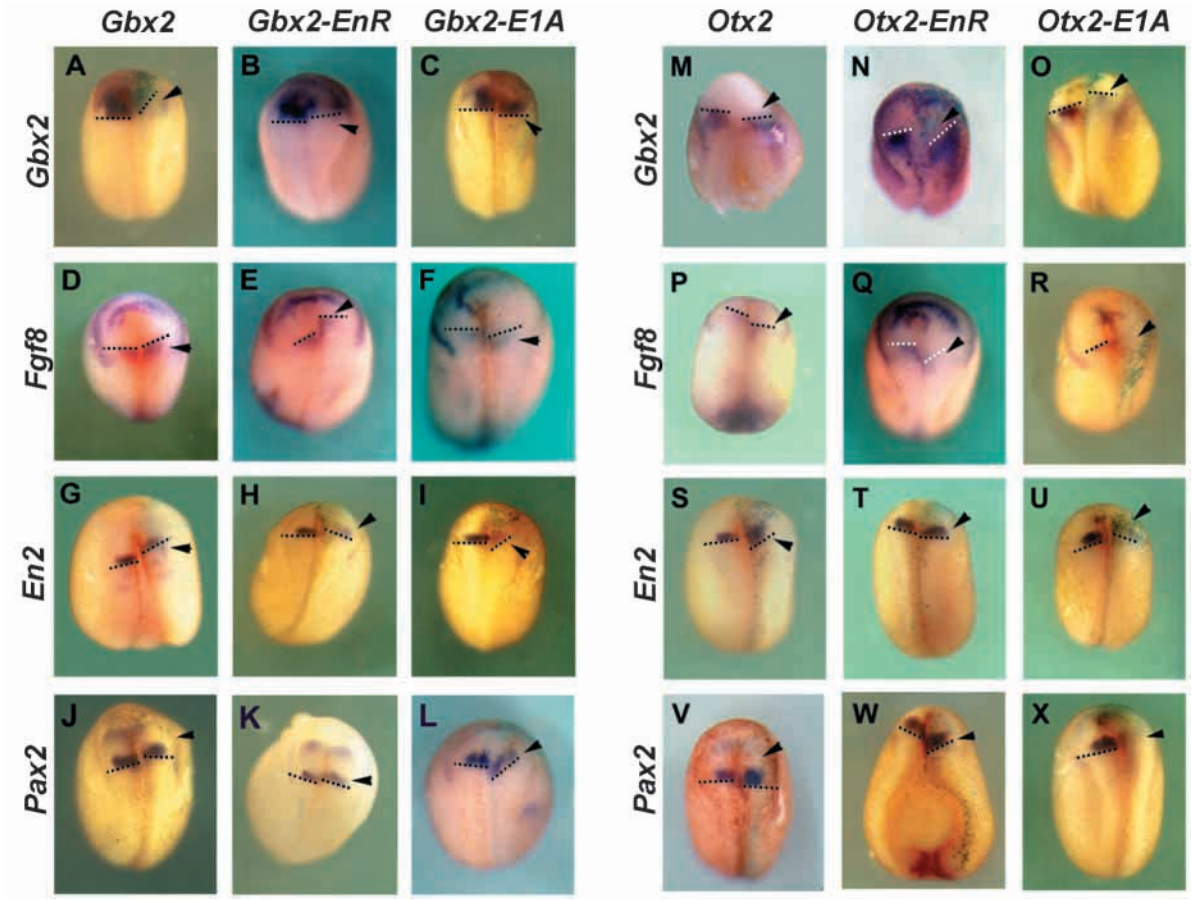


Fig. 2. *Otx2* and *Gbx2* participate as transcriptional repressors in the positioning of the isthmus organizer. Embryos were injected in one blastomere of two-cell stage embryos with 2 ng of *Gbx2* (A,D,G,J), 2 ng of the *Gbx2* repressor fusion (*Gbx-EnR*) (B,E,H,K) or 0.3 ng *Gbx2* activator fusion (*Gbx2-E1A*) (C,F,I,L) mRNAs. The expression of *Otx2*, *Fgf8*, *En2* and *Pax2* were analyzed at stage 17 and the injected sides were detected by X-Gal stain. (A-C) *Otx2* expression is inhibited in embryos injected with *Gbx2* or *Gbx2-EnR* mRNAs (A,B, broken lines), while is displaced caudally in those injected with *Gbx2-E1A* mRNA (C, broken lines). (D-F) A rostral shift of *Fgf8* isthmic expression territory is observed upon *Gbx2* or *Gbx2-EnR* overexpression (D,E, broken lines), and inhibition and caudal shift of this expression domain occurs in *Gbx2-E1A*-injected embryos (F, arrowhead). (G-I) *En2* is displaced anteriorly in *Gbx2*- or *Gbx2-EnR*-injected embryos (G,H, broken lines), while is repressed and shift caudally in those injected with *Gbx2-E1A* mRNA (I, broken lines). (J-L) *Pax2* expression is displaced rostrally in embryos injected with *Gbx2* or *Gbx2-EnR* mRNAs (J,K, broken lines), while a caudal shift occurs in *Gbx2-E1A*-injected embryos (L, broken lines). The injection at the two-cell stage of 5 ng of *Otx2* (M,P,S,V), 2 ng of *Otx2-EnR* (N,Q,T,W) or 1 ng of *Otx2-E1A* (O,R,U,X). The expression of *Gbx2*, *Fgf8*, *En2* and *Pax2* were analyzed at stage 17. (M-O) Overexpression of *Otx2* or *Otx2-EnR* mRNAs produce repression and caudal shift of *Gbx2* (broken lines) and injection of *Otx2-E1A* mRNA caused an anterior shift and diffusion of *Gbx2* (O, broken lines). (P-R) *Fgf8* is shifted posteriorly in embryos injected with *Otx2* or *Otx2-EnR* mRNAs (P,Q, broken lines) while injection of *Otx2-E1A* mRNA causes inhibition of the isthmus expression of *Fgf8* (R, arrowhead). (S-U) *En2* is shifted caudally in *Otx2* and *Otx2-EnR* injected embryos (S,T, broken lines), while there is a decrease in *En2* expression with an anterior displacement in embryos injected with *Otx2-E1A* mRNA (U, broken lines). (V-X) *Pax2* is shifted caudally in *Otx2*- and *Otx2-EnR*-injected embryos (V,W broken lines), while its expression decrease in the *Otx2-E1A*-injected embryos (X, arrowhead). Arrowheads point to the injected sides. Each experiment was performed at least twice with a minimum of 30 embryos. The percentage of effect for each experiment was ~ 70%.

during development (Fig. 1E,F) and it corresponds to the region where *En2* is expressed (Gómez-Skarmeta et al., 1998). *En2* is expressed mainly in the posterior midbrain and overlaps a small region of the *Gbx2* expression domain (Fig. 1L,O). The *Xiro1-Gbx2* early co-expression domain is broader than the region shared by *Xiro1* and *Otx2* and seems to be larger than the presumptive rhombomere one territory (Fig. 1D,G,M). Later on, during neurulation, expression patterns of *Gbx2* and *Xiro1* change drastically, maintaining their colocalization in part of the spinal chord and in rhombomere one (Fig. 1H,I,N,O).

At the gastrula stage, a clear intermingled population of cells expressing *Otx2* and *Gbx2* can be observed (Fig. 1A',M). It is important to note that at the early neurula stage, the time that *Fgf8* begins to be expressed (Fig. 1J,K,N), a faint overlap between the *Otx2* and *Gbx2* territories exists (Fig. 1B,N). The early *Fgf8*-expressing domain within the neural plate overlaps the faint *Otx2*-expressing region, within the *Gbx2* territory (Fig. 1J,N).

