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Xiro-1 Controls Mesoderm Patterning by Repressing *bmp-4* Expression in the Spemann Organizer

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ABSTRACT **The *Iroquois* genes code for homeodomain proteins that have been implicated in the neural development of *Drosophila* and vertebrates. We show here for the first time that *Xiro-1*, one of the *Xenopus Iroquois* genes, is expressed in the Spemann organizer from the start of gastrulation and that its overexpression induces a secondary axis as well as the ectopic expression of several organizer genes, such as *chordin*, *goosecoid*, and *Xlim-1*. Our results also indicate that *Xiro-1* normally functions as a transcriptional repressor in the mesoderm. Overexpression of Xiro-1 or a chimeric form fused to the repressor domain of Engrailed cause similar phenotypes while overexpression of functional derivatives of *Xiro-1* fused with transactivation domains (VP16 or E1A) produce the opposite effects. Finally, we show that *Xiro-1* works as a repressor of *bmp-4* transcription and that its effect on organizer development is dependent on BMP-4 activity. We propose that the previously observed down regulation of *bmp-4* in the dorsal mesoderm during gastrulation can be explained by the repressor activity of *Xiro-1* described here. Thus, *Xiro-1* seems to have at least two different functions: control of neural plate and organizer development, both of which could be mediated by repression of *bmp-4* transcription.**

Key words: *Xiro-1*; mesoderm; Spemann organizer; BMP-4

INTRODUCTION

Iroquois genes code for transcription factors of the three amino acid loop extension (TALE) superclass of homeobox genes (Bürglin, 1997). In *Drosophila, caupolican, araucan*, and *mirror* are three highly homologous genes belonging to the *Iroquois* complex. These genes are thought to establish a "pre-pattern" that governs the spatially localized expression of the proneural *achaete-scute* genes, which in turn determine the site at which neural precursors arise (Gómez-Skarmeta and Modolell, 1996; Leyns et al., 1996). In *Drosophila,* the *Iroquois* genes have also been implicated in other processes, such as vein formation (Gómez-Skarmeta and Modolell, 1996), formation of the eye (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopulus et al., 1998; Cavodeassi et al., 2000) and specification of the dorsal head structure and dorsal mesothorax (Diez del Corral et al., 1999; Cavodeassi et al., 2000). Vertebrate homologues to the *Drosophila Iroquois* genes have been identified in *Xenopus* (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998), mouse (Bosse et al., 1997; Cohen et al., 2000), chick (Goriely et al., 1999), and zebra fish (Tan et al., 1999).

The expression patterns of the *Iroquois* genes suggest that they are involved in neural development. Functional studies in *Xenopus* have shown that these genes are able to control the expression of proneural genes such as *Xash-3* and *neurogenin* (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998). Thus, injection of *Xiro* mRNAs promotes the ectopic expression of these proneural genes, associated with an expansion of the neural plate and a change in shape of the embryo. It has been suggested that *Xiro* mRNA injection causes a direct effect on the ectoderm (Gómez-Skarmeta et al., 1998, 2001), although a possible effect on the mesoderm has not been ruled out completely. The expression of the zebra fish *Iroquois* gene, *Ziro3*, begins during gastrulation in the dorsal axial mesoderm that then develops into the notochord (Tan et al., 1999). Later, the expression is limited to the chordo-neural hinge in the tail bud. *Ziro3* expression also occurs in the central nervous system, excluding the telencephalon. In mouse, *Iroquois* genes are expressed in neural and mesodermal tissues (Bosse et al., 1997).

The present study analyzes the role of *Xiro* in mesodermal development. In *Xenopus*, the mesoderm is formed in the equatorial sector of the blastula, by cells that have responded to two maternal agents (reviewed in Harland and Gerhart, 1997). Maternal activities such as dorsal β-catenins and vegetal VgT cooperate at

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stage 9, when mesoderm induction takes place, to set up a zygotic dorsal to ventral gradient in the endoderm, composed of multiple *Xenopus* nodal-related factors (*Xnrs*). At high nodal-related concentrations, which require a functional β-catenin pathway on the dorsal side of the embryo, the Spemann organizer is induced in overlying cells by early gastrula. On the ventral side, VgT and Vg1 lead to the production of low levels of nodal-related signals, and induction of the ventral mesoderm (Angius et al., 2000). At the gastrula stage, the organizer secretes a variety of zygotic proteins that act as antagonists to various members of the BMP and Wnt ligand families, which are secreted by cells of the competence domain surrounding the organizer. BMPs and Wnts favor ventral development, while Noggin, Chordin, Frzb, and Cerberus, secreted by the organizer, bind the BMP and Wnt ligands, interfere with their signaling, and promote dorsal fate (Bouwmeester et al., 1996; Piccolo et al., 1997; Zimermann et al., 1996, Wang et al., 1997; Leyns et al., 1997; Hoppler and Moon, 1998). It thus appears that the dorso-ventral polarity of the mesoderm is mediated by the establishment of a BMP and Wnt activity gradient, which specifies different cell fates along its axis.

