The Wnt-activated Xiro1 gene encodes a repressor that is essential for neural development and downregulates Bmp4

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SUMMARY

In the early Xenopus embryo, the Xiro homeodomain proteins of the Iroquois (Iro) family control the expression of proneural genes and the size of the neural plate. We report that Xiro1 functions as a repressor that is strictly required for neural differentiation, even when the BMP4 pathway is impaired. We also show that Xiro1 and Bmp4 repress each other. Consistently, Xiro1 and Bmp4 have complementary patterns of expression during gastrulation. The expression of Xiro1 requires Wnt signaling. Thus, Xiro1 is probably a mediator of the known downregulation of Bmp4 by Wnt signaling.

Key words: Xenopus, iroquois, Wnt signaling, BMP4, Homeodomain repressor

INTRODUCTION

Vertebrate neural tissue is generated by an inductive process. In Xenopus embryos, the Spemann organizer, which is located in the dorsal mesoderm, secretes neural inducers such as Noggin, Chordin and Follistatin that diffuse to the adjacent ectoderm and promote its neural development (Smith and Harland, 1992; Lamb et al., 1993; Smith et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994; Sasai et al., 1995; Fainsod et al., 1997). In the last few years, it has become clear that neural induction is mediated by interference with BMP4 signaling. The neural inducers bind to BMP4, which is present in the ectoderm, and prevent its interaction with BMP4 receptors, thus blocking signaling and allowing the acquisition of the default neural fate of the ectoderm (Piccolo et al., 1996; Zimmerman et al., 1996; reviewed by Harland and Gerhard, 1997; Sasai and De Robertis, 1997; Weinstein and Hemmati-Brivanlou, 1999). In the absence of neural inducers, BMP4 can signal and promote the epidermal fate (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995).

Bmp4 is initially expressed in the whole embryo. During gastrulation, expression disappears from the dorsal side of the embryo, including the dorsal mesoderm and the prospective neural plate (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995). It has been postulated that this downregulation is a consequence of the interference of neural inducers with BMP4 signaling, as in both Xenopus and zebrabish, BMP4 appears to regulate positively its own expression (Jones et al., 1992; Hammerschmidt et al., 1996; Piccolo et al., 1997; Nguyen et al., 1998). However, Baker et al. have found that Noggin, while interfering with BMP4 function, is unable to repress Bmp4 expression in Xenopus (Baker et al., 1999). Instead, the repression seems to depend on Wnt signaling. This signaling is mediated by blocking the activity of glycogen synthase kinase 3 (Gsk3), which, in the absence of the signal, phosphorylates β-catenin and promotes its degradation. The inhibition of Gsk3 activity thus stabilizes β-catenin, which forms complexes with members of the family of LEF/TCF DNA-binding proteins. The complexes facilitate the nuclear localization of these factors and, therefore, the activation of target genes (reviewed in Moon and Kimelman, 1998).

Wnt signaling cooperates with the neural inducers to specify posterior versus anterior neural identity. Thus, while in competent ectoderm the inducers upregulate anterior neural genes, in combination with Wnt signaling, they activate posterior neural genes and repress anterior ones (McGrew et al., 1995). Conversely, interference with Wnt function reduces expression of posterior neural genes and upregulates anterior ones (McGrew et al., 1997). Moreover, LEF/TCF-binding sites within the engrailed 2 promoter are required for the Wnt-dependent activation of this neural gene (McGrew et al., 1999). On the other hand, it has recently been found that, in the absence of neural inducers, mouse and Xenopus Wnt3 and Wnt8 (and downstream components of the Wnt-signaling pathway) can neuralize competent ectoderm (animal caps) and, in whole embryos, induce expansion of the neural plate (Baker et al., 1999). Upon sperm entry, cortical rotation activates Wnt signaling along most of the dorsal side of the embryo. As a consequence, β-catenin is detected in the nuclei of cells of the dorsal ectoderm, mesoderm and endoderm, and persists through the gastrula stages (Schneider et al., 1996; Larabell et al., 1997; Rowning et al., 1997). Hence, it appears that the Wnt
pathway activates an unknown factor that is responsible for downregulating Bmp4 in the prospective neural plate (Baker et al., 1999).