Xiro1 encompasses the *Fgf8*-expressing domain (Fig. 1K,N) and as mentioned before, the *Otx2* and *Gbx2*-expressing domains.

Otx2 and *Gbx2* participate as repressors in positioning the isthmus

In the mouse, these homeoproteins have been implicated in the

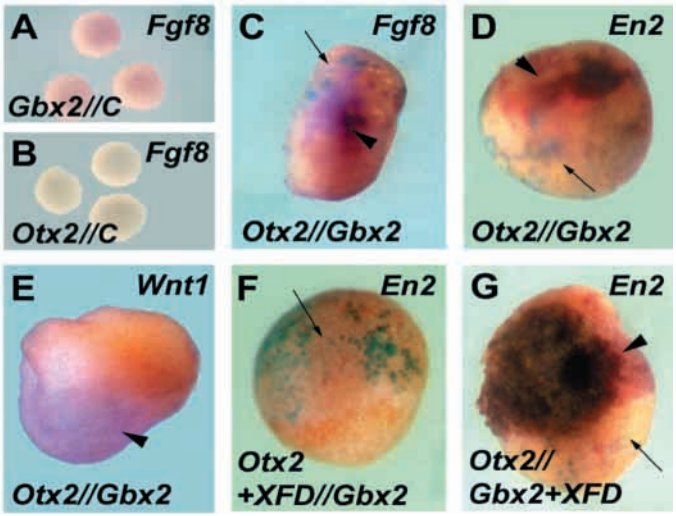


Fig. 3. The interaction between *Otx2* and *Gbx2* induce the isthmus. Embryos were injected with different mRNAs (5 ng of *Otx2*, 2 ng of *Gbx2*, 0.3 ng of β-galactosidase, 1 ng of *XFD*) at the one-cell stage. Animal caps were dissected at stage 10 and cultured as conjugates. (A,B) No *Fgf8* expression was detected in conjugates of control uninjected animal caps with *Gbx2*-injected ones (A, 0%, *n*=20) or with *Otx2*-expressing caps (B, 0%, *n*=23) at stage 17. (C-E) Conjugates of *Otx2*- with *Gbx2*-expressing caps performed at stage 10 can induce *Fgf8* (C; 69%, *n*=45), *En2* (D; 93%, *n*=109) and *Wnt1* (E; 65%, *n*=17) (arrowheads) at stage 17. β-Galactosidase (arrow) was co-injected with *Otx2* (C) or with *Gbx2* (D). *Fgf8* was induced in the *Gbx2* injected cap and *En2* in the *Otx2*-expressing cap as shown by the X-Gal staining. (F) Conjugate of *Otx2+XFD*- and *Gbx2*-expressing caps. *En2* induction was blocked when *XFD* was co-expressed with *Otx2* (arrow in F shows X-Gal staining in the *Otx2+XFD* animal cap, 22% of expression, *n*=37). (G) Conjugate of *Otx2*- and *Gbx2+XFD*-expressing caps. *XFD* co-injected with *Gbx2* did not block the induction of *En2* (arrowhead, 95% of expression, *n*=47). Arrow in G shows X-Gal in the *Gbx2+XFD* cap.

positioning of the isthmus. It has been postulated that they antagonize the transcription of each other and in this manner, generate the sharp border between *Otx2* and *Gbx2* expression territories, thus defining the position of the *Fgf8*-expressing domain (Millet et al., 1999; Broccoli et al., 1999; Katahira et al., 2000). To examine if they have similar functions in *Xenopus* midbrain-hindbrain boundary formation, and whether they act as activators or repressor, we fused their homeodomains with activator (E1A) and repressor (EnR) domains and compared the effects of overexpressing the corresponding mRNAs (*Gbx2-E1A*, *Gbx2-EnR*, *Otx2-E1A* and *Otx2-EnR*) with that caused by the wild-type *Gbx2* and *Otx2* mRNAs counterparts injections. Embryos were injected with the corresponding mRNA in one blastomere at the two-cell stage together with β-galactosidase mRNA, fixed at neurula stages, and analyzed for the expression of *Otx2*, *Gbx2*, *Fgf8*, *En2* and *Pax2*. Figure 2 shows that overexpression of *Gbx2* or *Gbx2-EnR* mRNAs shifts the expression of *Otx2* to more anterior positions or inhibits its expression (Fig. 2A,B), whereas the opposite effect was observed in *Gbx2-E1A*-injected embryos (Fig. 2C). The new limit created by the overexpression of *Gbx2* or its repressor construct repositioned *Fgf8* expression towards a more anterior position (Fig. 2D,E). This anterior shift was also observed in the cases of *En2* and *Pax2* expressions (Fig. 2G,H,J,K). By contrast, injection of *Gbx2-E1A* mRNA produced a posterior diffusion and expansion of *Fgf8* expression (Fig. 2F), similar to that observed on *En2* and *Pax2* expressions (Fig. 2I,L). This indicates that *Gbx2* acts as a repressor and that the activator fusion constructs interfere with *Gbx2* function.

Otx2 participates as a transcriptional repressor in the positioning of the isthmus organizer as defined by the effect observed for the injection of the wild-type transcript and the repressor construct. Thus, in embryos injected with *Otx2* or *Otx2-EnR* mRNAs, *Gbx2* is repressed and shifted posteriorly (Fig. 2M,N). *Pax2* and *En2* moved in accordance caudally (Fig. 2S,T,V,W), while *Fgf8* was shifted posteriorly and sometimes disappeared from the injected side in embryos injected with the wild type or repressor construct (Fig. 2P,Q). Conversely, *Otx2-E1A* expanded *Gbx2* into the forebrain region (Fig. 2O) and decreased its expression. *Fgf8*, *En2* and *Pax2* were inhibited or diffused and shifted anteriorly (Fig. 2R,U,X). Thus, *Otx2* and *Gbx2* work as transcriptional repressors and they repress each other.

The interaction between *Otx2* and *Gbx2* expressing cells is enough to induce the isthmus organizer

Data from chick experiments have shown that tissue from rhombomere 1 or tissue electroporated with a *Gbx2*-expressing vector induces an ectopic isthmus when transplanted into the *Otx2* expression domain (Marin and Puelles, 1994; Katahira et al., 2000). We analyzed whether the interaction between cells over expressing *Otx2* and *Gbx2* was enough for the induction of markers of the isthmus. Embryos were injected with *Otx2* or *Gbx2* mRNAs at the one-cell stage. At stage 10, their animal caps were explanted. When *Otx2*- or *Gbx2*-injected caps were conjugated with control uninjected animal caps, no isthimc markers were induced (Fig. 3A,B for *Fgf8*, expression data for *En2* and *Wnt1* not shown). However, when caps expressing *Otx2* were conjugated with those expressing *Gbx2*, the expression of *Fgf8*, *En2* and *Wnt1* was observed (Fig. 3C,D,E). In Fig. 3C, the *Otx2*-expressing cap was co-injected with β-galactosidase mRNA as a lineage tracer, which allowed us to conclude that *Fgf8* expression appeared in the *Gbx2* cap. In Fig. 3D, the *Gbx2*-expressing cap was co-injected with β-galactosidase mRNA; therefore, the expression of *En2* occurred within the *Otx2* cap. We have used this in vitro assay to determine whether FGF signal pathway is strictly required in the *Otx2*-expressing tissue for *En2* activation, or whether it is necessary in the *Gbx2* region for activation of a relay signal that promotes *En2* activation in the adjacent *Otx2*-expressing territory. For that, we co-expressed *Otx2* or *Gbx2* with a dominant negative form of the FGF receptor (XFD), conjugated these caps with caps expressing *Gbx2* or *Otx2*, respectively, and analyzed their ability to express *En2*. Fig. 3F,G show that *En2* is completely inhibited when FGF signaling is impaired in the *Otx2* territory, but is not affected when this pathway is blocked in the *Gbx2* region. This indicates that the induction of *En2* is promoted by the activation of the FGF signal pathway in the *Otx2*-positive cells, probably caused by the FGF8 molecules produced in the *Gbx2* cap.

