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

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

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 expression in the anterior neuroectoderm and heart (Bao et al., 1999; Bellefroid et al., 1997), 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; Díez 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; Díez 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'-AGGCGGTAATTCGCT-CAGCC-3'/5'-CACCTCTCGAGCTCATTCCC-3' and 5'-ACCTG-GACTGAAATTCAAGTAC-3'/5'-TTCGTCGACTCGTTGCG-3' respectively. EcoRI and XhoI sites (underlined) were used to fuse them to the engrailed repressor domain (EnR) or the E1A transactivator domain in the pCS2-MT-EnR and pCS2-MT-EnR vectors (reviewed by Cavodeassi et al., 2001). The fragments generated were digested with EcoRI and XhoI restriction enzymes and cloned in pBS SKII and were subsequently sequenced. To obtain the E1A fusion proteins the pCS2-MT-EnR-E1A vector and the homeodomain fragments were double digested with EcoRI and XhoI restriction enzymes and then ligated together. The EnR fusion constructs were generated by exchanging the E1A domain, excised with XhoI and KpnI from the pCS2-MT-EnR-E1A or pCS2-MT-EN-R-E1A with the EnR-coding sequence, excised with the same enzymes, from the pCS2-MT-EN-R 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...
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
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-expression domain (Millet et al., 1999; Broccoli et al., 1999; Katabira 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. 2L). 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; Katabira 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 isthmic 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.

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. 5I). Interference with Xiro1 function at early gastrula by injecting HD-GR-E1A and HD-GR repressed Otx2 (Fig. 5E,F), Gbx2 (Fig.

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 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%.
**Xiro1 in mid-hindbrain boundary formation**

**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 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 causal 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. 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 0.5 ng 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 et al., 1999).
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 supports 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 isthmus 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 Xiro1-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 (Gavalas 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 (Wassermann 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 known 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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