Xiro1 is a member of the Iroquois (Iro) family of TALE class homeoproteins, which are present in all the animal kingdom (Bürglin, 1997). They were first identified as prepatterning factors necessary for the expression of proneural and proneural genes in Drosophila (Gómez-Skarmeta et al., 1996; Leyns et al., 1996). Since then, they have been implicated in other processes in Drosophila, such as formation of the eye (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998; Cavodeassi et al., 1999) and specification of dorsal head structures and dorsal mesothorax (Díez del Corral et al., 1999; Cavodeassi et al., 2000), and, in chicken, specification of the ventricle chamber of the heart (Bao et al., 1999). In Xenopus three members of the Iro family have been identified, Xiro1-3. Similarly to their Drosophila counterparts, they seem to control the expression of proneural genes such as Xash3 and Xangr1 (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998). Thus, injection of Xiro mRNAs promotes ectopic expression of these proneural genes, an effect associated with an expansion of the neural plate, which occurs at least in part at the expense of the neural crests.

In this work we report that Xiro1 acts as a transcriptional repressor that is strictly required for neural development, even when Bmp4 signaling is impaired. Our data also indicate that there is a reciprocal inhibition between Xiro1 and Bmp4. Moreover, Xiro1 is activated by Wnt signaling. Xiro1 is probably a mediator of the repression of Bmp4 by Wnt in the dorsal ectoderm.

MATERIALS AND METHODS

Plasmid constructions

To prepare the EnR-Xiro and the VP16-Xiro constructs, we used the EnR-Mix.1 and VP16Mix.1 DNAs (Lemaire et al., 1998). The Mix.1 homeodomain was removed with BamHI and NotI digestion, and most of the Xiro1 cDNA was inserted using a BamHI site (Xiro1 cDNA position 440) and a NotI site in the polylinker 3′ of the cDNA. The resulting fusion proteins contain most of the Xiro1 coding region except the first 41 amino acids. To generate the HD-GR, HD-GR-EnR, HD-GR-domain was obtained by PCR using the oligonucleotides 5′-GAGCTC CCCTCTG-3′ and 5′-GAATTC CTCTCTTAC-3′ or 5′-XhoI site in the polylinker 3′ of the cDNA. The resulting fragment was digested with BamHI and NotI digestion, and most of the Xiro1 cDNA (from position 440) and a XhoI repressor domain of Engrailed (EnR) or the activation domain of the viral protein VP16 with a fragment.

Gel mobility-shift assay

GST-Xiro1 was generated by inserting most of the Xiro1 cDNA (from a BamHI site at position 440 to a HindIII site in the polylinker 3′ of the cDNA) into PGEX-KG digested with BamHI and HindIII. This GST-Xiro1 fusion contains all Xiro1 amino acids except for the amino terminal 41 residues. Protein purification was performed as previously described (Gómez-Skarmeta et al., 1996); GST-Xiro1 or GST-Ara homeodomain (Gómez-Skarmeta et al., 1996) were incubated with a 315 bp 32P-labeled PCR fragment of the Bmp4 promoter (obtained using the oligonucleotides 5′-CAGCTGACCGTCTTACTCC-3′ and 5′-CTACGTCAACTCCAGCCCC-3′) in binding buffer (20 mM Heps pH 7.9, 50 mM KCl, 1 nM MgCl2, 1 mM EDTA, 5% glycerol, 0.07 mg/ml poly [dI-C]) and 0.3 mg/ml bovine serum albumin). After 30 minutes at room temperature, 10 μl of the 15 μl reaction volume were loaded on a 4% polyacrylamide (60/1, acrylamide/bisacrylamide ratio) gel containing 5% glycerol.

DNA sequencing

DNA sequencing was performed with ABI chemistry in an automatic DNA sequencer using T3 and T7 oligonucleotides. Custom synthesized oligonucleotides were obtained from ISOGEN (Bioscience BV, Maarsenen, The Netherlands).

Whole-mount in situ hybridization, X-Gal and antibody staining

Antisense RNA probes were prepared from different cDNAs using digoxigenin or fluorescein (Boehringer Mannheim) as labels. Standards were prepared, hybridized and stained as described (Harland 1991). Double in situ hybridization was performed as described (Gómez-Skarmeta et al., 1998). X-Gal staining was performed as previously described (Coffman et al., 1993). Antibody staining was performed (Turner and Weintraub, 1994) after in situ hybridization and bleaching of the embryos using mouse monoclonal anti-Myc from BabCo.