Xiro1 participates in positioning the isthmus organizer

In *Xenopus*, *Xiro1* expression precedes that of *Gbx2*, which

appears within the *Xiro1* expression domain, and overlaps with the *Otx2*-midbrain expressing territory. This prompted us to examine whether *Xiro1* participates in the midbrain-hindbrain boundary formation. To that end, we analyzed the effect of overexpressing *Xiro1* mRNA and its derivatives over the midbrain-hindbrain boundary at early neurula, when the isthmus begins to be established (Fig. 5), and at mid neurula (Fig. 4), when the midbrain-hindbrain boundary has been refined and reached its final configuration. Injection of *Xiro1* mRNA increased the expression of *Gbx2* and displaced its rostral limit posteriorly (Fig. 4, Fig. 5B). Accordingly, the midbrain expression domain of *Otx2*, shifted to a more caudal position (Fig. 4D, Fig. 5A). In addition, at the stages analyzed *Pax2* was expanded and displaced caudally in embryos injected with *Xiro1* mRNA (Fig. 4J, Fig. 5C). A posterior displacement was also observed for *Fgf8* expression (Fig. 4G). This indicates that *Xiro1* could participate at the initial events during isthmus establishment through the activation of *Gbx2*, but also may modulate *Otx2* and *Pax2* expression.

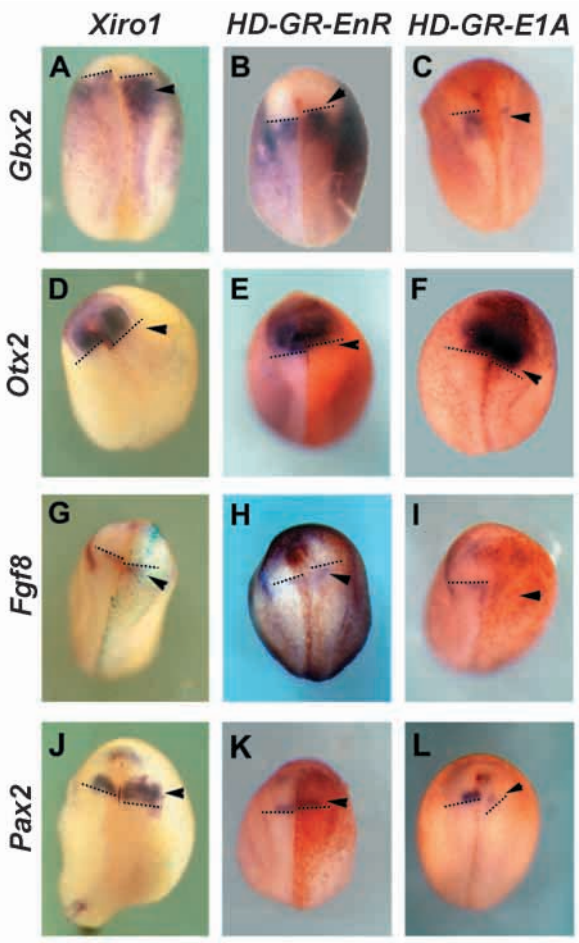


Fig. 4. *Xiro1* participates in the positioning of the isthmus organizer. Embryos were injected in one blastomere at the two-cell stage with 2 ng of *Xiro1* mRNA (A,D,G,J), 0.5 ng of *HD-GR-EnR* (B,E,H,K) or *HD-GR-E1A* (C,F,I,L); the inducible constructs were induced around stage 12.5. The injected side is marked by X-Gal stain in the *Xiro1*-injected embryos and by Myc staining in the case of the inducible constructs. (A) *Xiro1* overexpression promotes an expansion and caudal shift of *Gbx2*. (B) *HD-GR-EnR* mRNA injection causes expansion and anterior shift of *Gbx2* expression. (C) *Gbx2* is repressed in embryos injected with *HD-GR-E1A* mRNA. (D) In embryos injected with *Xiro1* mRNA *Otx2* midbrain expression domain is expanded caudally. (E) However, injection of *HD-GR-EnR* mRNA caused an anterior shift of the *Otx2* expression domain. (F) A caudal expansion of *Otx2* when *HD-GR-E1A* mRNA is overexpressed. (G) *Fgf8* expression is displaced posteriorly in embryos injected with *Xiro1* mRNA. (H) Overexpression of *HD-GR-EnR* promotes an expansion and anterior shift of the isthmus domain of *Fgf8*. (I) This domain is repressed in *HD-GR-E1A*-injected embryos. (J) In embryos injected with *Xiro1* mRNA, *Pax2* is expanded. (K) *HD-GR-EnR* mRNA injection causes an anterior shift of *Pax2* expression. (L) *Pax2* is repressed and shifted caudally in embryos injected with *HD-GR-E1A* mRNA. Broken lines show the described effects. Arrowheads indicate the injected sides. Each experiment was performed at least twice with a minimum of 45 embryos. The percentage of effect for each experiment was ~70%.

Previous studies have implicated *Xiro1* in the repression of *Bmp4* expression in the neural plate and dorsal mesoderm during gastrulation (Glavic et al., 2001; Gómez-Skarmeta et al., 2001). Thus, the effects of overexpressing *Xiro1* on *Gbx2* and *Otx2* may be an indirect consequence of mesoderm alteration earlier during development, which then affects neural plate patterning. To overcome these possible early effects, we used *Xiro1* inducible chimeras. Overexpression of *Xiro1* homeodomain fused to an inducible module and to a EnR repressor domain (*HD-GR-EnR*) has been shown to produce similar effects to that caused by overexpression of wild type *Xiro1* (Glavic et al., 2001; Gómez-Skarmeta et al., 2001). By contrast, overexpression of a similar fusion with no transcriptional module (*HD-GR*) or with an activator domain (*HD-GR-E1A*) interferes with *Xiro1* function (Glavic et al., 2001; Gómez-Skarmeta et al., 2001). These constructs allowed us to modify *Xiro1* function at different stages of development.

When the *HD-GR-EnR* fusion protein was induced at late gastrula stage in injected embryos *Gbx2* expression was increased but, in contrast to *Xiro1* injected embryos, its rostral limit was shifted anteriorly (Fig. 4B). Moreover, *Otx2* expression was displaced rostrally rather than expanded posteriorly (Fig. 4E) and the isthmus domain of *Fgf8* and *Pax2* expression was shifted anteriorly (Fig. 4H,K). In the case of *HD-GR-E1A* overexpression, the opposite effects were observed, that is, inhibition of *Gbx2* and posterior expansion of the *Otx2* expression domain (Fig. 4C,F). Notice that the inhibition of *Xiro1* function with *HD-GR-E1A* completely represses *Fgf8* (Fig. 4I) and decrease and shift posteriorly the expression of *Pax2* (Fig. 4L) and *En2* (not shown).