Recently, *Xiro-1* was shown to function as a transcriptional repressor in the ectoderm (Gómez-Skarmeta et al., 2001). In contrast, *Irx4*, a chick *Iroquois* gene, was shown to act as a transcriptional activator required for heart development (Bao et al., 1999), while *Drosophila Iroquois* genes have been proposed to act as transcriptional activators of proneural genes (Gómez-Skarmeta and Modolell, 1996). Thus, it seems that the *Iroquois* genes encode for transcription factors that can act either as activators or repressors, depending on the gene family member or the species under study. In *Xenopus*, while *Xiro-1* repress *bmp-4* expression in the prospective neural plate region (Gómez-Skarmeta et al., 2001), both *Xiro-1* and *bmp-4* are coexpressed in the prospective placode region (Gómez-Skarmeta et al., 1998), making it difficult to propose *Xiro-1* as a repressor of *bmp-4* in that region. Thus, *Iroquois* repressor and/or activator activity seems to be context dependent, a context that is likely defined not only by gene or species specificity, but also by tissue specificity. There are several examples where the transcriptional activity of a transcription factor cannot be defined clearly as an activator or as a repressor, because it depends on the tissue where it is expressed and the target genes analyzed. Thus, for example, the *Xslug* gene is a repressor of *Xsnail* in the dorsal mesoderm but it is an activator of *Xsnail* in the neural crest (LaBonne and Bronner-Fraser, 2000; Mayor et al, 2000). The *Drosophila* dorsal protein can act as a transcriptional activator of *twist* and as a transcriptional repressor of *zen* (Jiang et al., 1992). In the light of these data, we decided to analyze which was the transcriptional activity of *Xiro-1* in the mesoderm, a novel region of expression of this gene.

In this study, we show for the first time that *Xiro-1* is expressed in the dorsal meso-endoderm, in the region of the Spemann organizer. We have analyzed the function of *Xiro-1* in the mesoderm and whether this gene acts as a transcriptional repressor or activator in this tissue. In order to perform functional studies, we have used functional derivatives of *Xiro-1*. Briefly, most of the Xiro-1 protein or just its homeodomain were fused either with the transactivation domain of the viral-derived VP16 protein (Friedman et al., 1988) or the E1A protein (Marine et al., 1997), or with the repressor domain (EnR) of the *Drosophila* Engrailed protein (Jaynes and O'Farrell, 1991). To control the timing of expression, we used a chimera that incorporated the glucocorticoid-binding domain of the glucocorticoid receptor (Kolm and Sive, 1995). Our results show that the derivatives fused to the EnR domain produce the same effect on mesoderm development as wild type *Xiro-1* mRNA, whereas those fused to activation domains cause the opposite effect, suggesting a role for *Xiro-1* as a transcriptional repressor in the mesoderm. We also show that *Xiro* is able to induce the formation of a secondary axis and to control the expression of organizer genes. Finally, we show that *Xiro* works as a repressor of *bmp-4* transcription and that its effect on organizer development is dependent on BMP-4 activity.

RESULTS

Xiro-1 Is Expressed in the Spemann Organizer and Its Derivatives

It has been reported previously that *Xiro-1* expression is localized in the neural plate but that its expression is initiated in the dorsal ectodermal region of the gastrula embryo. We have performed a careful study of this early expression. We detected *Xiro-1* expression in the dorsal meso-endoderm region of a stage 10 gastrula *Xenopus* embryo (Fig. 1A). Although this expression was too weak to be detected in the complete embryo, it became visible when the embryo was sectioned into halves. In order to determine the region of the dorsal mesoderm in which *Xiro-1* was expressed, we performed in situ hybridization of stage 10+ embryos for *Xiro-1, chordin (chd), goosecoid (gsc),* and *cerberus (cer)* genes, and then sectioned the embryos in two (Fig. 1B–E). *Xiro-1* was found to be expressed in the involuted marginal zone, not adjacent to the blastopore, in a region that overlapped with the most anterior expression of *chd*, and the posterior expression of *cer* and *gsc*. The mesodermal expression continued after gastrulation, where *Xiro-1* was easily observed in the notochord of the sectioned embryo (Fig. 1F), as well as in the somites of stage 25 embryos (Fig. 1G,H). At the tailbud stage, *Xiro-1* expression could be detected in somites, neural tube and notochord. (Fig. 1G,H).

