XIPOU 2 is a potential regulator of Spemann’s Organizer

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SUMMARY

XIPOU 2, a member of the class III POU-domain family, is expressed initially at mid-blastula transition (MBT) and during gastrulation in the entire marginal zone mesoderm, including Spemann’s Organizer (the Organizer). To identify potential targets of XIPOU 2, the interaction of XIPOU 2 with other genes co-expressed in the Organizer was examined by microinjecting XIPOU 2’s mRNA into the lineage of cells that contributes to the Organizer, head mesenchyme and prechordal plate. XIPOU 2 suppresses the expression of a number of dorsal mesoderm-specific genes, including gsc, Xlim-1, Xotz2, noggin and chordin, but not Xnot. As a consequence of the suppression of dorsal mesoderm gene expression, bone morphogenetic factor-4 (Bmp-4), a potent inducer of ventral mesoderm, is activated in the Organizer. Gsc is a potential target of XIPOU 2. XIPOU 2 is capable of binding a class III POU protein binding site (CATTAAT) that is located within the gsc promoter, in the activin-inducible (distal) element. Furthermore, XIPOU 2 suppresses the activation of the gsc promoter by activin signaling. At the neurula and tailbud stages, dorsoanterior structures are affected: embryos displayed microphthalmia and the loss of the first branchial arch, as detected by the expression of pax-6, Xotz2 and en-2. By examining events downstream from the Wnt and chordin pathways, we determined that XIPOU 2, when overexpressed, acts specifically in the Organizer, downstream from GSK-3β of the Wnt pathway and upstream from chordin. The interference in dorsalizing events caused by XIPOU 2 was rescued by chordin. Thus, in addition to its direct neuralizing ability, in a different context, XIPOU 2 has the potential to antagonize dorsalizing events in the Organizer.

Key words: XIPOU 2, POU domain, Spemann’s Organizer, Xenopus

INTRODUCTION

During early cleavage stages, a maternal set of intercellular signaling molecules produced by the dorsovegetal blastomeres of an embryo will determine the pattern of tissues found in the late blastula stage Xenopus embryo. Candidates for these maternal signals are capable of directly inducing mesoderm, and one promising candidate is vg1, a transforming growth factor β (TGF β) superfamily member (Thomsen and Melton, 1993; Kessler and Melton, 1995). Other signaling molecules do not directly induce mesoderm but are capable of modifying the type of mesoderm that is produced, and include noggin (Smith et al., 1993) and wnt (Sokol et al., 1991; Smith and Harland, 1991; Christian et al., 1991). All of these signaling molecules can rescue UV ventralized embryos and induce a complete secondary axis (reviewed by Kessler and Melton, 1995). One major consequence of these signaling events is to produce Spemann’s Organizer (the Organizer; Spemann, 1938, reprinted 1967).

A second wave of embryonic inducers is synthesized beginning at mid-blastula transition (MBT), the onset of zygotic transcription, and continues to be synthesized during gastrulation. These inducers, produced by the Organizer, regulate important events of early gastrulation by dorsalizing and patterning the mesoderm (Cunliffe and Smith, 1994), and by neuralizing and patterning the ectoderm to form neural tissue (Ruiz i Altalba, 1994; Green, 1994; Harland, 1994; Chitnis and Kintner, 1995). There are several candidate signaling molecules that are localized in Spemann’s Organizer and exhibit some Organizer-like functions or can modulate Organizer function. These putative inducing molecules include several members of the TGF β superfamily, including nodal-related signals (Smith et al., 1995; Jones et al., 1995), which induce mesoderm, and ADMP (Moos et al., 1995), which can modulate the dorsalizing activity of the Organizer. Three secreted molecules, follistatin, an antagonist of activin (Hemmati-Brivanlou et al., 1994), chordin (Sasai et al., 1994, 1995) and noggin (Lamb et al., 1993) can act as direct neural inducers. Additionally, chordin and noggin can dorsalize ventral mesoderm.

In addition to factors produced by the Organizer, there are also potent ventralizing signals produced bythe ventral marginal zone of gastrulating Xenopus embryos, such as Bmp-4 (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Schmidt et al., 1996) and a number of transcriptional regulators that mediate the action of Bmp-4, including vent-1 (Gawantka et al., 1995), Xom (Ladher et al., 1996), Vox (Schmidt et al., 1996) and PV1 (Ault et al., 1996). Bmp-4 can eliminate the expression of all dorsal markers when it is expressed on the dorsal side of a Xenopus embryo (Schmidt et al., 1996). Both chordin and noggin bind directly to Bmp-4, inactivating Bmp-4, thus accounting for their dorsalizing and neutralizing properties (Piccolo et al., 1996; Zimmerman et al., 1996).
In addition to signaling molecules and secreted molecules, there are a number of potential transcriptional regulators that are activated at MBT and expressed in the Organizer. XlPOU2 is one of many potential transcriptional regulators that is expressed in the dorsal lip, including Xnot (von Dassow et al., 1993), gsc (Blumberg et al., 1991), Xlim-1 (Taira et al., 1992), XFKH1/pintalavis (Dirksen and Jamrich, 1992; Ruiz i Altaba and Jessell, 1992), XANF-1 (Zaraisky et al., 1995), Xotx2 (Blitz and Cho, 1995; Pannese et al., 1995) and Xbra (Smith et al., 1991), and, based on their expression patterns (Vodicka and Gerhart, 1995), it is probable that these gene products act in collaboration with each other to define Organizer function at the level of transcriptional control. In addition, XlPOU2’s expression in the endoderm during gastrulation (Witta et al., 1995) may overlap with that of siamois (Lemaire et al., 1995), a homeobox gene and a proposed mediator of the late blastula Organizer (Carnac et al., 1996). These transcriptional regulators can act as downstream determinants to the signaling pathways and, like the signals themselves, have Organizer-like properties. For example, gsc (Cho et al., 1991), XANF-1 (Zaraisky et al., 1995) and Xnot-2 (Gont et al., 1996) induce a partial secondary axis, and siamois (Lemaire et al., 1995) induces a complete secondary axis, when misexpressed in the ventrovegetative region of the embryo. We have previously identified a POU domain gene, XlPOU2, that has direct neuralizing capability and appears to be downstream from the neural inducer, noggin. When misexpressed in uncommitted ectoderm, XlPOU2 is capable of switching cells towards a neural fate (Witta et al., 1995). Interestingly, although expressed in the Organizer, XlPOU2 does not produce a secondary axis when injected into the ventrovegetal marginal zone, and it is not an immediate response gene to the mesoderm inducer, activin.