The different effects of *Xiro1* and *HD-GR-EnR* on the isthmus positioning could be a consequence of an early requirement of *Xiro1* for *Otx2* expression that is no longer observed when the inducible construct is activated at late gastrula or early neurula stages. Indeed, *Xiro1* is necessary for neural plate formation and activates *Otx2* in animal caps (Gómez-Skarmeta et al., 2001). To address this point more directly, *Xiro1* derivatives were activated at early gastrula stage (stage 10) or late gastrula (stage 12) and their effects were examined by the time when the initial *Fgf8* expression is detected (stage 14). Induction of *HD-GR-EnR* at stage 10 produced similar effects to that observed in *Xiro1* injected embryos, that is, *Otx2* expression was displaced caudally (Fig. 5D), *Gbx2* expression was expanded and its anterior limit was moved posteriorly (Fig. 5D,G). In addition, *Pax2* was shifted caudally in these embryos (Fig. 5J). Interference with *Xiro1* function at early gastrula by injecting *HD-GR-E1A* and *HD-GR* repressed *Otx2* (Fig. 5E,F), *Gbx2* (Fig.

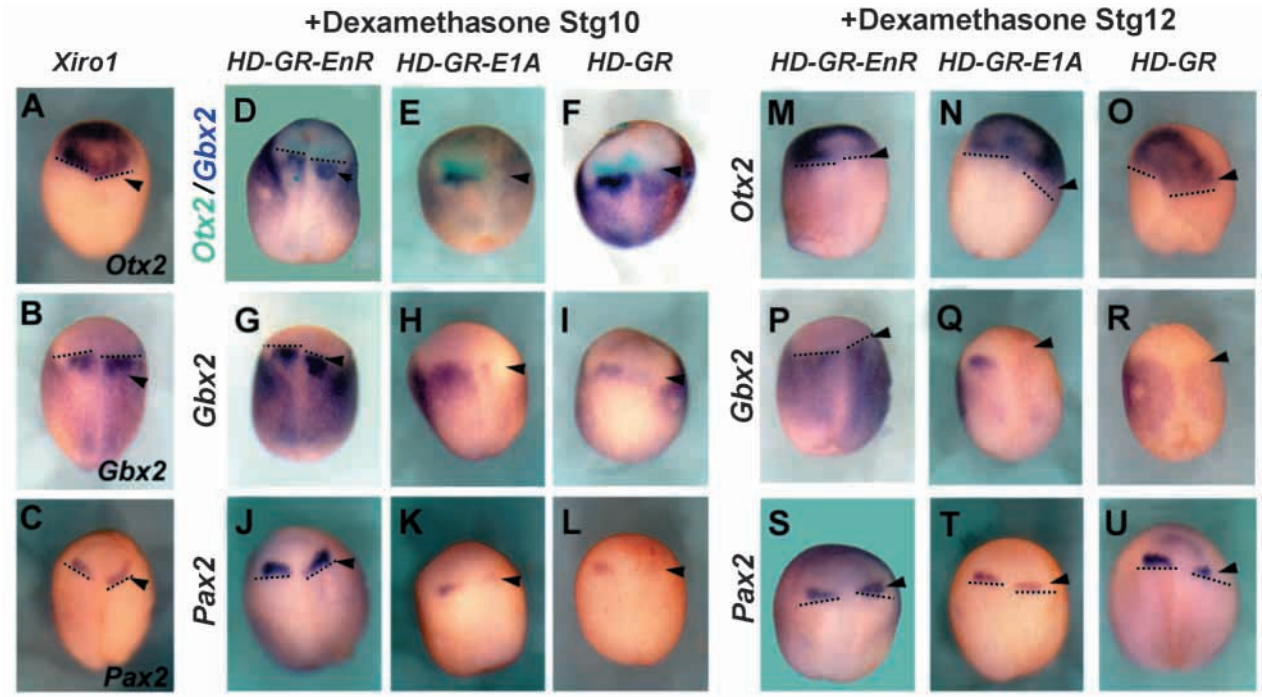


Fig. 5. *Xiro1* controls the expression of *Otx2* and *Gbx2* at different developmental stages. Embryos were injected in one blastomere at two-cell stage with 2 ng of *Xiro1* mRNA (A-C), 0.5 ng of *HD-GR-EnR* (D,G,J,M,P,S), 0.5 ng of *HD-GR-E1A* (E,H,K,N,Q,T) or 0.5 ng of *HD-GR* (F,I,L,O,R,U) and the expression of *Otx2*, *Gbx2* and *Pax2* were analyzed at early neurula stage (stage 14). Activation of the inducible constructs was achieved by adding dexamethasone at stage 9.5-10 (D-L) or at stage 12-12.5 (M-U). Embryos injected with *Xiro1* mRNA show a caudal expansion of *Otx2* (A, broken lines), expansion and caudal shift of *Gbx2* (B, broken lines), and *Pax2* is displaced caudally (C, broken lines). (D-I) *Otx2* (green) and *Gbx2* (purple) were expanded and shifted caudally in embryos injected with *HD-GR-EnR* mRNA (D,G, broken lines). *HD-GR-E1A* and *HD-GR* repressed *Otx2* and *Gbx2* expression when activated at stage 9.5-10 (E,H and F,I, arrowheads). A caudal shift of *Pax2* expression is observed in embryos injected with *HD-GR-EnR* when activated at stage 9.5-10 (J, broken lines). The injection of both *HD-GR-E1A* and *HD-GR* repress *Pax2* midbrain expression domain (K,L, arrowheads). (M-O) *Otx2* midbrain territory is inhibited and shifted rostrally in embryos injected with *HD-GR-EnR* mRNA (M, broken lines). A caudal expansion in *Otx2* expression is produced by *HD-GR-E1A* and *HD-GR* overexpression and activation at stage 12-12.5 (N,O, broken lines). (P-R) *Gbx2* expression is expanded anteriorly in embryos injected with *HD-GR-EnR* mRNA and activated at stage 12-12.5 (P, broken lines), while the injection of *HD-GR-E1A* and *HD-GR* mRNAs promote repression of *Gbx2* (Q,R, arrowheads). (S-U) Embryos injected with *HD-GR-EnR* and activated at stage 12-12.5 causes an anterior shift of *Pax2* expression (S, broken lines), while *HD-GR-E1A* and *HD-GR* produce repression and caudal displacement of *Pax2* expression when activated at stage 12-12.5 (T,U, broken lines). Arrowheads indicate the injected sides. Each experiment was performed at least twice with a minimum of 20 embryos. The percentage of effect for each experiment was ~70%.

5E,F,H,I) and *Pax2* expression (Fig. 5K,L). This is probably due to suppression of neural plate fate by early interference with Xiro1 function (Gómez-Skarmeta et al., 2001). At early neurula, and similar to what is observed at mid neurula (Fig. 4B,E,K), activation of *HD-GR-EnR* at late gastrula displaced the *Otx2* (Fig. 5M) and *Gbx2* (Fig. 5P) expression domains anteriorly. In addition, *Gbx2* expression is also expanded (Fig. 5P). Accordingly, *Pax2* expression shifted rostrally in these embryos (Fig. 5S). Conversely, activation *HD-GR-E1A* and *HD-GR* at stage 12, which do not affect neural plate formation (Gómez-Skarmeta et al., 2001), expanded *Otx2* expression (Fig. 5N,O), while *Gbx2* was decreased (Fig. 5Q,R). *Pax2* expression was inhibited and shifted posteriorly by these treatments (Fig. 5T,U).