Xiro-1 Is Able to Induce a Secondary Axis and to Control Mesodermal Patterning by Acting as a Repressor

Xiro-1 function was studied by injecting mRNAs encoding several chimeric forms of the Xiro-1 protein

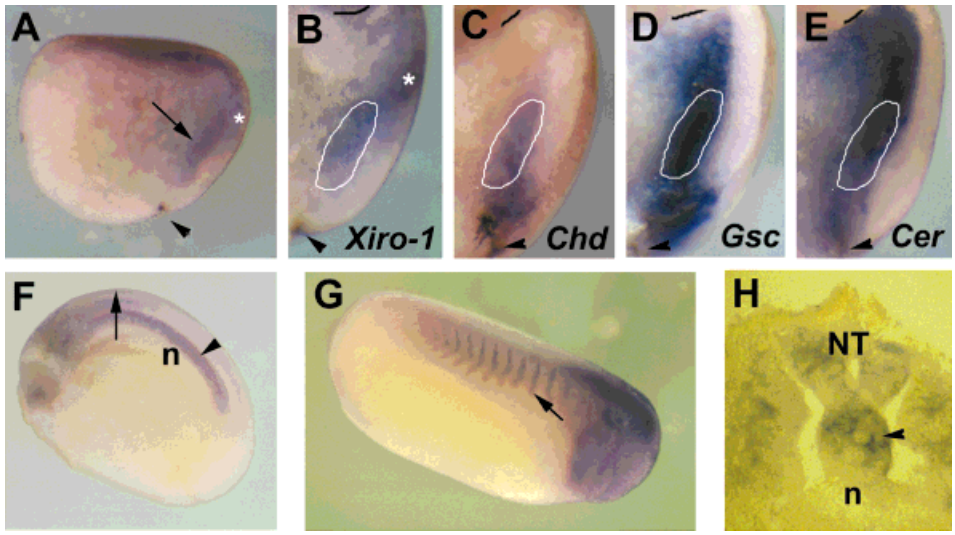

Fig. 1. *Xiro-1* expression. **A:** Saggital sections of a stage 10+ embryos were analyzed by in situ hybridization for the expression of *Xiro-1* (arrow). Arrowhead: blastopore; asterisk: ectodermal expression. **B–E:** Embryos were fixed at stage 10+ and after in situ hybridization, were sectioned into halves to examine internal gene expression. The border of the blastocoel cavity is indicated by a black line (top); the arrowhead indicates the dorsal blastopore lip (bottom). B: *Xiro-1* expression in the involuting marginal zone is indicated by a white line; asterisk: ectodermal expression. C: *Chd* expression; D: *Gsc* expression; E: *Cer* expression. The expression of *Xiro-1* is indicated in all three cases (C–E) to show its overlapping expression with *Chd, Gsc*, and *Cer*. **F:** After gastrulation, *Xiro-1* expression is detected in the ectoderm (arrow), notochord (n, arrowhead), (saggital section stage 17). **G:** At stage 25, *Xiro-1* is located in the neural tube and can easily be seen in the somites (arrow). **H:** A section from a stage-25 embryo following in situ hybridization for *Xiro-1*. Expression is observed in the notochord (arrowhead), somites and neural tube (n: notochord; NT: neural tube).

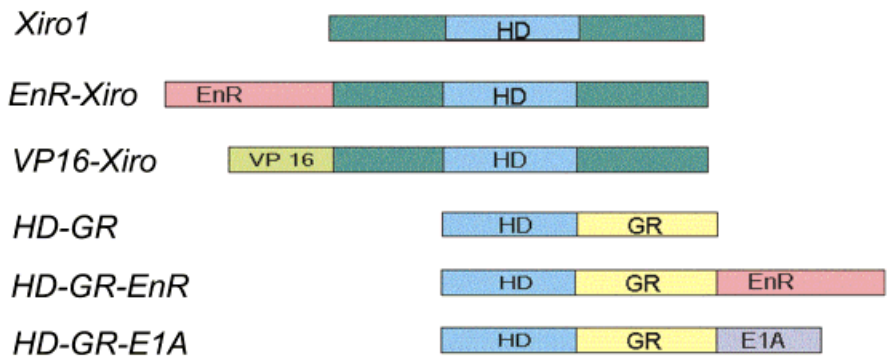

Fig. 2. Schematic representation of wild-type and *Xiro1* constructs. The different colors in the constructs indicate the different regions of the genes and its relative position within the quimeric proteins. The homeodomain (HD) is indicated in light blue. The repressor domain of *engrailed* (EnR) and the VP16 transactivator domain (VP16) are indicated in pink and green, respectively. The inducible proteins include the glucocorticoid binding domain (yellow) and the E1A activator domain (purple).

(Fig. 2). Fusion proteins (Gómez-Skarmeta et al., 2001) were constructed either using the repressor domain of Engrailed (EnR) or the activation domain of the viral protein VP16, and a *Xiro-1* fragment comprising most of the protein (constructs: *EnR-Xiro* and *VP16-Xiro*). Additional constructs employing only the Xiro-1 homeodomain (HD) were also made as well as inducible proteins through fusion with the glucocorticoid-binding domain (GR) of the glucocorticoid receptor (*HD-EnR, HD-E1A, HD-GR-EnR* and *HD-GR-E1A*).