In this study, we have addressed the function of XlPOU2 in the Organizer by three means. First, by overexpressing XlPOU2 in the dorsal and ventral marginal zones of the embryo, potential interacting genes were identified. The expression of several important Organizer-specific genes during gastrulation including gsc, Xlim-1, Xbra, chd and noggin were suppressed, resulting in the activation of Bmp-4 in the dorsal lip. In later development, these events led to the restricted disruption of the eye and first branchial arch development. Second, using in vitro studies, at least one potential target gene of XlPOU2, gsc, was identified. XlPOU2 binds specifically to the activin-inducible and Wnt-inducible elements within the gsc promoter. In a functional assay, XlPOU2 suppresses the activation of the gsc promoter by activin. Third, to identify the temporal and spatial window of XlPOU2’s function, the interaction of XlPOU2 with the dorsaling signaling pathways of Wnt and chordin was examined. XlPOU2 acted downstream from GSK-3β in the Wnt signaling pathway and upstream from the chordin, thus narrowing XlPOU2’s window of action between MBT and early gastrulation. Thus, in addition to its neuralizing ability, XlPOU2 has the potential to modulate, specifically, the function of the Organizer, lending credence to the hypothesis that transcriptional regulators co-expressed in the Organizer can interact with each other to define Organizer function.

MATERIALS AND METHODS

Microinjection studies

Eggs were fertilized in vitro, dejellied with 2% cysteine, pH 7.8, and allowed to develop in 0.1x MMR until the 4-cell stage (Newport and Kirschner, 1982). At the 4-cell stage, embryos were transferred into 2% Ficoll/0.5x MMR and arranged on a grid. At the 8- to 16-cell stage, 200 to 1000 pg of mRNA (in 3 nl) was injected into one or two ventrovegetal, or dorsovegetal blastomeres. CaprM RNA was prepared from linearized templates and mRNAs were injected as follows: 200 pg of mRNA synthesized from plasmids encoding the open reading frame of XlPOU2, SP64T XlPOU2, or a truncated form of XlPOU2, SP64T tXlPOU2 (Witta et al., 1995); 1 ng of mRNA synthesized from the plasmid, SP64T POU domain only; 1 ng of mRNA synthesized from a plasmid encoding a mutant glycogen synthase kinase-3β, 3βKM (He et al., 1995); 200 pg of mRNA synthesized from a plasmid encoding chordin, pSP35-chd (Sasai et al., 1994); 700 pg mRNA synthesized from a plasmid encoding a mutant Xlim-1, 3m-Xlim1 (Taira et al., 1995).

Lineage analysis

To follow the lineage of the injected blastomeres, 200 pg of β-galactosidase mRNA was co-injected with the various mRNAs (Detrich et al., 1990). Embryos were allowed to develop until the gastrula, neurula, tailbud or tadpole stage, and fixed with MEMFA for 1 hour at room temperature (Harland, 1991). Fixed embryos were stained with red-gal, a substrate for β-galactosidase (Research Organics) (Turner and Weintraub, 1994). Following the β-galactosidase staining, embryos were rinsed in PBS, refixed in MEMFA for 2 hours at room temperature, dehydrated in ethanol and stored at ~20°C. The lineage tracer in all injected embryos was checked to ensure that the proper lineage was injected before proceeding to the in situ hybridization analysis. In many cases, the red-gal-staining product is obscured by the intense blue reaction product from the alkaline phosphatase reaction.

In situ hybridization

The whole-mount in situ hybridization procedure was performed as described previously (Harland, 1991; Knecht et al., 1995). Embryos were staged according to Nieuwkoop and Faber, 1967. RNA antisense probes were prepared from linearized templates as described previously (Witta et al., 1995). At the gastrula stage, the expression of gsc (Blumberg et al., 1991), Xlim1 (Taira et al., 1992), chordin (Sasai et al., 1994), Xpo (Sato and Sargent, 1991), Xbra (Smith et al., 1991), Xnot (von Dassow et al., 1993), PV1 (Ault et al., 1996) and Bmp-4 (Fainsod et al., 1994) was examined. At the neurula and tailbud stages, the expression of pax-6 (Espineth et al., 1995), Xotx2 (Blitz and Cho, 1995) and en-2 (Hemmati-Brivanlou et al., 1991) was examined. Following the in situ hybridization analyses, cleared embryos were photographed in dark field using a Zeiss Axioshot microscope and Kodachrome 40 film or Kodak 320T film, or uncleared embryos at the gastrula stage were photographed using a Zeiss dissecting scope.