These results suggest that *Xiro1* upregulates *Otx2* expression at early gastrula and *Gbx2* at early neurula. Thus, in *Xiro1*-injected embryos or in embryos in which *HD-GR-EnR* is activated at early gastrula, *Otx2* is ectopically expressed at a more caudal position. This causes posterior displacement of *Gbx2* and of the midbrain-hindbrain boundary. In addition, *Xiro1* has a positive effect of on *Gbx2*, which causes an expansion of *Gbx2* expression domain. By contrast, in embryos injected with *HD-GR-EnR* and induced at late gastrula, only *Gbx2* is activated. Gbx2 then represses *Otx2* and shifts the isthmus organizer anteriorly.

In order to define the specificity of the phenotypes described for the gain and loss of *Xiro1* function and to further define *Xiro1* transcriptional activity, we performed rescue experiments. As described above, dominant negative forms of *Xiro1* (*HD-GR-E1A* and *HD-GR*) inhibit *Gbx2* expression (Fig. 5H,I,Q,R). Co-injection with *Xiro1* rescued completely the *Gbx2* expression when the dominant negative was induced at the early or late gastrula stages (Fig. 6B,C and 6H,I respectively). The *Xiro1* dominant negatives (*HD-GR-E1A* and *HD-GR*) produced an inhibition or a caudal expansion of *Otx2*, depending whether they were induced at the early or late gastrula stage, respectively (Fig. 5E,F,N,O). Both phenotypes were rescued by co-injection with *Xiro1* (Fig. 6E,F,K,L). Co-injection of *HD-GR-EnR* and *Xiro1*, when hormone was added at early gastrula, caused *Gbx2* upregulation associated with a caudal displacement of *Gbx2* and *Otx2* (Fig. 6A,D). This effect is identical to that observed in *Xiro1*-injected embryos. When *HD-GR-EnR* was activated at late gastrula, *Gbx2* is upregulated but the isthmus position was

not altered (Fig. 6G,J). This indicates that the posterior displacement of the isthmus, which is caused by Xiro1-mediated activation of *Otx2* in early gastrula, is counteracted by the anterior displacement, because of *Gbx2* activation by *HD-GR-EnR* at early neurula. These data further support the fact that Xiro1 behaves as a transcriptional repressor capable of promoting the expression of *Otx2* at early gastrula and of *Gbx2* at late gastrula.

We next examined whether the effects of dominant negative forms of Xiro1 on *Otx2* (Fig. 7A) and *Fgf8* expression were consequence of the suppression of *Gbx2* expression in the injected embryos (Fig. 7C). Indeed, this was the case for the caudal limit of *Otx2*, as co-injection of *HD-GR* and *Gbx2* was sufficient to generate embryos with a normal *Otx2* expression pattern (Fig. 7A,B). Although the co-injection of *HD-GR* and *Gbx2* rescued the normal expression of *Otx2* it did not rescue *Fgf8* expression (Fig. 7D). We conclude, that *Xiro1* function is necessary for *Fgf8* induction independent of *Gbx2* and *Otx2* expression.

To clarify the epistatic relationships between the genes involved in the positioning of the isthmus organizer, we performed animal cap assays and the conjugate experiments described previously. In the embryo, *Otx2* and *Xiro1* expression domains overlap; thus, we tested whether *Otx2* was capable of inducing *Xiro1* expression. Indeed, *Otx2* overexpression activated *Xiro1* expression in animal caps (Fig. 8B). The ability of *Xiro1* to activate *Otx2* has been reported previously (Gómez-Skarmeta et al., 2001). *Gbx2* is initially expressed within the *Xiro1* territory and *Xiro1* overexpression induces *Gbx2* in the embryo. Thus, we asked whether *Xiro1* could also promote *Gbx2* expression in an animal cap assays where other signals presents in the embryo are absent. *Xiro1* activity effectively induced the expression of *Gbx2* in competent ectoderm, while *Gbx2* was unable to induce *Xiro1* expression (Fig. 8A,C). Next we analyzed the relationships between *Xiro1* and *Gbx2* using conjugate experiments. If *Xiro1* was able to promote *Gbx2* expression, then conjugates of *Otx2*-expressing cells and *Xiro1* expressing cells should produce the induction of *Fgf8* and *En2*. Fig. 8D,E show that this is indeed the case. The interaction between tissue expressing *Otx2* and tissue expressing *Xiro1* was enough to induce the isthmus organizer markers *Fgf8* and *En2* in the *Gbx2*- and *Otx2*-expressing caps, respectively.

Xiro1 and *Otx2* activate each other and the corresponding genes are co-expressed in the midbrain territory in which *En2* is activated. We have examined if *Xiro1* is required in the *Otx2* expression domain for *En2* expression. To that end, the inducible dominant negative form of *Xiro1* was co-injected with *Otx2*, the corresponding animal caps were conjugated with caps expressing *Gbx2* and the expression of *En2* was analyzed. Fig. 8F shows that *Xiro1* function is indispensable for the induction of *En2*.

DISCUSSION

Conserved mechanisms of positioning the isthmic organizer between chick/mouse and *Xenopus*: *Otx2* and *Gbx2* activities