In vitro RNA synthesis

All DNAs were linearized and transcribed as described (Harland and Weintraub, 1985) with GTP cap analog (New England Biolabs). SP6, T3 or T7 RNA polymerase were used. After DNase treatment, RNA was extracted with phenol-chloroform, column purified and precipitated with ethanol. mRNAs for injection were resuspended in water.

RT-PCR analysis

Embryos were injected with the corresponding mRNA in the animal region at one cell stage, caps were explanted at stage 9, and aged until stage 19-20 when RNA was extracted. The RNA was treated with DNase and processed for RT-PCR as described ( Gawantka et al., 1995).

Embryos, explants and microinjection of mRNA

Xenopus embryos were obtained and animals caps were prepared as described previously (Gómez-Skarmeta et al., 1998) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Synthetic mRNAs were injected into embryos at the one- or two-cell stage using 8-12 nl of vehicle.

RESULTS

Xiro1 acts as a repressor

To determine whether in Xenopus early embryogenesis Xiro1 acts as a repressor or an activator, we constructed several chimeric forms of the Xiro1 protein. We generated fusions of either the repressor domain of Engrailed (EnR) or the activation domain of the viral protein VP16 with a fragment.
comprising most of the Xiro1 protein or just the Xiro1 homeodomain (EnR-Xiro, VP16-Xiro, HD-EnR, respectively). If a transcription factor acts as an activator, overexpression of either its wild-type form or a chimeric form comprising its DNA-binding domain fused to an activation domain such as VP16 should have similar effects, while the equivalent chimera bearing EnR should function as a dominant negative. In contrast, if the transcription factor is a repressor, its overexpression or that of the chimeric protein bearing EnR should have similar effects, while the equivalent chimera bearing VP16 should function as a dominant negative (see, for example, Lemaire et al., 1998; Latinkic and Smith, 1999). These different forms of Xiro1 proteins were overexpressed by injecting their corresponding mRNAs in one blastomere at the two-cell stage embryo, together with β-galactosidase mRNA (0.3 ng) as a marker to identify the injected side of the embryo. The effects on the neural plate were monitored at neurula stage 17 by examining the accumulation of the neural marker Sox2 mRNA (Mizuseki et al., 1998a). Injection of either Xiro1 mRNA (1 ng), EnR-Xiro mRNA (0.5 ng), or HD-EnR mRNA (0.5 ng) similarly expanded the neural plate (Fig. 1A, 94% of embryos, n=105; Fig. 1B, 92%, n=54; and Fig. 1C, 83%, n=36, respectively). Moreover, many embryos (50%, n=18; Fig. 1C, inset) injected with HD-EnR mRNA displayed isolated groups of neural cells in the non-neural ectoderm. Anti-Myc staining revealed that these cells expressed HD-EnR autonomously. Similar isolated groups of neural cells were observed with EnR-Xiro mRNA (not shown).

In contrast, injection of VP16-Xiro mRNA (2 ng) reduced neural differentiation (64%, n=28; Fig. 1D), which indicates that VP16-Xiro acts as a dominant negative. This effect was specific, since in many embryos co-injection of 1 ng of Xiro1 and 2 ng of VP16-Xiro mRNAs canceled the individual effects of either mRNA (46%, n=39; Fig. 1E). Taken together, these data indicate that Xiro1 is a repressor.

We further investigated the function of Xiro1 by fusing its homeodomain (HD) with an hormone-inducible region (GR), either alone or including the EnR or the E1A activator domain (Bellefroid et al., 1996; Marine et al., 1997). These fusion proteins should be active upon the exogenous addition of the hormone dexamethasone (DEX) (Kolm and Sive, 1995). In these experiments, the localization of the chimeric proteins was detected by staining with anti-Myc antibody. Embryos injected with HD-GR-EnR mRNA (0.5 ng) and treated with DEX at stage 9 showed expansion of the neural plate (83%, n=24; Fig. 1F). The effects were dependent on DEX (100%, n=13; Fig. 1F, inset). In contrast, embryos injected with 0.5 ng of either HD-GR or HD-GR-E1A mRNA and treated with DEX at stage