RT-PCR analysis

1 μg of total RNA, isolated from injected embryos at stages 10.5-11 was digested with deoxyribonuclease I (Life Technologies) at 37°C for 15 minutes and reverse transcribed (RT) using Superscript II (Life Technologies). One tenth of the RT reaction was used as a template in the polymerase chain reaction (PCR). The conditions for the PCR were as follows: denaturation (94°C, 0.5 minutes), annealing (55°C, 0.5 minutes) and elongation (72°C, 0.5 minutes), 25 cycles, using a Perkin-Elmer 9600 cyt. A trace amount of [α-32P]dCTP (1.5 μCi) was added to each of the reactions. The primers used in the PCR were as follows: gsc, forward primer (R) 5’-CAACTGGAGAGCTCGGA-3’, reverse primer (R) 5’-TCTTATCTTACAGGACC-3’, Xbra, F 5’-GGATCTTATCTACCCTCCT-3’, R 5’-GTGATCTTCTGAGCC-3’; mix.1, F 5’-AATGCTCTCAAGCGAGAGG-3’, R 5’-GTGTGCTACGTGACACGAGA-3’, Xwnt-8, F 5’-AGATGACGGCATTCCAG-3’, R 5’-TCTCTTCCATCTTACCA-3’; noggin, F 5’-AGTTCCAGATGAATGCTCT-3’, R 5’-AGTCCAAATGACTGCAAC-3’, Efa, F 5’-CAATGGCTGCTGATGATGC-3’, R 5’-ACCTGCTTGTGACACAGC-3’; Xlim1, F 5’-CAATGGCTGCTGATGATGC-3’, R 5’-ACCTGCTTGTGACACAGC-3’ (Hemmati-Brivanlou et al., 1994); chordin,
F 5'-GCACCAAACTGAATCTCTGGTGAATCGA-3', R 5'-GCACTGACTCG
GCAATGGAATGGC-3', Xpo, F 5'-GCTATGCATGCTCTTATAGGCA-3', R 5'-CAGTTCATCAGTTATACACAGGG-3', Xlim-I, F 5'-GAAGATGGA-CACTGAGTGGTGTGG-3', R 5'-CAGTCGGTCATGGTTGCATTC-3', BMP-4, F 5'-AAACACTGGCCGAGCAGCATC-3', R 5'-AAGTC
CAGCGTAAACAGTC-3' (Moos et al., 1995); PV1, F 5'-AAGGAT
GATAAAAGCGAAGGTTAT-3', R 5'-GATTGAGTTGATGCAGT
GTTGATCTCCATCA-3' (Ault, personal communication). One tenth of each PCR was subjected to electrophoresis using a 6% tris-borate gel and the dried gel was exposed to Kodak X-Ray film overnight.

Gel mobility shift analysis
Synthetic oligonucleotides complementary to the distal (CCCCAT
TTTCCTAATGGGAGTTGATGATGTTTATACACAGGG-3') and proximal (GGTTTGTCTAACTGGAATGGAATGG-3') elements of the gsc promoter or mutant elements of these sequences (CCCCAAGCTTGAGGCGCCCTGAC
CAATTAG; CCCCAGCTCAGTTACTACAGGTTGACCAATTA
CCCACTAATGCGGCGGACCAATTTAG; mutagenesis in the proximal element) or (GGTTTGTCTAATGGGAGTTGATGATG
TTTATACACAGGG-3', R 5'-AAACACTGGCCGAGCAGCATC-3', R 5'-AAGTCAGCGTAAACAGTC-3', mutations in the proximal element), as originally characterized by Watabe et al. (1993), were used as probes for mobility gel shift assays. Both the distal and proximal elements of the gsc promoter contain the wild-type, class III POU domain protein binding motif, CATnTAAT, n = 0, 2, or 3 (Li et al., 1993; this motif is depicted in bold lettering above). Oligonucleotides were end-labeled with [γ-32P]-ATP using T4 DNA kinase (Life Technologies/BRL), annealed and gel-purified. Mobility shift assays were performed with 5 µl of programmed rabbit reticulocyte lysate (Promega) in 20 mM Hepes (pH 7.9), 1 mM dithiothreitol, 10% (v/v) glycerol, 2 mM MgCl2, 0.1 mg/ml BSA and 2 µg of poly(dI-dC) (Pharmacia) in a total volume of 20 µl. The efficiency of the synthesis of the truncated XIPOU 2 or full-length XIPOU 2 proteins was monitored and shown to be nearly identical using 35 S-Met incorporation. Reactions were incubated for 10 minutes on ice, and then the radiolabeled, double-stranded oligonucleotides were added (1×104 cts/minute). The reaction continued for 20 minutes at room temperature. The formation of specific DNA-protein complexes were confirmed in competition assays by incubating the programmed lysate with a 50-fold molar excess of a competitor for 15 minutes on ice before adding a labeled probe. Samples were loaded onto a 5% polyacrylamide gel and run with 0.5x TBE buffer for 2 hours at 100 V. Gels were dried and exposed to Kodak XAR-5 film with intensifying screens at -80°C.