In recent years, new insights have been reported by numerous

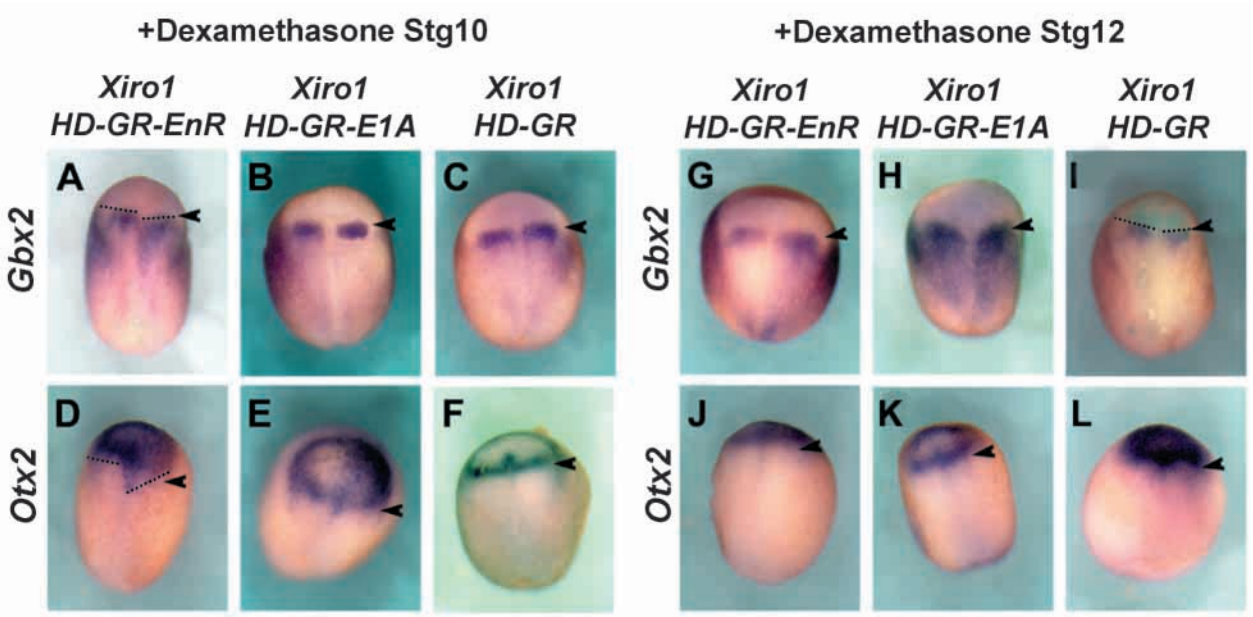


Fig. 6. Rescue experiments. Embryos were injected in one blastomere at the two-cell stage with *Xiro1* (1 ng) and *HD-GR-EnR* (0.5 ng) (A,D,G,J), or *HD-GR-E1A* (0.5 ng) (B,E,H,K), or *HD-GR* (0.5 ng) (C,F,I,L). The inducible constructs were activated around stage 10 (A-F) or 12.5 (G-L) and the expression of *Otx2* and *Gbx2* were analyzed at early neurula stage. Embryos injected with a mixture of *Xiro1* and *HD-GR-EnR* and activated around stage 10 show an expansion and caudal shift of *Gbx2* (A, broken lines, 90%, *n*=27) and a caudal expansion of *Otx2* midbrain expression domain (D, broken lines, 70%, *n*=23). The overexpression of *Xiro1* with *HD-GR-E1A* or with *HD-GR* and activation at stage 10 rescue the expression of both *Otx2* and *Gbx2* (B,E and C,F, respectively). The expression of *Otx2* and *Gbx2* is rescued in the embryos injected with mixtures of *Xiro1* with *HD-GR-EnR* (G,J), with *HD-GR-E1A* (H,K) or with *HD-GR* (I,L) activated at stage 12-12.5. Broken lines show the displacements of gene expression. Arrowheads indicate the injected sides. The percentage of rescue of normal expression for each experiment was ~75%.

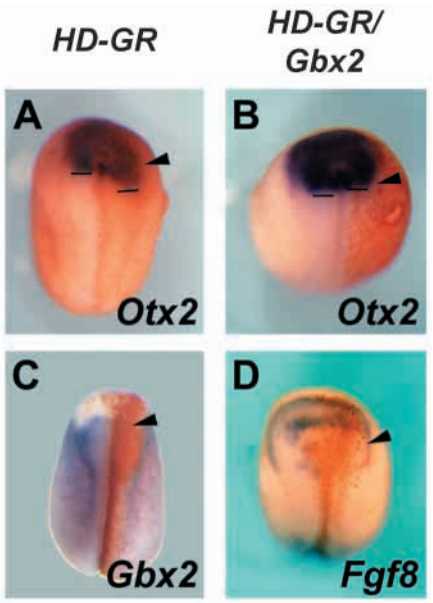


Fig. 7. *Gbx2* rescue *Otx2* but not *Fgf8* expression. Embryos were injected in one blastomere at the two cells stage with 0.5 ng of *HD-GR* (A,C), or with 0.5ng of *HD-GR* and 1 ng of *Gbx2* (B,D). The inducible constructs were induced around stage 12.5 and the injected side was detected by the Myc immunostaining. (A) Caudal expansion of the *Otx2* midbrain domain (black lines) in embryos injected with *HD-GR* mRNA. (B) A nearly normal *Otx2* expression is restored with the co-expression of *Gbx2* and *HD-GR* (black lines). (C) Injection of *HD-GR* produced a complete inhibition of *Gbx2*. (D) The co-injection of *HD-GR* and *Gbx2* did not rescue the expression of *Fgf8*, even though it produced a nearly normal *Otx2* expression. Arrowheads show the injected sides and point the effects described above. Each experiment was carried out at least twice with a minimum of 54 embryos. The percentage of effect (or rescue) for each experiment was ~70%.

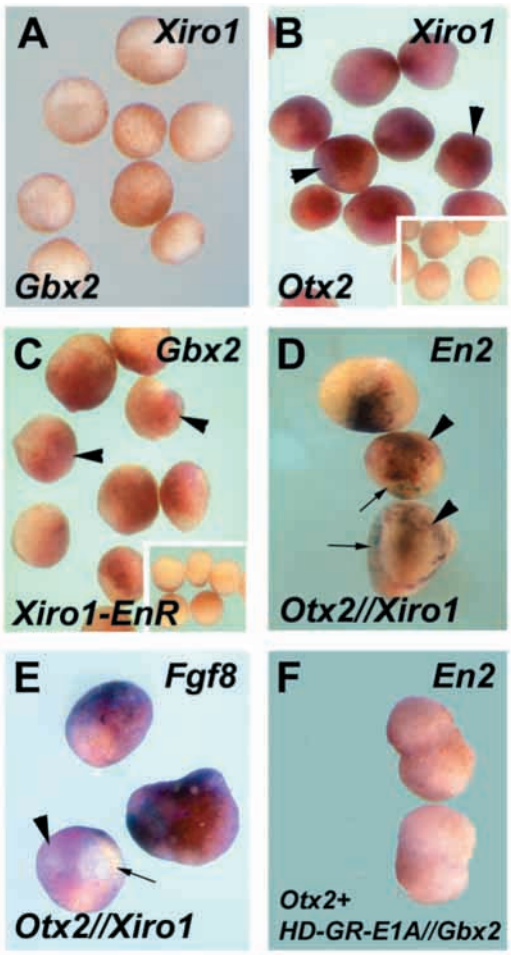


Fig. 8. Role of *Xiro1* on isthmic organizer in vitro. Embryos were injected at one-cell stage with the mRNAs described, the animal caps were explanted and conjugated at stage 10 and cultured until the equivalent of stage 17. At this stage the gene expression was assayed. (A) Injection of 2 ng of *Gbx2* mRNA do not induce *Xiro1* expression (0%, *n*=36). (B) In caps injected with 5 ng of *Otx2* mRNA, *Xiro1* expression is induced (arrowheads, 65%, *n*=23; inset shows uninjected animal caps). (C) Caps injected with 2 ng *Xiro1-EnR* mRNA express *Gbx2* (arrowheads, 57%, *n*=46; inset shows uninjected animal caps). (D) *Otx2*(5 ng)//*Xiro1*(2 ng) conjugates express *En2* (arrowheads, 90%, *n*=30) in the *Otx2* territory (arrow indicates the X-Gal stain in the *Xiro1*-expressing caps). (E) *Fgf8* also is induced in these conjugates (arrowhead, 71%, *n*=34, arrow shows the X-Gal stain in the *Xiro1* caps). (F) Interference with *Xiro1* function with *HD-GR-E1A* (0.5 ng) at stage 12 suppressed *En2* expression in the *Otx2* expressing cap (40%, *n*=33).

studies about the regulatory genetic mechanisms that underlie the specification of the isthmic organizer at the mid-hindbrain boundary (Broccoli et al., 1999; Liu et al., 1999; Martínez et al., 1999; Millet et al., 1999; Shamin et al., 1999) and the molecular nature of its morphogenetic activity (Crossley et al., 1996; Meyers et al., 1998; Reifers et al., 1998; Martínez et al., 1999; Shamin et al., 1999). Studies in chick, mouse and zebrafish have converged to show that mutually repressive interactions between homeodomain transcription factors of the Otx and Gbx class position this organizer in the neural primordia (Rhinn and Brand, 2001).

We have shown here that similar mechanisms are conserved in *Xenopus* and we have used the advantages of this system to further study this inductive process. We have analyzed the pattern of expression of *Otx2* and *Gbx2* genes from the gastrula until the neurula stages in *Xenopus* embryos. Our results show that at late gastrula, the posterior limits of *Otx2* overlaps with the anterior limits of *Gbx2*. At the early neurula, the expression domains of these genes start to separate although still a faint overlap is detected. It is at this stage when the expression of *Fgf8* is initiated in the overlapping region. A similar expression pattern was recently described for chick (Garda et al., 2001). Finally, at the mid neurula stage, the boundary between the *Gbx2* and *Otx2* expression domains becomes sharp and no overlap is detected.