Fig. 1. Xiro1 is a repressor. All embryos were injected in one blastomere at the two-cell stage and fixed at stage 17, when Sox2 mRNA distribution was analyzed. Views are dorsal and arrowheads point to the midline. (A,B,D,E) The injected side was determined by X-gal staining (0.3 ng of β-galactosidase mRNA). (C,F,G-L) Overexpressed protein distribution determined by anti-Myc staining. Embryos injected with 1 ng of Xiro1 or 0.5 ng of EnR-Xiro or HD-EnR mRNA expanded the Sox2 expression domain (A-C, respectively). While Xiro1 overexpression only promoted neural tissue near to the endogenous neural plate, HD-EnR neuralized clusters of isolated cells within the epidermis that autonomously expressed the injected mRNA (C, inset). (D) VP16-Xiro mRNA (2 ng) decreased Sox2 expression. (E) This effect was rescued by co-expressing VP16-Xiro mRNA (2 ng) and Xiro1 mRNA (1 ng). (F) A hormone inducible Xiro homeodomain fused to the EnR domain mRNA (HD-GR-EnR, 0.5 ng) expanded the neural plate upon addition of DEX at stage 9. The effect was not observed in the absence of DEX (inset). (G-L) Embryos injected with HD-GR or HD-GR-E1A mRNAs (0.5 ng). Addition of DEX at stage 9 (or 6 not shown) strongly impaired neural differentiation in embryos injected with either HD-GR or HD-GR-E1A mRNAs (G-J, respectively). No effect was observed in the absence of DEX (insets). (H,K) DEX addition at stage 13 caused weak or no reduction of the neural plate. (I,L) Epidermal fate, as determined by expression of keratin, is induced in the neural plate when DEX was added at stage 6 (I; inset shows a control embryo injected with 0.3 ng of β-galactosidase mRNA) but not at stage 9 (L, green shows keratin and pink shows Sox2).
6 or 9 showed a reduction of Sox2 expression (95%, n=19; Fig. 1G; and 90%, n=22; Fig. 1J, respectively). The effect required DEX (100%, n=12; Fig. 1G inset; and 100%, n=16; Fig. 1J inset, respectively). These results confirm those described above and indicate that the Xiro1 homeodomain interferes with Xiro1 function. This is probably due to the competition for Xiro1-binding sites in the regulatory region of target genes of Xiro1. This interference required most of or the entire homeodomain, since overexpression of a truncated Xiro protein that lacked the third helix of the homeodomain (plus the C terminus) did not affect Sox2 expression (Gómez-Skarmeta et al., 1998).

To determine the temporal requirement of Xiro1 function for neural acquisition, we activated the HD-GR or HD-GR-E1A antagonists by DEX addition, either before gastrulation (stage 6 or 9), at midgastrula (stage 11) or at the early neurula (stage 13). While early activation strongly reduced Sox2 expression (Fig. 1G,J,L) in most of the embryos, suppression of neural tissue was intermediate (60-65%; n=23 or 26, not shown) or weak (15-20%; n=23 or 24; Fig. 1H and K) at the later stages. These data indicate that proper formation of the neural territory requires Xiro1 function already at the gastrula stage. Interestingly, the repression of neural fate by HD-GR or HD-GR-E1A is associated with an expansion of epidermal tissue, as determined by expression of keratin (Jonas et al., 1985) when DEX was added at stage 6 (72-76%, n=18 or 21; Fig. 1I and not shown) but not when the hormone was added at stage 9 or later (100%, n=21; Fig. 1L). This suggests that neural to epidermal transformation can occur only when Xiro1 function is interfered with from its start (see Fig. 5E). The identity of the cells in which Xiro1 function is removed at stage 9 is unknown, as several neural or mesoderm markers fail to be expressed in these cells (below and not shown).

Overexpression of HD-EnR mRNA (0.5 ng) affected additional neural markers. Nrp1 (Knecht et al., 1995) was ectopically expressed (93%, n=15; Fig. 2A) and expressed in isolated groups of neural cells that autonomously expressed HD-EnR within the epidermis (47%, n=15; Fig. 2A inset). Identical effects were observed with the proneural gene Xngnr1 (Ma et al., 1996) (not shown). The domains of expression of the anterior neural marker Otx2 (Blitz and Cho, 1995; Pannese et al., 1995) and the proneural gene Xash3 (Ferreiro et al., 1994; Turner and Weintraub, 1994) were also expanded but only within the neural plate (60-50%, n=15-18; Fig. 2C and E, respectively). Similar results were found by injecting Xiro1 mRNA (1 ng) except that Xiro1 did not promote neural fate far away from the endogenous neural territory (Gómez-Skarmeta et al., 1998 and data not shown). In contrast, injection of HD-GR mRNA (0.5 ng) suppressed expression of Nrp1, Otx2, Xash3 and Xngnr1 (54-75%, n=13-15; Fig. 2B, D and F, respectively, and not shown). HD-EnR and HD-GR did not affect the mesoderm markers Shh and XMyoD (Ekker et al., 1995; Hopwood et al., 1989) (HD-EnR: 100%, n=14 and 15, respectively; Fig. 2G,I; HD-GR: 100%, n=18 and 19; Fig. 2H and J, respectively). This indicates that the effect of Xiro1 in neural development is independent of mesoderm.