Analysis of the gsc promoter
For the analysis of the gsc promoter, XIPOU 2 or β-galactosidase capped mRNA (200 pg/blastomere) was injected into four animal pole blastomeres at the 8-cell stage with 25 pg/blastomere of −226gsc/Luc DNA (Watabe et al., 1995). For examining the activin-inducible element within the gsc promoter, stage 8 animal cap ectoderm was incubated in 5-50 units/ml of purified bovine activin A (Biosource Int'l.). For examining the wnt-inducible element within the gsc promoter, animal pole blastomeres (8-cell stage) were co-injected with Xwnt-8 mRNA, as described in Watabe et al. (1995), and animal cap ectoderm was prepared at stage 8. For each set of experiments, six samples (five animal caps/sample) were harvested when sibling embryos reached stage 11 and then analyzed for luciferase activity (Promega).

RESULTS

XIPOU 2 suppresses the expression of dorsal mesoderm-specific genes and activates Bmp-4 in the dorsal lip

XIPOU 2 is expressed in the Organizer in early gastrulation, in the entire marginal zone mesoderm during mid-gastrulation, and in the dorsal mesoderm in late gastrulation. To identify potential interacting genes, XIPOU 2 was overexpressed in the dorsal or ventral marginal zones, and embryos were examined for alterations in the expression of several mesoderm markers during gastrulation. XIPOU 2 mRNA was injected into two dorsovegetal blastomeres at the 8-cell stage. Injected embryos were evaluated at the gastrula stage (stage 10.5 or stage 11) for the expression of the following markers: gsc, Xlim-1, chordin,
mix.1, Xwnt8, Xbra, Xpo, noggin, Xnot, PV.1 and Bmp-4 (Blumberg et al., 1991; Taira et al., 1992; Sasai et al., 1994; Rosa, 1989; Smith and Harland, 1991; Smith et al., 1991; Sato and Sargent, 1991; Smith and Harland, 1992; von Dassow et al., 1993; Ault et al., 1996, Fainsod et al., 1994, respectively). The most dorsal region of the Organizer was the most severely affected. A pronounced suppression in the expression of several markers of the Organizer, including chordin, gsc, Xlim-1 and noggin was detected by an in situ hybridization analysis and an RT-PCR assay (Figs 1B,FJ, 2A). The expression of the predominantly endodermal marker, mix.1, also was suppressed (Fig. 2A), and the most dorsal expression of Xbra was reduced, to a small degree, as shown by in situ hybridization analysis (Fig. 1L). At least one organizer-specific marker, Xnot, was unchanged (Fig. 1N). Interestingly, a pronounced activation of Bmp-4, a marker indicative of a ventralization of mesoderm, was detected in the Organizer (Figs 1D,1D’, 2B), although we did not observe the activation of three ventral mesoderm markers, Xpo, Xwnt-8 or PV.1 (Fig. 1H (Xpo); Fig. 3A-C).

To determine if XIPOU's actions were confined to dorsal mesoderm, we examined the ability of XIPOU 2 to suppress genes expressed in the ventrolateral mesoderm. In these experiments, XIPOU 2 mRNA was microinjected in two ventrogeatal cells at the 8-cell stage. The expression of ventral mesodermal markers, Bmp-4, PV.1, Xpo and Xwnt-8, and the general mesodermal marker, Xbra, was determined at stages 10.5-11. In these embryos, the expression of the three ventral mesodermal markers, Bmp-4, PV.1 and Xwnt-8 was not altered [Fig. 1T(PV.1); Fig. 2B,C]. However, in these injected embryos, we observed that, during gastrulation, the expression of Xbra (a general mesodermal marker) and Xpo (a ventrolateral mesodermal marker), was reduced slightly (Fig. 1R; Xpo, not shown). The decrease in the expression of Xbra was observed only in the in situ hybridization analysis, but not by the RT-PCR analysis. The ubiquitous marker, Efla, was unchanged in embryos after a dorsal or ventral injection of XIPOU 2 mRNA (Fig. 1P; ventral injection, not shown). We conclude that XIPOU 2 has the potential to suppress a selective set of genes in the dorsal and ventrolateral mesoderm. In the dorsal mesoderm, overexpression of XIPOU 2 leads to the activation of Bmp-4, ventralizing the embryo. Ultimately, injected XIPOU 2 mRNA affects only the head Organizer in Xenopus development.

The ability of XIPOU 2 to activate Bmp-4 in the dorsal lip is indirect

Does XIPOU 2 activate Bmp-4 directly? The overexpression of XIPOU 2 in the ventral marginal zone did not lead to an induction of Bmp-4 above the endogenous levels (Fig. 2B, lanes 3 and 4), so it is unlikely that XIPOU 2 can directly activate Bmp-4. To examine further this question, XIPOU 2 was expressed in the lineage that gives rise to the animal pole ectoderm by microinjecting XIPOU 2 mRNA into four animal pole blastomeres at the 8-cell stage. Uncommitted ectoderm was dissected at stage 8, animal cap ectoderm was harvested when sibling controls reached stage 10.5 and the induction of Bmp-4 was determined by an RT-PCR analysis. Bmp-4 is normally expressed in animal cap ectoderm at low levels at stage 10.5 (Fainsod et al., 1994) and these levels were not increased with the injection of XIPOU 2 mRNA (Fig. 2B, lanes 6-7). Note that in this experiment, as expected, Xbra is not present and levels of Xwnt-8 are unchanged (Fig. 2B, lanes 6-8). Therefore, it is likely that the elevation in Bmp-4 that was observed in the embryos after overexpressing XIPOU2 in the dorsal marginal zone resulted from a severe suppression of dorsal mesoderm gene activity, an event that can cause a premature activation of Bmp-4 (Fainsod et al., 1994).