Injection of *Xiro-1, EnR-Xiro*, or *HD-GR-EnR* at the 2-cell stage was found to cause a partial secondary axis (Table 1). The frequency of the secondary axis was higher with the *HD-GR-EnR* construct than with *EnR-Xiro*, showing a higher efficiency for the inducible system, which is probably related to the higher stability of the GR protein compared to the Xiro protein. Injection of *HD-GR-E1A* inhibited the development of dorso-anterior structures, and the embryos exhibited absence of eyes and other cephalic structures and reduced cement glands (not shown). To explain the secondary axis induced by *Xiro-1* and taking into account that *Xiro-1* is expressed in the Spemann organizer, we decided to analyze whether the expression of organizer genes was affected by these treatments. Embryos were injected with *Xiro-1* or *EnR-Xiro* mRNA in the equatorial region of one blastomere of 2-cell stage embryos, and the expression of the *chd* and *gsc* genes was analyzed at the gastrula stage. Injection of *Xiro-1* or *EnR-Xiro* was seen to induce ectopic expression of the dorsal markers *chd* and *gsc* on the injected side (Fig. 3A–D, Table 1). The observation that *Xiro-1* and its repressor forms produced similar effects suggested that *Xiro-1* could function as a repressor in the mesoderm. To further analyze this possibility, we tested the effect of VP16 and E1A constructs. The expression of *chd*, *gsc*, and

TABLE 1. Effect of *Xiro1* in Mesodermal Patterning

	% of induction (number of embryos)[a]			% of repression (number of embryos)[a]		
	Secondary axis	*chordin*	*gsc*	*Xvent-1*	*Xwnt-8*	*bmp-4*
Xiro1	23 (n = 149)	37 (n = 104)	34 (n = 95)	89 (n=162)	91 (n=172)	95 (n=41)
EnR-Xiro	22 (n = 44)	38 (n = 132)	54 (n = 131)	92 (n=158)	97 (n=129)	98 (n=53)
	% of inhibition (number of embryos)[b]					
VP16-Xiro	63 (n = 67)	63 (n = 89)	60 (n = 62)	—	—	—

[a]Injections were made in one blastomere of 4 cell stage embryos in the presumtive ventral (a) or dorsal (b) marginal zone.

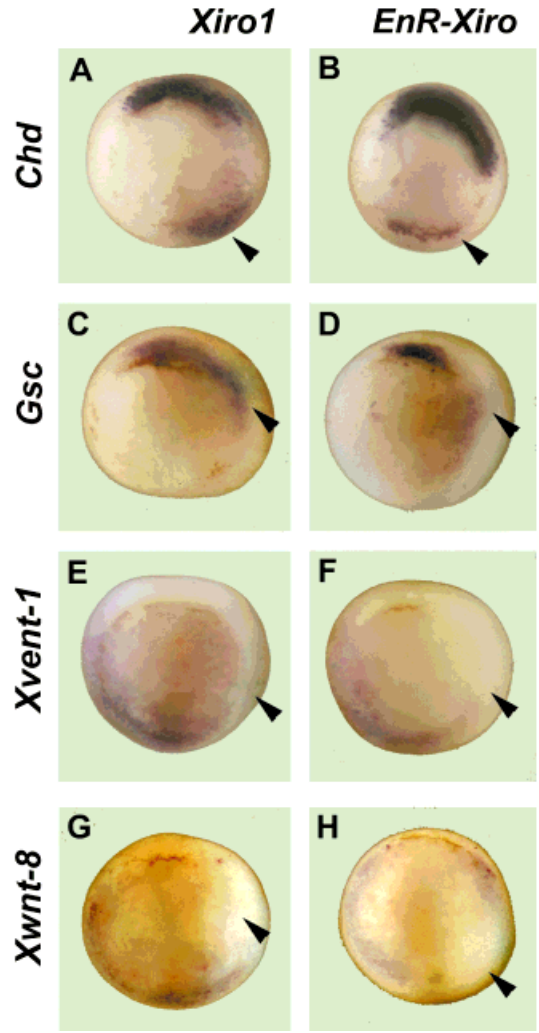

Fig. 3. *Xiro-1* regulates the expression of mesodermal genes by repression. When injected into one blastomere of 2-cell stage embryos, in the presumptive ventro-lateral mesodermal region, 2 ng of either *Xiro-1* (**A,C,E,G**) or *EnR-Xiro* (**B,D,F,H**) produced the ectopic expression of *chordin* (A and B: arrowheads, A: 37% n = 104 and B: 38% n = 132) and *goosecoid* (C and D: arrowheads, C: 34% n = 95 and D: 54% n = 131). Ventral genes were inhibited by these treatments. Inhibition of *Xvent-1* (E) and *Xwnt-8* (G) by *Xiro-1* was observed in 89 and 91% of cases, respectively (E and G: arrowheads, E: n = 162 and G: n = 172) while *EnR-Xiro* caused an inhibitory effect in 92% (F: arrowhead, n = 158) and 97% (H: arrowhead, n = 129) of cases, respectively. All figures shown are vegetal views of stage 10.25 embryos with the dorsal region at the top. Dexametasone treatment was started at stage 5 until the embryos were fixed.