Xiro1 promotes neural fate in the absence of mesoderm

We further examined whether Xiro1 can promote neural fate in the absence of mesoderm. Embryos were injected at the one-

![Fig. 2](image-url)
animal caps is similar to that observed upon injection of an mRNA (0.25 ng) encoding a dominant negative form of the BMP receptor (dnBMPR) (Sasai et al., 1995; Suzuki et al., 1995; Xu et al., 1995; 100%, n=26; Fig. 3C). We tested whether the dominant negative Xiro1 interferes with the dnBMPR-promoted neuralization by co-injecting dnBMPR (0.25 ng) and VP16-Xiro mRNAs (2 ng). Under this condition most caps did not express Sox2 (89%, n=28; Fig. 3D). Similar antagonistic effects were found by co-injecting HD-GR mRNA (0.5 ng) upon addition of DEX (not shown). These results were confirmed by examining by RT-PCR for the presence of mRNAs of neural and mesodermal markers in similarly injected or un.injected caps (Fig. 3E). Moreover, in contrast to the anterior neural identity of caps injected with dnBMPR

![Fig. 3.](image)

**Fig. 3.** Xiro1 promotes neural fate in the absence of mesoderm. Embryos were injected with different mRNAs at the one-cell stage, animal caps were excised at blastula stage and cultured until stage 19, when the presence of Sox2 mRNA was analyzed. (A) Caps are neutralized by Xiro1 mRNA (2 ng). Sox2 expression is not detected in uninjected caps (inset). (B) Similar results were found by injecting EnR-Xiro mRNA (2 ng). Inset shows Sox2 staining in stage 19 whole embryos. (C) Caps injected with dnBMPR mRNA (0.25 ng) are also neutralized. (D) This neutralization is impared by co-injecting dnBMPR (0.25 ng) and VP16-Xiro (2 ng) mRNAs. (E) Caps were prepared from embryos injected with 2 ng of Xiro1, EnR-Xiro, HD-EnR or 0.25 ng of dnBMPR with or without 2 ng of VP16-Xiro mRNAs and were cultured until stage 19 when RT-PCR was performed to detect XMyoD, Sox2, Otx-2, Krox20 and H4. Note that Xiro1, HD-EnR and EnR-Xiro mRNAs activated anterior (XAG1 and Otx2) and posterior (Krox20) neural markers while dnBMPR mRNA only activates anterior markers. VP16-Xiro mRNA reduced Sox2 expression in embryos injected with dnBMPR mRNA. ND, not determined.

![Fig. 4.](image)

**Fig. 4.** Xiro1 and Bmp4 have complementary patterns of expression and repress each other. Embryos were hybridized in situ for either Xiro1, Bmp4 or Sox2 mRNAs, as indicated. (A-J) Dorsal views at stage 10. (B) Dorsal view at stage 11. (C,D) Lateral views at stage 11. (E,F) Vegetal views at stage 10.5. (I) Dorsal view at stage 16. (A) At early gastrula, Xiro1 and Bmp4 domains of expression overlap in the dorsal ectoderm (arrowheads). (B-D) A little later, their expression domains become largely complementary. (B) Double in situ to detect Xiro1 and Bmp4 mRNAs. Arrowheads in C,D point to the limits of Xiro1 and Bmp4 expression domains. (E) Xiro1 mRNA (2 ng) repressed Bmp4 expression (arrowhead). (F) VP16-Xiro mRNA (2 ng) induced ectopic expression of Bmp4 in the dorsal ectoderm and mesoderm (arrowhead). (G,H) Xiro1 was expressed in caps taken from embryos injected with dnBMPR (0.25 ng) or Noggin (0.03 ng) mRNAs. (I) Xmxs1 mRNA (2 ng) repressed Xiro1 at neurula stages. (J) This repression is also observed at early gastrula stages (top). Probably as a consequence, Sox2 (bottom) is also repressed. Arrowheads point to the injection site, as determined by X-gal staining. At this stage, both Xiro1 (A top) and Sox2 are expressed in most, if not all, of the prospective neuroectoderm of wild-type embryos.
mRNA, caps expressing Xiro1, EnR-Xiro or HD-EnR mRNAs also induced more posterior genes such as Krox20 (Fig. 3E). We conclude that Xiro1 can promote the neural fate in the absence of mesoderm and that the dominant negative form of Xiro1 interferes with the neuralizing ability of dnBMPR.