XIPOU 2 interacts with the activin and Wnt responsive elements in the gsc promoter and suppresses the activation of gsc by activin

Since our experiments suggested that the suppression of gsc occurs prior to the activation of Bmp-4 in the dorsal lip, we asked whether the suppression of gsc expression was a direct action of XIPOU 2 on the transcription of gsc. The suppression of gsc by XIPOU 2 was investigated by examining two elements within the gsc promoter (Watabe et al., 1995). Using a gel mobility shift assay, we determined that XIPOU 2 can bind specificaly to both the distal and proximal elements within the gsc promoter (Fig. 3, lanes 3 and 19). When mutations are made within the distal (M1 through M3) or proximal elements, XIPOU 2 can no longer bind (lanes 7 and 11) or XIPOU 2’s ability to bind is severely reduced (lanes 15 and 23). These mutations fall within the sequences CATTAAT,
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1. Prefex binding site for class III POU domain proteins (Li et al., 1992) or ATT A, a binding site for homeobox proteins (Hoe y and Levine, 1988), and are the most likely binding sites of XIPOU 2.

2. After demonstrating that XIPOU 2 could bind the distal and proximal elements of the gsc promoter, we determined whether XIPOU 2 could suppress the ability of either activin or Wnt to activate the gsc promoter. For these experiments, we used the construct −226gsc/Luc, a construct that contains both the Wnt-inducible (proximal) and activin-inducible (distal) elements (Watabe et al., 1995). In these experiments, XIPOU 2 mRNA or control mRNA, β-galactosidase, was co-injected into four animal pole blastomers at the 8-cell stage with −226gsc/Luc DNA. In some experiments, Xwnt-8 mRNA was co-injected. Animal pole ectoderm was dissected at stages 8-9 and incubated with or without purified activin until approximately 5 hours later when sibling controls reached the mid-gastrula stage (stage 11). XIPOU 2 suppressed only the activin-induced activation of the gsc promoter (Fig. 4). There was no significant change in the activity of Wnt to activate the gsc promoter after the injection of XIPOU 2 mRNA (data not shown). Luciferase activity, indicative of the activin-induced activation of the gsc promoter, was reduced by greater than 90%. We conclude that XIPOU 2 has the potential to suppress gsc transcription directly. XIPOU 2 bound specifically to both the distal and proximal elements, but XIPOU 2 acting alone could suppress only the activin-induced activation of the gsc promoter. Therefore, gsc is a potential target of XIPOU 2’s action.

The loss of dorsal gene expression results in a suppression of the eye and facial structures

After observing a suppression in a select group of dorsal and ventral genes during gastrulation, we looked for phenotypic changes in embryos at later stages in development. After over-expressing XIPOU 2 in the dorsal marginal zone, embryos were evaluated for phenotypic changes by looking for alterations in the expression of genes specifically expressed in the head, eye and brain. 200 pg of XIPOU 2 mRNA was co-injected with β-galactosidase mRNA into one dorsovegetal blastomere of the 16-cell-stage embryo (Fig. 5A). The lineage of this blastomere contributes to the endoderm and the Organizer at the gastrula stage, and to the endoderm, the dorsal mesoderm and the head.

Luciferase activity

βgal/−226 gsc-luc
XIPPOU 2/−226 gsc-luc
βgal/−226 gsc-luc (+activin)
XIPPOU 2/−226 gsc-luc (+activin)

Fig. 4. XIPOU 2 suppresses the activin-induced activation of the gsc promoter. This analysis was performed as described in Materials and Methods. Each experiment was replicated 2-3 times.
The expression of pax-6, a paired box gene expressed in the eye and CNS, at the neurula stage, was examined. At this stage, injected embryos displayed a reduction of the eye field on the side ipsilateral to the blastomere injected with XlPOU2 (Fig. 5C). At the tailbud stage, asymmetry in head development was detected by a whole-mount in situ hybridization technique using Xotx2 and en-2 as markers (Blitz and Cho, 1995; Pannese et al., 1995; Hemmati-Brivanlou et al., 1991). A reduction in the eye field was observed using the marker Xotx2 (Fig. 5E). A defect in the first branchial arch was detected using the marker en-2 (Fig. 5G), while no phenotypic changes were detected in the CNS component of pax-6, Xotx2 or en-2 (Fig. 5C,E,G). No changes were observed in embryos injected with a control mRNA, tXlPOU2 (Fig. 5D,F,H), or an mRNA encoding the POU domain alone, or a related POU family member, XlPOU1 (data not shown). No phenotypic changes were observed in embryos in which two ventrovegetal blastomeres of the 8-cell-stage embryo were injected with XlPOU2 mRNA (data not shown).

Is XlPOU2’s action restricted to the Organizer or does it act later in the prechordal plate? The interaction of XlPOU2 with the signaling pathway of Wnt and the secreted factor, chordin, was examined. We investigated, first, whether XlPOU2 could interfere with the formation of the Organizer or, second, whether XlPOU2 could interfere with the ability of the Organizer to produce signaling molecules. We examined the ability of XlPOU2 to interfere with secondary axis formation produced by expressing, mutant GSK-3β, or chordin in the ventral marginal zone.