We analyzed the transcriptional activity of *Otx2* and *Gbx2* by making fusion derivatives with activator or repressor domains (Friedman et al., 1988; Jaynes and O'Farrell, 1991). Our results indicate that *Otx2* and *Gbx2* are likely to be transcriptional repressors, as the same phenotype, assayed by the expression of several genes, is obtained when wild-type and repressor constructs are overexpressed, and the opposite effects are observed in embryos injected with the activator constructs. Thus, the injection of *Gbx2* or its repressor construct shifts the expression of *Otx2*, *Fgf8*, *Pax2* and *En2* towards a more anterior position. This is similar to that observed in a transgenic mouse embryo that expresses *Gbx2* under the *Wnt1* promoter (Millet et al., 1999), or by misexpression experiments in chick (Katahira et al., 2000) and zebrafish (Rhinn and Brand, 2001). By contrast, overexpression of *Otx2* or its repressor construct produces the same phenotype as that observed in mutant mouse embryos that express *Otx2* under the *En1* promoter (Broccoli

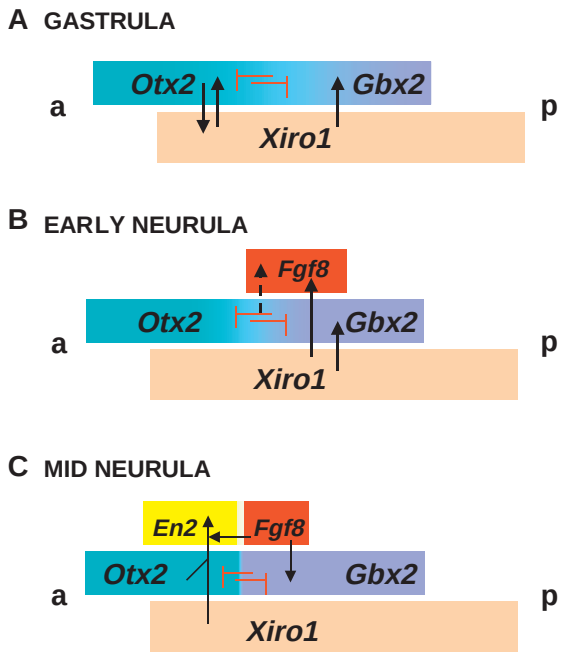


Fig. 9. A model for the induction and positioning of the isthmus organizer. (A) Gastrula. *Xiro1* encompasses *Gbx2* expressing domain and the presumptive midbrain territory of *Otx2* and participate in the activation of both genes (arrows). In addition, *Otx2* also activates *Xiro1* expression in the midbrain. At this stage *Otx2* and *Gbx2* expression domains overlap in the prospective isthmus and the mutual repressive activities between the corresponding proteins begin (red lines) (B) Early neurula. The expression domains of *Otx2* and *Gbx2* start to separate although a faint overlapping is still detected. At this stage, *Xiro1* is no longer able to activate *Otx2*. In addition, *Fgf8* expression, and therefore the establishment of the isthmus, begins as a result of the overlapping domain created by *Otx2* and *Gbx2* (broken arrows) and the activity of *Xiro1* in this region (arrow). (C) Mid neurula. A sharp boundary between *Otx2* and *Gbx2* arises, which is probably due to an equilibrium reached by their cross-inhibitory activities (red lines). The interaction between *Otx2* and *Gbx2* maintains *Fgf8*, which reinforces the expression of *Gbx2* in the caudal face of the isthmus (arrow). In addition, *Fgf8* induces *En2* expression in the competent territory defined by the co-expression of *Otx2* and *Xiro1*. a, anterior; p, posterior.

et al., 1999), or in the chick embryo where *Otx2* was ectopically expressed in the hindbrain (Katahira et al., 2000): a posterior shift of the isthmic organizer genes. It should be noted that in some injected embryos, the expression of *Gbx2*, *Fgf8*, *Pax2* or *En2* is almost completely absent. This observation could be explained by the existence of a limited competent region in which these genes can be expressed. In other vertebrates, graft transplantations and implantation experiments using FGF8 loaded beads have shown that such a competent region for isthmic organizer induction exists (Martínez et al., 1991; Bally-Cuif and Wassef, 1994; Marin and Puelles, 1994; Crossley et al., 1996; Martínez et al., 1999). It should be noted that the size of the midbrain, and in consequence the area of competence, in *Xenopus* embryos is much smaller than in chick or mouse, and the probability of being in the area of competence is therefore lower in *Xenopus*.

Taken together, these observations suggest that, as in other organisms, a mutual repression between Gbx2 and Otx2 occurs in *Xenopus*. This interaction defines the positioning of the limit of expression of these two transcription factors and the positioning of the isthmic organizer, as detected by the expression of *Fgf8*, *Wnt1*, *Pax2* and *En2*.

All previous experiments concerning the interaction between *Otx2* and *Gbx2* in the specification of the isthmic organizer have been carried out in whole animals, where the possibility of additional signals coming from different regions of the embryos have not been directly ruled out. We have found that conjugating animal caps expressing *Otx2* with animal caps expressing *Gbx2* is sufficient for the induction of isthmic markers such as *Fgf8*, *En2* and *Wnt1*. Interestingly, the expression of *Fgf8* is induced in the *Gbx2*-expressing cells, while the induction of *En2* is found in the *Otx2*-expressing cells, which is similar to the pattern observed in whole embryos. This novel in vitro assay for the induction of the isthmic organizer support previous observations in zebrafish and mouse. In mutants that lack notochord, the anterior-posterior polarity at the mid-hindbrain boundary is correctly specified, indicating that the induction of this border does not require signals from the axial mesoderm (Halpern et al., 1993; Talbot et al., 1995; Ang and Rossant, 1994; Weinstein et al., 1994). However, we cannot rule out the possibility that, in the embryo, other factors, in addition to *Otx2* and *Gbx2*, are required to induce some of the elements of the isthmic organizer. Indeed, supporting this possibility, in mouse there is some initial *En2* expression that is independent of the Otx-Gbx boundary (Acampora et al., 1997). Our results also suggest that a signal produced in the *Gbx2*-expressing cells, which is likely to be Fgf8, acts on the *Otx2*-expressing cells in order to induce *En2* and *Wnt1*. Thus, interference with Fgf signaling by overexpressing a dominant negative Fgf receptor (XFD) in the *Otx2* territory suppressed *En2* expression. Although there is evidence that XFD is able to block several members of the Fgf family of receptors (Amaya et al., 1991), the simplest interpretation of our results is that XFD is blocking the Fgf8 signal produced by the *Gbx2* cells. Indeed, it has been proposed that Fgf8 is the mediator of the organizing activity and is required for the maintaining of the expression of the isthmic markers (Reifers et al., 1998; Crossley et al., 1996; Heikinheimo et al., 1994). Our in vitro assay supports this idea and introduces a new in vitro assay system to analyze other signals involved in the induction of the isthmic organizer.

Role of *Xiro1* on the positioning of the isthmic organizer

Previous work has shown that *Xiro1* functions as a transcriptional repressor in the Spemann organizer and in the neural plate (Glavic et al., 2001; Gómez-Skarmeta et al., 2001). We show that *Xiro1* is required for the expression of several isthmic organizer genes, and in this process acts as a repressor. In addition, *Xiro1* can acts at different stages of development, regulating the expression of different genes and, as a consequence, the isthmus position.