**Mutual antagonism between Xiro1 and BMP4**

The neuralizing ability of Xiro1 in the absence of mesoderm suggests that Xiro1 interferes with BMP4 signaling. Since Xiro1 acts as a repressor, it may downregulate Bmp4 expression and in this way promote neural differentiation. To investigate this possibility, we first compared the patterns of expression and in this way promote neural differentiation. To investigate this possibility, we first compared the patterns of expression of Xiro1 and Bmp4. At stage 10, these patterns overlap in the prospective neural territory (Fig. 4A). However, a little later the patterns become mostly complementary, as seen at stage 11 (Fig. 4B-D). This situation persists until the end of gastrulation, when Bmp4 expression becomes restricted to the more ventral mesoderm and that of Xiro1 resolves into two bands located at each side of the midline (Gómez-Skarmeta et al., 1998). These data are compatible with a downregulation of Bmp4 by Xiro1 in the dorsal ectoderm. Indeed, embryos injected with Xiro1 mRNA (2 ng) showed a clear repression of Bmp4 at the injected side (100%, n=27; Fig. 4E). In contrast, embryos injected with VP16-Xiro mRNA (2 ng) displayed ectopic expression of Bmp4 in the dorsal region of the embryo (100%, n=23; Fig. 4F).

To determine whether BMP4 can repress Xiro1 we isolated animal caps from embryos injected with either 30 pg of Noggin or 0.25 ng of dnBMPR mRNAs, both of which are known to interfere with BMP4 signaling (reviewed in Weinstein and Hemmati-Brivanlou, 1999). In both cases, Xiro1 was expressed in these caps (as detected by in situ hybridization, Fig. 4G,H, and by RT-PCR, not shown), but it was not expressed in caps from un.injected embryos (see Fig. 7A, inset and Gómez-Skarmeta et al., 1998). Conversely, the increased BMP4 signaling caused by injection of 2 ng of Xmsx-1, a downstream component of BMP4 pathway (Suzuki et al., 1997), repressed both Xiro1 (82%, n=34; Fig. 4J) and Sox2 (74%, n=27; Fig. 4J). These data indicate that BMP4 signaling represses Xiro1 and, probably as a consequence, blocks neural fate as determined by Sox2 expression.

We have also examined whether in whole embryos HD-GR can counteract the excess neuralization produced by dnBMPR mRNA. Embryos were injected in one blastomere of the two-cell stage with dnBMPR (0.25 ng) and β-galactosidase (0.3 ng) mRNAs or with dnBMPR (0.25 ng) and HD-GR mRNAs (0.5 ng). dnBMPR mRNA caused an expansion of the Sox2 domain (97%, n=30; Fig. 5A). As expected, dnBMPR mRNA also expanded the domain of Xiro1 (87%, n=15; Fig. 5B). However, in embryos co-injected with dnBMPR and HD-GR mRNAs neural fate was strongly suppressed upon addition of DEX at.

**Fig. 5.** Xiro1 is strictly required for neural differentiation. Embryos were injected with the indicated mRNAs in one blastomere at the two-cell stage and either Sox2 or Xiro1 mRNA distribution was determined at stage 17. The injection side was determined by X-gal staining (A,B,D), as these embryos were co-injected with β-galactosidase mRNA (0.3 ng). Anti-Myc staining revealed overexpression domain in C. Arrowheads point to the midline. (A,B) dnBMPR mRNA (0.25 ng) expands the domains of Sox2 and Xiro1 expression. (C) Neural differentiation is strongly impaired by co-injection of dnBMPR (0.25 ng) and HD-GR (0.5 ng) mRNAs. (D) Neural plate expansion is rescued in embryos co-injected with Xiro1 (1 ng) and Bmp4 (0.25 ng) mRNAs (compare with Fig. 1A). (E) RT-PCRs from embryos at different stages performed to detect Xiro1, Bmp4, Sox2 and, as a control, H4. Note that Xiro1 and Bmp4 are detected at stage 9, before the onset of expression of Sox2.