### Table 1. Interaction of XlPOU2 with GSK-3β, a component of the Wnt-like dorsalizing pathway, and with chordin

<table>
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<th>Complete 2^o axis</th>
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<th>73%</th>
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<td>96%</td>
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<td>40%</td>
<td>4%</td>
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**n** 16 30 25 27 30 60

VA, ventroanimal blastomeres; VV, ventrovegetal blastomeres.

**Fig. 5.** The overexpression of XlPOU2 leads to the suppression of the eye and facial structures as evaluated using a whole-mount in situ hybridization technique. One D2.1 blastomere of a 16-cell-stage embryo (A) was injected with 200 pg each of XlPOU2 and β-galactosidase mRNAs (C, neurula stage; E and G, tailbud stage). Control embryos were injected with 200 pg of tXlPOU2 mRNA (B and D, neurula stage; F and H, tailbud stage). The expression of *pax-6* (C,D), *Xotx2* (E,F) and *en-2* (G,H) is shown. (C) The expression of *pax-6* in the central nervous system appears reduced on the injected side because of the plane of the embryo as it was photographed.

**XIPOU2 suppresses the wnt-like signal when co-injected with the mutant GSK-3β**

Does XlPOU2 interfere with the Wnt signaling pathway and at what step of the pathway does this occur? In *Drosophila*, genetic studies have identified potential members in the Wnt signaling pathway, including *zeste-white3/shaggy* (*zw3/shaggy*) (Siegfried et al., 1992). GSK-3β, the *Xenopus* homolog of *zw3/shaggy* has been identified (Pierce and Kimelman, 1995). Inactivation of GSK-3β in UV-ventralized *Xenopus* embryos or in the ventral region of an embryo induces an Organizer, as detected by the expression of the Organizer-specific genes, *gsc* and *Xnot* (He et al., 1995; Pierce and Kimelman, 1995). The induced Organizer promotes the formation of the prechordal plate, the notochord and the axial mesoderm, and induces the overlying ectoderm to become neuroectoderm, events leading to the formation of a complete secondary axis. To determine whether XlPOU2 might interfere with the action of GSK-3β, a component of the Wnt pathway, XlPOU2 mRNA was co-injected with a dominant negative, kinase-dead mutant of GSK-3β (GSK-3β KM) (He et al., 1995) into a pair of ventrovegetal blastomeres. We detected a significant reduction in the ability of the GSK-3β KM to produce a secondary axis in these injected embryos: only 12% of the XlPOU2-injected embryos developed a complete secondary axis, 48% of these embryos developed a truncated secondary axis (Fig. 6C,C'; Table 1), while 40% of these embryos failed
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**Fig. 6.** Interaction of XIPOU 2 with GSK-3β, a component of the Wnt-like dorsalizing pathway. In a control experiment, two ventroanimal blastomeres of a 16-cell-stage embryo were injected with 200 pg of tXIPOU 2 mRNA and 200 pg of β-galactosidase (β-gal) mRNA and two ventrovegetal blastomeres were injected with 1 ng of GSK-3β KM mRNA. Views A and A’ show a tadpole from this experimental control group; [A, a dorsal perspective showing the secondary axis (red) and A’, a side view of the same tadpole]. Another group of embryos were injected similarly, but with 200 pg of XIPOU 2 mRNA instead of tXIPOU 2 mRNA (B,B’, a dorsal and side perspective, respectively, show a tadpole from this experimental group). In other experiments, two ventrovegetal blastomeres from embryos at the 16-cell stage were injected with 200 pg of XIPOU 2 mRNA, 200 pg of β-galactosidase, and 1 ng of GSK-3β KM mRNA (C,C’, a dorsal and side perspective, respectively, show a tadpole from this experimental group). Note that the reaction product from lineage tracer is dark red in all three views. The nearly 100% contribution of the injected lineage to the secondary axis in views A and B was readily detected.

Fig. 7. XIPOU 2 does not disrupt chordin signaling and chordin rescues the phenotype induced by the overexpression of XIPOU 2. Two ventrovegetal blastomeres of embryos at the 16-cell stage were injected with 200 pg of chordin and 200 pg of β-galactosidase mRNAs. (A) A tadpole from this experiment. In another experiment, embryos were injected similarly, with 100 pg of chordin, 200 pg of XIPOU 2, and 200 pg of β-gal mRNAs. (B) A tadpole from this experimental group. The lineage from the injected blastomeres (dark red) contributes heavily to the secondary axis (A,B,B’, a section from the secondary axis shown in view B). (C-E) Tadpoles from chordin ‘rescue’ experiments. (C) Tadpole from a control group, in which two dorsovegetal blastomeres from embryos at the 16-cell stage were injected with 200 pg each of tXIPOU 2 and β-galactosidase mRNAs. (D) A tadpole from an experimental group similarly injected as in C, but with 200 pg of XIPOU 2 instead of tXIPOU 2. There are severe abnormalities in the head region of these embryos. The most severe phenotype from this experimental group is a ‘headless embryo’. (E) A tadpole from an experimental group that had been ‘rescued’ by injecting 200 pg of chordin mRNA.

to develop any secondary axis at all. In contrast, 75% of the control embryos developed a complete secondary axis (Fig. 6A,A’; Table 1). Thus, XIPOU 2 can interfere with secondary axis formation, specifically disrupting events downstream from GSK-3β within the Wnt signaling pathway.