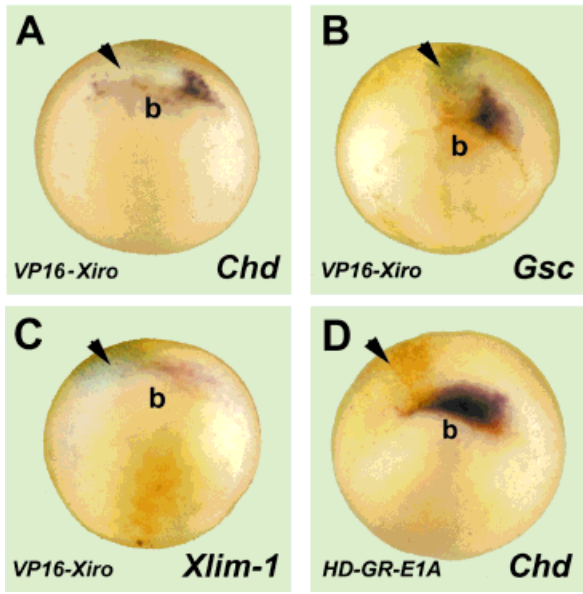

Fig. 4. *Xiro-1* activator fusion proteins cause repression of dorsal mesodermal genes. Overexpression of 2 ng of *VP16-Xiro* in the presumptive dorsal mesoderm region, in one blastomere of 2-cell stage embryos, represses the expression of *chordin* (**A:** arrowhead, 63% n = 89), *goosecoid* (**B:** arrowhead, 60% n = 62) and *Xlim-1* (**C:** arrowhead, 79% n = 76). The dorsal injection of 0.5 ng of *HD-GR-E1A* and Dexametasone treatment causes the same inhibition of *chordin* (**D:** arrowhead, 83% n = 32) and *goosecoid* (83% n = 30, not shown) expression (b: blastopore lip).

Xlim-1 was analyzed in embryos injected with *VP16-Xiro* and *HD-GR-E1A* (Fig. 4, Table 1). As these genes are normally expressed in the dorsal mesoderm, the injection was directed at the equatorial region of the dorsal side of the 2-cell stage embryo. Injection of both constructs inhibited the expression of *chd, gsc*, and *Xlim-1* genes (Fig. 4A–D, arrowheads). The similarity in the effect of *Xiro-1* and *EnR-Xiro* (inducible or not) and the opposite effect of the *HD-GR-E1A* and *VP16-Xiro* constructs, suggested that *Xiro-1* was likely to be a transcriptional repressor in the mesoderm.

As *Xiro-1* induces the expression of dorsal mesodermal genes into ventral mesoderm, we investigated the expression of ventral mesodermal genes. Embryos were injected with *Xiro-1* or *EnR-Xiro* in the equatorial region of one blastomere of 2-cell stage embryos, fixed at the gastrula stage, after which the expression of the

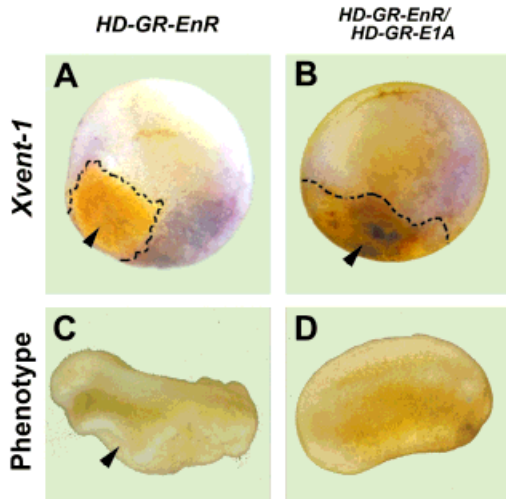

Fig. 5. The effects of *EnR-Xiro* are mediated via its homeodomain. The inhibition of *Xvent-1* caused by the injection of 0.5 ng of *HD-GR-EnR* (**A:** arrowhead, 94% n = 54) can be rescued by its coinjection with an equivalent amount of *HD-GR-E1A* (**B:** arrowhead, 22% n = 119). Myc immuno-staining is shown enclosed in a black line. The same result is observed upon analysis of the partial secondary axis produced by the injection of 0.5 ng of *HD-GR-EnR* (C arrowhead, 37% n = 30). Coinjection of equal amounts of *HD-GR-EnR* and *HD-GR-E1A* rescued the partial secondary axis phenotype (D, 0% n = 32).

TABLE 2. Effects of *Xiro* Can Be Rescued by Its Dominant Negative and *bmp-4*

	% of induction (number of embryos)	% of repression (number of embryos)
	Secondary axis	*Xvent-1*
HD-GR-EnR	35 (n = 80)	92 (n = 77)
HD-GR-EnR/HD-GR-E1A	0 (n = 72)	22 (n = 119)
HD-GR-EnR/BMP-4	0 (n = 90)	17 (n = 73)

ventral markers *Xvent-1* and *Xwnt-8* was analyzed (Fig. 3E–H). Both injections inhibited the expression of the *Xvent-1* and *Xwnt-8* markers. The ectopic expression of dorsal mesodermal genes induced by *Xiro-1* and the repression of ventral genes could explain the secondary axis induced by this gene.