**Fig. 6.** Xiro1 and Drosophila Ara bind to a Bmp4 promoter fragment. Xiro1 (lanes 1-8) bind to a 315 bp fragment of the Bmp4 promoter in a concentration dependent way. Different complexes are formed (arrowheads). The complexes are impaired in the presence of a 50 molar excess of unlabeled Bmp4 promoter fragment. Similarly, Ara (lanes 9 and 10) can also bind to this fragment and this binding is interfered by an excess of non labeled DNA. Presence of competitor DNA is denoted by a + sign. Amounts of added protein are indicated in µg.
**DISCUSSION**

**Xiro1 protein functions as a transcriptional repressor**

Our results indicate that Xiro1 acts as a transcriptional repressor. Thus, the intact protein and two derivatives containing the transcription repressor domain EnR fused to either most of the Xiro1 protein or just the Xiro1 homeodomain caused (upon overexpression) similar effects, namely, enlargement of the neural plate, as determined by Sox2 or Bmp4 becomes detectable and before Sox2 is expressed (Fig. 5E).

**Xiro1 binds to the Bmp4 promoter**

In gel retardation assays, we found that the Xiro1 protein binds in vitro to a 32P-labeled 315 pb fragment of the Bmp4 promoter (nucleotides −338 to −23, Metz et al., 1998) in a concentration-dependent manner and that the binding is blocked by an excess (50-fold) of non-labeled fragment (Fig. 6). Moreover, the homeodomain of the *Drosophila* Iroquois proteins Ara or Caup can also bind to this Bmp4 promoter fragment (Fig. 6 and not shown). Several DNA/Xiro1 or DNA/Ara-HD complexes were observed (Fig. 6, arrowheads). This could be due to several Xiro1/Ara binding sites within this promoter fragment or to multimerization of the GST-Xiro1 or GST-Ara-HD polypeptide. These results suggest that Xiro1 repression of Bmp4 is direct.

**Xiro1 is activated by Wnt signaling**

The downregulation of Bmp4 in the dorsal mesoderm and in the prospective neural plate depends on Wnt signaling (Baker et al., 1999). This signaling probably activates a repressor that downregulates Bmp4. Since the above data suggested that Xiro1 might be such a repressor, we examined whether Wnt signaling activated Xiro1. To that end, we injected one-cell stage embryos in the animal region with either *dnGsk-3* or *mΔβ-catenin* mRNAs (1 ng). These respectively encode a dominant negative form of Gsk-3 and a truncated mouse β-catenin that lacks the first 177 amino acids and promotes neural fate in animal caps (Yost et al., 1996; Baker et al., 1999). Animal caps from these embryos strongly expressed Xiro1 (Fig. 7A,B). Expression was absent in uninjected caps (Fig. 7A, inset). Moreover, mΔβ-catenin could not promote neural fate in the presence of 0.5 ng of *HD-GR* mRNA and upon the addition of DEX (Fig. 7C,D). Xiro1 was also ectopically expressed in embryos injected with an mRNA (1 ng) that encode the *Xenopus* β-catenin (Yost et al., 1996) (Fig. 7E).

If, as indicated by these experiments, Wnt signaling promotes Xiro1 expression, a decrease in this signal should downregulate Xiro1. Indeed, Xiro1 expression at early gastrula and neurula stages was reduced in embryos injected with 2 ng of Gsk3 mRNA (80%, n=20; Fig. 7F, top) and Fig. 7G). Congruently, these embryos also showed repression of Sox2 at gastrula stage (78%, n=23; Fig. 7F, bottom). Xiro1 was also repressed in embryos injected with an mRNA (1 ng) encoding a dominant negative form of *Xenopus TCF-3* (Molenaar et al., 1996) (Fig. 7H). We conclude that Wnt signaling activates Xiro1, which in turn participates in the repression of Bmp4 at the dorsal ectoderm of gastrula embryos.