Does XIPOU 2 interfere with signals produced by the secondary Organizer? As in the previous experiment, GSK-3β KM mRNA was injected into a pair of ventroanimal blastomeres. However, in these experiments, XIPOU 2 mRNA was injected into a pair of ventroanimal blastomeres, whose lineage will contribute to the dorsal mesoderm, prechordal plate and neural tube in forming a complete secondary axis. In this case, XIPOU 2 did not interfere with the ability of the GSK-3β KM to form a secondary axis (Fig. 6B,B’; Table 1). Thus, XIPOU 2 did not interfere with the signals produced and secreted by the secondary Organizer.

Chordin can rescue the phenotype that arises when XIPOU 2 is overexpressed in the Organizer

Does XIPOU 2 interfere with the ability of chordin to produce
a secondary axis? To answer this question, XIPOU 2, chordin and β-galactosidase mRNAs were injected together into two ventrogevelast blastomeres of the 16-cell-stage embryo. Using β-galactosidase as a lineage tracer, we determined that the injected lineage contributed to the induced secondary axis in 93% of the injected embryos (Fig. 7B,B'); no differences were detected in control embryos that were co-injected with chordin and β-galactosidase mRNAs alone (96% of the injected embryos displayed a secondary axis) (Fig. 7A). These data indicated that XIPOU 2 did not interfere with chordin’s ability to form a secondary axis (Table 1). Therefore, one explanation is that XIPOU 2 acts upstream of chordin, since it did not interfere with chordin’s ability to form a secondary axis. If chordin acts downstream from XIPOU 2, then the overexpression of chordin should rescue the phenotype that we observed when XIPOU 2 is overexpressed in the dorsal marginal zone. Chordin can antagonize the action of Bmp-4 by directly binding to Bmp-4 and inactivating it (Sasai et al., 1995; Piccolo et al., 1996). Since the XIPOU 2-injected embryos were ventralized and had abnormal elevations of Bmp-4 within the dorsal lip during early to mid-gastrulation, we predicted that chordin should antagonize the actions of Bmp-4 and, thus, rescue the phenotype. Our results support this premise since most of the embryos in which dorsogevelast blastomeres were co-injected with XIPOU 2 and chordin mRNAs developed normal facial features (93% of the embryos developed normal facial features, n=29, Fig. 7E), in contrast to embryos injected with XIPOU 2 mRNA alone (only 20% of the embryos developed normal facial features, n=25, Fig. 7D). We conclude that chordin can fully rescue the phenotype that arises from the overexpression of XIPOU 2 in the dorsal marginal zone and, therefore, chordin must act downstream from the action of XIPOU 2, possibly acting by inactivating Bmp-4 in the dorsal lip. The observed phenotype was not rescued by co-injecting up to 700 pg of a mutant Xlim-1 (3m) (Taira et al., 1995) or 1 ng of GSK-3β KM (data not shown).

DISCUSSION

In Caenorhabditis elegans, Drosophila and mouse, genetic studies have shown that POU genes are required for the determination of specific neuronal lineages in the developing nervous system (Finney and Ruvkun, 1990; Yang et al., 1993; Bhat and Schedl, 1994; Nakai et al., 1995; Schonemann et al., 1995; Weinstein et al., 1995) or Schwann cell differentiation (Bermingham et al., 1996; Jaegle et al., 1996). Like other class III POU domain genes, XIPOU 2 is expressed predominantly in the developing nervous system (Witta et al., 1995). XIPOU 2 is downstream from the neural inductive signaling pathway for noggin and has direct neutralizing activity in ectoderm, perhaps accounting for its action during neural induction (Witta et al., 1995). In Xenopus, prior to its expression in the developing nervous system, XIPOU 2 is activated at MBT, making it one of the earliest expressed zygotene POU genes. During gastrulation, XIPOU 2 is expressed throughout the marginal zone mesoderm, including the Organizer, and it is in this context that we have addressed a potential function for XIPOU 2. Identifying gene products that might interact with XIPOU 2 in the Organizer has been a major focus of our studies.

What are the genes that XIPOU 2 might interact with in the Organizer? In the dorsal mesoderm of the amphibian, there are numerous transcriptional regulators that are co-expressed with XIPOU 2 (ie. gsc, Xlim-1, Knot, XANF-1, Xbra and XFKH1/pintalavis), which have the potential to interact with XIPOU 2. We have identified at least one direct target of XIPOU 2, gsc. XIPOU 2 appears to be a potent suppressor of activin-induced gsc transcription. Although XIPOU 2 binds to the proximal element (the Wnt-inducible element) of the gsc promoter in vitro (Fig. 3, lane 19), the XIPOU 2 protein cannot act alone to alter transcription of gsc. As more Organizer-specific genes are characterized, at the level of the promoter, it will be possible to identify more potential targets of XIPOU 2.

XIPOU 2 has the potential to suppress a selective number of genes expressed in both the dorsal and ventral mesoderm, although it perturbs only the function of the head Organizer. Phenotypically, only head structures rostral to the otic vesicle were affected. The effect of XIPOU 2 is likely to be specific, since the injection of a related family member, XIPOU 1, into dorsogevelast blastomeres of embryos at the 8- to 16-cell stage does not result in any morphological phenotype.