In order to show the specificity of the effect of injecting the activator and the repressor *Xiro-1* constructs, we performed a rescue experiment. One blastomere of 2-cell stage embryos was injected with *HD-GR-EnR* or a mixture of *HD-GR-EnR* and *HD-GR-E1A* and treated with Dex at the blastula stage. In both constructs, a Myc tag was included so as to localize injected cells by Myc immunostaining. We analyzed both the expression of *Xvent-1* and the formation of a secondary axis. As described before, *HD-GR-EnR* repressed *Xvent-1* expression (Fig. 5A) and induced a secondary axis (Fig. 5C). However, both effects could be reversed by the coinjection of *HD-GR-E1A* (Fig. 5B,D, Table 2), indicating the specificity of the described effects.

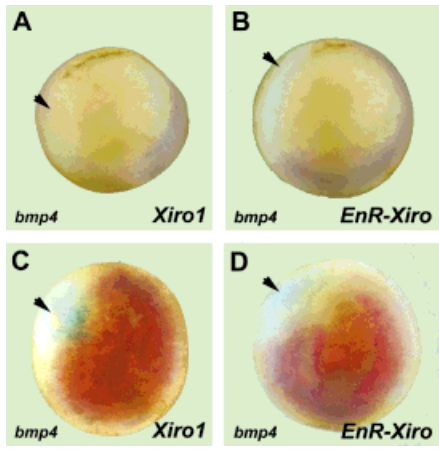

Fig. 6. *Xiro-1* represses *bmp-4* expression. Embryos were analyzed for *bmp-4* expression (**A,B:** vegetal view; **C,D:** animal view). Overexpression of 2 ng of *Xiro-1* or *EnR-Xiro* repressed the expression of *bmp-4* when injected into one blastomere of 4-cell stage embryos. *Xiro-1* inhibited *bmp-4* expression in the ventro-lateral mesoderm and ectoderm (A and C: arrowheads, 95% n = 41). *EnR-Xiro* behaved in the same manner, repressing *bmp-4* expression in 98% of cases (B and D: arrowheads, n = 53).

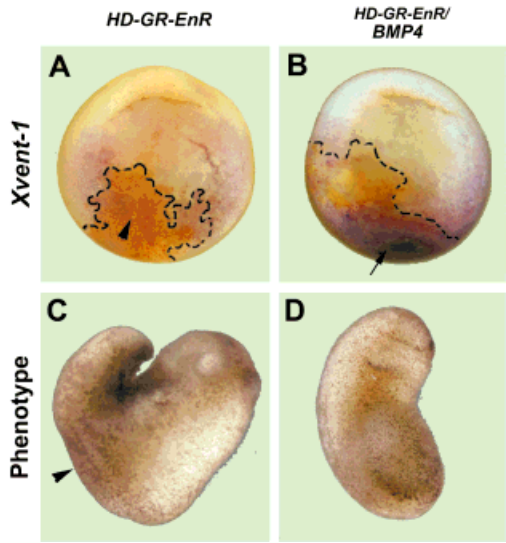

Fig. 7. The phenotype generated by *HD-GR-EnR* can be rescued by co-injecting with *bmp-4*. The injection of 0.5 ng of *HD-GR-EnR* into one ventral blastomere of a 4-cell stage embryo inhibited the expression of *Xvent-1* (**A:** arrowhead, 91% n = 23). Myc staining has been marked with a black line. **B:** Rescue of *Xvent-1* expression by coinjection with equal amounts (0.5 ng) of *HD-GR-EnR* and *bmp-4*. Arrow shows rescue of *Xvent-1* expression. The secondary axis generated by injecting 0.5 ng of *HD-GR-EnR* (**C:** arrowhead, 37% n = 30) seems to be due to the inhibition of *bmp-4*, since the injection of both *bmp-4* and *HD-GR-EnR* rescued the secondary axis phenotype (**D:** 0% n = 44).

Our results show that the repressor construct produces an effect similar to injection of *Xiro-1* and an opposite effect to that of the activator constructs, indicating that *Xiro-1* is likely to work as a transcriptional repressor in the mesoderm. Our results also show that

Xiro-1 induces dorsal mesodermal genes and inhibits ventral mesodermal genes, and as a consequence generates a secondary axis.