**Stage 9 (100%, n=34; Fig. 5C). In addition, we found that the excess neutralization caused by overexpressing Xiro1 mRNA (1 ng) was also partially rescued by co-expressing 0.25 ng of Bmp4 mRNA (80%, n=21; compare Fig. 5D with Fig. 1A). Taken together, these results indicate a mutual antagonism between Xiro1 and Bmp4 and that, in the absence of BMP4 signaling, Xiro1 is strictly required for neural differentiation.**

We determined the onsets of expression of Xiro1, Bmp4 and Sox2 by RT-PCR. Xiro1 is initially expressed at stage 9, when


*Xiro1* downregulates *Bmp4* and is essential for neurogenesis

Acquisition of the neural fate requires exclusion of BMP4 signaling from the dorsal ectoderm (reviewed in Harland and Gerhard, 1997; Sasai and De Robertis, 1997; Weinstein and Hemmati-Brivanlou, 1999). We now find that *Xiro1* probably participates in this exclusion by directly repressing *Bmp4* at the gastrula stage. Indeed, ectopic *Xiro1* represses *Bmp4* and dominant negative *Xiro1* ectopically activates *Bmp4* in the dorsal mesoderm and ectoderm. Moreover, *Xiro1* or the *Drosophila* Araucan or Caupolican, which functionally behave as *Xiro1* in expanding neural territory (not shown), bind to a 315 bp *Bmp4* promoter fragment that directs stronger expression of a reporter gene in ventral than in dorsal domains of gastrula embryos. In addition, this promoter fragment participates in this exclusion by directly repressing *Bmp4*. Thus, it seems that *Iro* proteins can be either activators or repressors.

Neuronal induction in *Drosophila* depends on the presence of BMP4 signaling. Neural inducers (Baker et al., 1999) therefore, this pathway should promote neural fates in animal caps (Mizuseki et al., 1998a; Mizuseki et al., 1998b). *Zic1* seems to be a transcriptional activator and, therefore, it should not be responsible for *Bmp4* downregulation, at least directly (reviewed in Sasai, 1998; Weinstein and Hemmati-Brivanlou, 1999). *SoxD* is initially expressed in all of the ectoderm, and it is only restricted to the neuroectoderm by mid-gastrulation (Mizuseki et al., 1998b). Thus, *Xiro1* is so far the only known repressor that is expressed at the appropriate place and time to downregulate *Bmp4* in the dorsal ectoderm.

It should be stressed that *Xiro1* may not be strictly required for neural induction. However, by helping repress *Bmp4*, *Xiro1* may make the removal of BMP4 more complete and independent of the presence of the neural inducers in the whole neuroectoderm, thus ensuring proper neural fate acquisition. However, *Xiro1* seems indispensable for the acquisition of neural fate. In both animal caps and whole embryos, neuralization does not occur if *Xiro1* function is inhibited, even when BMP4 signaling is blocked. These data also indicate that *Xiro1* represses factors other than BMP4 that prevent neurogenesis.

Wnt signaling promotes *Xiro1* expression

While neural inducers direct anterior neural development, the presence of Wnt signaling causes these inducers to activate posterior neural genes (McGrew et al., 1995; McGrew et al., 1999). Hence, Wnt participates in the patterning of the neural plate. However, Wnt can also induce neuralization of competent ectoderm in the absence of neural inducers (Baker et al., 1999). Moreover, the repression of *Bmp4* that occurs during gastrulation depends on Wnt signaling and not on neural inducers (Baker et al., 1999). Therefore, this pathway should activate a repressor of *Bmp4* expression. Our data suggest that *Xiro1* is one such repressor. Indeed, *Xiro1* is activated by Wnt signaling in animal caps and depletion of this signal reduces *Xiro1* expression in early gastrula dorsal ectoderm. A dominant negative *Xiro1* represses neural fate promoted by Wnt signaling. Both Wnt signaling and *Xiro1* induce anterior and posterior genes in animals caps. We thus propose that Wnt signaling, triggered by the cortical rotation, activates *Xiro1* (and possibly other repressors) after the mid-blastula transition. This activation is weak (Gómez-Skarmeta et al., 1998), probably because of the presence of BMP4 signaling. Neural inducers...
subsequently interfere with this signaling and Xiro1 expression is reinforced. This higher level of Xiro1 can then repress Bmp4. This could explain the initial partially overlapping domains of both genes. Its is also possible that Xiro1 represses Bmp4 in concert with other proteins that are not present at the very early gastrula.

We cannot exclude the possibility that the activation of Xiro1 promoted by Wnt is due to a downregulation of Bmp4 mediated by another repressor downstream of Wnt. One candidate is the nieuwkoid/dharma/bozozok homeobox gene, which (in zebrafish) is activated by Wnt signaling and can repress Bmp4 (Koss and Ho, 1999).

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