In overexpressing XIPOU 2 in the dorsal and ventral marginal zone, what can we learn about the function of the Organizer? First, despite the specific suppression of several important Organizer-specific genes, including chordin, gsc Xlim-1 and noggin (Figs 1B,E,2A), surprisingly, embryos develop a normal axis. The most extreme phenotype observed after overexpression of XIPOU 2 in the dorsal marginal zone was a headless embryo (observed 10% of the time), similar to the phenotype displayed in a mouse that is null for the Lim1 gene (Shawlot and Behringer, 1995). These data suggest that a severe reduction of Organizer-specific signaling molecules, such as noggin or chordin, or transcriptional regulators, such as gsc and Xlim-1, in the frog is not sufficient to eliminate Organizer activity. The data support the idea that there are a number of redundant signaling pathways that define Organizer function. The presence of Knot in the Organizer of injected embryos (Fig 1N) might explain why the trunk and tail axes were not disturbed, since Knot alone has Organizer function (Gont et al., 1996). Second, we can learn about the consequences of the activation of Bmp-4 in the dorsal lip, resulting from the loss of dorsal gene activity. Interestingly, although we detected the activation of Bmp-4 in the dorsal lip during gastrulation (Figs 1D,D', 2B, lane 3), we did not observe the subsequent induction of at least three ventralateral genes (Xwnt-8, PV.1 and Xpo) in the Organizer (Figs 1T, 2A, lane 2, 2B, lane 3 and 2C, lane 4). It is possible that the levels of Bmp-4 that are needed to induce these ventralateral markers in the dorsal lip (i.e., the abnormally high levels of Bmp-4 transcripts that are generated after an mRNA injection) were not achieved. It should be noted that Bmp-4 is not maintained in the dorsal lip of injected embryos and that, by late gastrulation, the levels of Bmp-4 in the whole embryo return to normal (data not shown). This temporal pattern of Bmp-4 expression may explain why the ‘ventralization’ of injected embryos is mild. Since XIPOU 2’s actions are ‘ventralizing’ in nature, it is not surprising that a phenotype was not observed when XIPOU 2 was overexpressed in the ventral marginal zone. It may seem contradictory that a gene with neutralizing activity can be a suppressor of Organizer function. However, the elevated levels of Bmp-4 in the dorsal lip of injected embryos are not conducive
with the formation of neural tissue but, rather, create an anti-
neuralizing condition. Neuralization occurs when Bmp-4 or
Bmp-7 signaling is disrupted (Wilson and Hemmati-Brivanlou,
1995; Sasai et al., 1995; Hawley et al., 1995; Piccolo et al.,
1996; Zimmerman et al., 1996), or when a dominant-negative,
truncated activin receptor is overexpressed (Hemmati-
Brivanlou and Melton, 1992), and the ‘default neural pathway’
is activated. Therefore, it is not surprising that XIPOU 2 does
not have the ability to generate neural tissue at the expense of
the existing mesoderm. In this respect, XIPOU 2 is similar in
its action to another transcription factor, a bHLH gene, neuro D
(Lee et al., 1995), which, like XIPOU 2, is capable of neu-
ralizing only ectoderm. On the contrary, the failure of XIPOU 2
to activate Bmp-4 in the uncommitted ectoderm is consistent
with its neuralizing activity.

The examination of events downstream from the Wnt-like
signaling pathway, and secreted factor, chordin, enabled us to
define further the action of XIPOU 2. These gain-of-function
studies suggest that XIPOU 2 must directly interfere with
the formation of the head Organizer, since XIPOU 2 can interfere
primarily with the action of head formation by a dominant-
negative GSK-3β mutant. Chordin is not activated immediately
at MBT but, instead, it is activated during early gastrulation by
the immediate-response gene, gsc (Sasai et al., 1994). Since
XIPOU 2 did not interfere with the action of chordin, chordin
most likely acts downstream from XIPOU 2. Because chordin
dhas neuralizing activity and can antagonize the action of Bmp-
4, it is reassuring to know that chordin can fully rescue the
phenotype generated by the overexpression of XIPOU 2.

What is the endogenous role of XIPOU 2 in the Organizer?
XIPOU 2’s endogenous role may be to dampen selectively, at

the transcriptional level, events downstream from general
mesoderm signaling pathways (Fig. 8), or events downstream
from a Wnt-like dorsalizing pathway (Fig. 5). XIPOU 2 might
regulate head Organizer function by suppressing genes such as
gsc and Xlim-1 (Figs 1F and 1J and 2A, lane 2), following their
induction at MBT. We have identified gsc as one direct target of
XIPOU 2 (Figs 3, 4). It is reasonable to believe that XIPOU 2
may be only one of several possible suppressors expressed in
the Organizer and that, ultimately, Organizer function will
reflect the transcriptional balance of many genes, including
signals, as well as transcriptional regulators, expressed in the
Organizer (for a review of Organizer-specific molecules, see
Lemaire and Kodjabachian, 1996). In Fig. 8, we have provided a
model summarizing our current understanding of how
endogenous XIPOU 2 may function in the Organizer.

In conclusion, we believe that XIPOU 2 has the potential to
modulate gene activity in the Organizer during gastrulation by
directly altering the transcription of Organizer-specific genes
such as gsc. XIPOU 2’s expression, beginning at MBT, and its
localized expression in the marginal zone mesoderm, including
Spemann’s organizer, throughout gastrulation, place it at the
right time and place to make this idea feasible. In future
studies, we hope to clarify XIPOU 2’s role, in vivo, more fully,
by knocking out the activity of XIPOU 2, in combination with
other genes that are expressed in the Organizer.

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