Xiro-1 Function Depends on *Bmp-4* Expression in the Mesoderm

BMP-4 has been shown to be a ventralizing agent that acts upstream of many of the genes analyzed in this study. In order to investigate whether *Xiro-1* was able to repress *bmp-4* expression, one blastomere of 4-cell stage embryos was injected with *Xiro-1* or *EnR-Xiro* and the expression of *bmp-4* analyzed. Injections were targeted either at the equatorial region or the animal hemisphere. A clear inhibition of *bmp-4* expression was observed on the injected side, in the presumptive mesoderm (Fig. 6A and B, Table 1) as well as in the presumptive ectoderm (Fig. 6C and D), confirming previous observations (Gómez-Skarmeta et al., 2001). As the inhibition of *bmp-4* is able to dorsalize the embryo and generate all the effects described here for *Xiro-1* overexpression, we analyzed whether these effects could be rescued by coinjection of *bmp-4* mRNA. Embryos were injected with *HD-GR-EnR* and treated with Dex as previously described, in order to induce the inhibition of *Xvent-1* and the secondary axis (Fig. 7A and C). However, upon coinjection of these embryos with *bmp-4* mRNA, *Xvent-1* expression was rescued and formation of a secondary axis was inhibited (Fig. 7B and D, Table 2). These results show that the effects induced by *Xiro-1* here described are dependent on *bmp-4* expression.

DISCUSSION

The *Xiro-1* and *-2* genes have been implicated in the control of neural plate development and have been shown to regulate the expression of proneural genes such as *Xash-3, ATH-3*, and *neurogenin* (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998). The effect on neural plate development was recently shown to be dependent on the repressor activity of *Xiro-1* on *bmp-4* transcription in the ectoderm (Gómez-Skarmeta et al., 2001). In this study, we have demonstrated a new function for the *Xiro-1* gene in dorsal mesoderm development.

We have shown for the first time that *Xiro-1* is expressed in the organizer region and that it is able to control the expression of organizer genes such as *chd* and *gsc*. The expression of *Xiro-1* in the involuting marginal zone, in a region that overlaps with the expression of other Spemann organizer genes (*chd, gsc, cer*), is compatible with a regulatory function in the expression of organizer genes. By making use of chimeric Xiro-1 proteins containing the transcriptional repressor or activator domains of known proteins, we were able to analyze whether *Xiro-1* functioned as an activator or repressor of mesodermal genes. *Xiro-1* and *EnR-Xiro* produced the same effects in injected embryos, while *VP16-Xiro* and *HD-GR-E1A* generated the opposite phenotypes, suggesting a likely role for *Xiro-1* as a transcriptional repressor (Lemaire et al., 1998; Latinkic and Smith, 1999).

The observation that *Xiro-1* acts as a transcriptional repressor that is also able to induce the expression of dorsal mesodermal genes suggests that it affects these genes indirectly. This effect could be mediated via the repression of another factor that in turn represses the expression of the dorsal genes. Such a factor is probably BMP-4. Indeed, *Xiro-1* inhibits the expression of ventral genes, including *bmp-4* transcription. Thus, it may be suggested that *Xiro-1* is expressed in the dorsal mesoderm where it helps repress *bmp-4* expression. *Xiro-1* injection in the ventral mesoderm would lead to decreased levels of BMP-4 activity, inducing the dorsalization of this tissue in a manner similar to that observed with the injection of a dominant negative form of the BMP receptor (Schmidt et al., 1995; Hoppler and Moon, 1998). Since interference with BMP-4 signaling has been shown to suppress *Xwnt-8* expression, Xiro-1 effect on *Xwnt-8* is likely to be due to reduction of BMP-4 activity (Schmidt et al., 1995; Hoppler and Moon, 1998).

Organizer signals induce heart, kidney, and somites in the dorsalization process. It has been proposed that this dorso-ventral pattern of mesoderm differentiation is induced by a gradient of BMP-4 activity generated in the mesoderm (Dosch et al., 1997; Eimon and Harland, 1999). This gradient arises from the interaction between BMP-4 and organizer secreted proteins such as Noggin and Chordin, which bind and prevent BMP-4 signaling (Zimmermann et al., 1996; Piccolo et al., 1996). Although *bmp-4* is expressed in the dorsal mesoderm at the start of gastrulation, its expression disappears from that region at the mid gastrula stage (Schmidt et al., 1995; Hemmati-Brivanlou and Thomsen, 1995). At that stage, the BMP binding proteins such as Noggin and Chordin are being secreted by the organizer, although it has been shown that their activity is unable to down regulate *bmp-4* expression (Baker et al., 1999). Our results show that *Xiro-1* represses *bmp-4* expression in the dorsal mesoderm, although we cannot rule out the possibility that this regulation could not be direct. While *bmp-4* is initially expressed in the entire marginal zone, *Xiro-1* is transcribed in the Spemann organizer, probably as a consequence of the inductive signals that induce the organizer itself. *Xiro-1* is then able to repress the transcription of *bmp-4* in a region of the dorsal mesoderm, and contribute to the dorsalization of this tissue. Baker et al. (1999) have shown that Wnt signals act as repressors of *bmp-4* transcription on the dorsal side of the embryo. It is interesting to note that *Xiro-1* was found to be activated by Wnt signals in the ectoderm region (Gómez-Skarmeta et al., 2001). It is tempting to propose that the Wnt signals involved in the activation of the Niewkoop center may be responsible for the induction of *Xiro-1* in the dorsal mesoderm. As *Xiro-1* expression does not encompass the entire region where *bmp-4* is down regulated, it is possible to propose that additional

factors also participates in *bmp-4* repression. One of these factors could be the homologue of the zebrafish gene *bozozok*, which promote anterior neuroectoderm formation through negative regulation of BMP2/4 and Wnt pathways (Melby et al., 2000; Sirotkin et al., 2000; Fekany-Lee et al., 2000).