Xiro1 is required for *Gbx2* expression

It is clear from our work that *Xiro1* expression precedes that of *Gbx2*, and that this gene is initially activated within the *Xiro1* domain. In embryos injected with *Xiro1* or an inducible repressor variant (*HD-GR-EnR*), *Gbx2* expression is expanded. By contrast, in embryos injected with an inducible dominant

negative form of *Xiro1* (*HD-GR*) or an inducible activator variant *(HD-GR-E1A*), *Gbx2* is downregulated. In addition, the expression of *Xiro1* in animal caps is enough to activate *Gbx2*. Taken together, these results strongly support the idea that *Xiro1* is required, as a repressor, for *Gbx2* expression in the isthmic organizer. Moreover, we have found that in embryos injected with *HD-GR-EnR*, activation of *Gbx2* expression was observed when dexamethasone was added at both early and late gastrula stages. This suggest that *Xiro*-mediated *Gbx2* activation occurs at late gastrula stage.

Xiro1 is required for *Otx2* expression

Xiro1 is co-expressed with *Otx2* in the midbrain (Gómez-Skarmeta et al., 1998) (this work). We have found a mutual positive regulation between these two genes. *Otx2* activates *Xiro1* in animal caps and *Xiro1* activates *Otx2* expression in whole embryos and in animal caps (this work) (Gómez-Skarmeta et al., 2001). *Otx2* activation was also observed in embryos injected with *HD-GR-EnR* and treated with Dex at early gastrula, but not when hormone was added at late gastrula. Moreover, interference with Xiro1 function with *HD-GR* or *HD-GR-E1A* downregulate *Otx2*. This indicates that *Xiro1* is required as a repressor for *Otx2* expression at early gastrula stage.

Xiro1 effects on isthmic positioning

The isthmic position is the result of the balance between Otx2 and Gbx2 mutual repression (Millet et al., 1999; Broccoli et al., 1999; Katahira et al., 2000). As *Xiro1* participates in the activation of both genes, it also help position the midbrain-hindbrain boundary. Overexpression of *Xiro1* cause, during gastrulation, ectopic activation of *Otx2* at more caudal positions. This promotes a posterior shift of the isthmic position, despite *Xiro1* also expanding *Gbx2* expression at neurula stage. This posterior displacement is also observed in embryos injected with *HD-GR-EnR* and treated with Dex at early gastrula, but not when hormone was added at late gastrula. In this late condition, *Xiro1* is not longer able to activate *Otx2*, but it can activates *Gbx2*, which displace the midbrain-hindbrain boundary anteriorly through *Otx2* downregulation.

We do not know how *Xiro1* could activate two different genes, *Otx2* and *Gbx2*, at different places and at different times. It may do so by acting in collaboration with other factors such as retinoic acid, Fgf or Wnt signaling, as they are involved in posteriorizing signals in the neural plate and in the expression of *Gbx2* (Gvalas and Krumlauf, 2000; Gamse and Sive, 2000).

Xiro1 is required for *Fgf8* expression

The effect of *Xiro1* on *Fgf8* expression is not completely explained by its effect on *Otx2* and *Gbx2*. Injection of *Xiro1* and *HD-GR-EnR* produced an enlargement in the domain of *Fgf8* expression. Part of this enlargement could be a consequence of a broader overlap between *Otx2* and *Gbx2*, as has been suggested for chick (Garda et al., 2001). Interference with *Xiro1* completely suppresses *Fgf8* expression. This is not due to absence of *Gbx2*, as the dominant negative form of *Gbx2* does not repress *Fgf8* expression. In addition, in embryos with impaired *Xiro1* function in which *Gbx2* expression is reconstituted, the expression of *Otx2*, but not that of *Fgf8*, is rescued. These results suggest that *Xiro1* is absolutely required for *Fgf8* expression and that *Gbx2* and *Otx2* are not sufficient for the activation of *Fgf8* expression. In agreement, in *Gbx2* null mice, *Fgf8* is initially expressed, although this expression is not maintained (Wassarmann et al., 1997). Thus, *Xiro1* may participate in this initial *Fgf8* activation.

We also used the in vitro assay developed here to test the role of *Xiro1* on the induction of the isthmic organizer. Conjugates of caps expressing *Otx2* and *Xiro1* are able to induce *En2* expression in the *Otx2* cap and *Fgf8* expression in the *Xiro1* cap, as expected if *Xiro1* is activating *Gbx2* expression that in turn interacts with the *Otx2* cap. In addition to this role of *Xiro1* on isthmus induction, we found that *Xiro1* activity was required in the *Otx2* cap, as co-expression of a dominant negative form of *Xiro1* in this cap blocks *En2* induction. Thus, the mutual interaction between *Otx2* and *Xiro1* produces the co-expression of these two genes, which is probably required to define the competent domain for the signals coming from the *Gbx2*-expressing cells. The cephalic limit in the expression of the Iro genes in chick and mouse correlates exactly with the region of the diencephalon that induces ectopic isthmic tissue in response to grafts of midbrain or beads soaked with Fgf8 (Bosse et al., 1997; Bosse et al., 2000; Cohen et al., 2000; Alvaro-Mallart, 1993; Crossley et al., 1996).

A model for the positioning of the isthmic organizer

We propose the following model for the positioning of the isthmic organizer in *Xenopus* (Fig. 9). In this model, some elements are similar to those found in mouse and chicken. At the gastrula stage (Fig. 9A), there is a reciprocal activation of *Otx2* and *Xiro1* in the caudal part of the midbrain. These interactions help to maintain the co-expression of these two genes which will be required for the competence of this territory to receive the signals that later will promote *En2* expression. During late gastrula-early neurula, *Xiro1* upregulates *Gbx2* (Fig. 9A,B). This produces an overlap in the expression of *Otx2* and *Gbx2* within the prospective isthmic territory. In this region, in part as a consequence of *Xiro1*, the expression of *Fgf8* in the prospective isthmic organizer is initiated (Garda et al., 2001) (Fig. 9B). *Fgf8* and *Gbx2* begin a positive crossregulation. Then, *Gbx2* and *Otx2* by mutual repression transform this interface into a sharp border (Fig. 9C). *Xiro1* is later required in the *Otx2* territory for *En2* (and probably for *Wnt1*) activation mediated by Fgf8 from adjacent *Gbx2*-expressing cells. The isthmic organizer is perpetuated by the mutual interaction of *Fgf8*, *En2* and *Wnt1*.

In our experiments, we induced higher levels of *Gbx2* activity, either by injecting *Gbx2* mRNA directly by overexpressing *Xiro1*, which up regulates *Gbx2*. Under these circumstances, the equilibrium in the mutual repression between *Otx2* and *Gbx2* is shifted in favor of *Gbx2*, which, by repressing *Otx2*, shifts the *Otx2-Gbx2* border into a more anterior position and with it all of the midbrain-hindbrain boundary.

Although we show evidence for this model in *Xenopus* embryos, the expression patterns of several Iro genes in mouse, chick and zebrafish are compatible with our model. In *Xenopus*, *Xiro1*, *Xiro2* and *Xiro3* are expressed in the midbrain-hindbrain boundary (Gómez-Skarmeta et al., 1998; Bellefroid et al., 1998). A recent report by Sato et al. (Sato et al., 2001) shows that *Irx2*-positive territory is able to respond to the Fgf8b signal in the isthmic organizer region of chick embryos. Future experiments

are required in these organisms to test the role of the Iro genes in the specification of the isthmic organizer.

It is interesting to note that in *Drosophila*, the Iro genes participate in the generation of organizer boundaries during imaginal disc development (reviewed by Cavodeassi et al., 2001). We have found a similar Iro function in vertebrate brain development. The restricted pattern of expression of several Iro genes in vertebrate rhombomeres, which are know to behave as compartment borders (reviewed by Lumsden and Krumlauf, 1996), raise the possibility that the Iro genes are common elements in the genetic pathways required for the generation of boundaries.

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