Both Gómez-Skarmeta et al. (2001) and ourselves have observed that *Xiro-1* induces the down regulation of *bmp-4* not only in the mesoderm but also in the ectoderm (Fig. 6). This decrease in BMP-4 leads to the neuralization of the ectoderm and the expansion of proneural gene expression described previously for *Xiro* injection studies (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998). Thus, the enlarged neural plate produced by the injection of *Xiro* mRNA described previously (Gómez-Skarmeta et al., 1998; 2001) is likely the result of the dorsalization of the mesoderm followed by the neural induction of the ectoderm in a bigger region. However, Gómez-Skarmeta et al. (2001) have reported that embryos injected with *HD-EnR* or *HD-GR-EnR* failed to show modification of the mesoderm. Conversely, our results show a clear modification of mesodermal patterning by *Xiro-1* overexpression and an induction of a secondary axis. An explanation for this discrepancy may be related to the site of injection. Gómez-Skarmeta et al. (2001) performed injections in the animal hemisphere whereas our injections were targeted at the equatorial region. In the present study, we compared both kinds of injections in specific experiments, as shown for example in Figure 6. It is clear that the differential repression of *bmp-4* in the ectoderm or mesoderm will depend on the site of injection. Thus, our results support a reinterpretation of previously published experiments (Bellefroid et al, 1998; Gómez-Skarmeta et al., 1998; 2001) and they are important in the correct interpretation of null mutations of the *Iro* genes performed in mouse.

Although our results support the conclusion that *Xiro-1* is a repressor of *bmp-4*, certain findings cannot be entirely explained by this model. If *Xiro-1* functioned only by repressing *bmp-4*, *Xiro-1* injections would generate the same phenotype as injections of a dominant negative form of the BMP-4 receptor. Although both treatments cause dorsalization, there are nonetheless some differences. Firstly, *Xiro-1* is unable to induce a complete secondary axis, and, secondly, *Xiro-1* causes a reduction in the expression of neural crest markers, while the dominant negative BMP-4 receptor induces the expansion of those genes (Marchant et al., 1998). A possible explanation for this discrepancy may be in the different mechanisms by which BMP-4 activity is repressed. Whereas *Xiro-1* regulates transcription, the dominant negative BMP-4 receptor regulates signal transduction, so that each treatment may give rise to very different levels of BMP-4 activity. The induction of neural crest cells has been shown to require very specific levels of BMP-4, which are unlikely to be reached through the inhibition of its transcription (Marchant et al., 1998; Nguyen et al., 1998). Alternatively, the observed differences could be explained through the action of *Xiro-1* on other targets. *Xiro-1* is expressed in the posterior region of the neural plate and is induced in the ectoderm by a combination of neural inducers and posteriorizing agents (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998). It is possible that *Xiro-1* could have a posteriorizing activity that may explain its inability to induce a secondary axis with a normal head.

MATERIALS AND METHODS

Plasmid Constructions, In Vitro RNA Synthesis, and Microinjection of mRNAs

Plasmid constructs are described in Gómez-Skarmeta et al. (2001). All cDNAs were linearized and transcribed, as described by 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. mRNA for injection was resuspended in DEPC-water and injected using 8–12-nl needles in 2- or 4-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), *chordin* (Sasai et al., 1994), *cerberus* (Bouwmeester et al., 1996), *goosecoid* (Cho et al., 1991), *Xlim-1* (Taira et al., 1992), *Xwnt-8* (Christian et al., 1991), *Xvent-1* (Gawantka et al., 1995), *bmp-4* (Hemmati-Brivanlou and Thomsen, 1995) were synthesized from cDNAs using digoxigenin (Boehringer Mannheim) as a label. Specimens were prepared, hybridized, and stained using the method of Harland (1991). X-Gal staining was performed according to Coffman et al. (1993). Antibody staining was performed after in situ hybridization of the embryos using mouse monoclonal anti Myc from BabCo, and according to the method described by Turner and Weintraub (1994). Histology was performed as described in Mayor et al. (2000).

Embryos and Dexametasone Treatments

Xenopus embryos were obtained as described previously (Gómez-Skarmeta et al., 1998) and staged according to Niewkoop and Faber (1967). Dexametasone treatment was performed as described by Kolm and Sive (1995). Dexametasone was included in the culture medium at stage 5 and maintained until the embryos were fixed.

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