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The *Xvent-2* homeobox gene is part of the *BMP-4* signalling pathway controling dorsoventral patterning of *Xenopus* mesoderm

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SUMMARY

We describe a novel *Xenopus* homeobox gene, *Xvent-2*, which together with the previously identified homeobox gene *Xvent-1*, defines a novel class of homeobox genes. vent genes are related by sequence homology, expression pattern and gain-of-function phenotype. Evidence is presented for a role of *Xvent-2* in the *BMP-4* pathway involved in dorsoventral patterning of mesoderm. (1) *Xvent-2* is expressed in regions that also express *BMP-4*. (2) *Xvent-2* and *BMP-4* interact in a positive feedback loop. (3) *Xvent-2* ventralizes dorsal mesoderm in a dose-dependent manner resulting in phenoytpes ranging from microcephaly to *Bauchstück* pieces, as does *BMP-4*. (4) Like *BMP-4* and *gsc*, *Xvent-2* and *gsc* are able to interact in a crossregulatory loop to suppress each other. (5) Microinjection of *Xvent-2* mRNA can rescue dorsalization by a dominant-negative BMP-4 receptor. The results suggest that *Xvent-2* functions in the *BMP-4* signalling pathway that antagonizes the Spemann organizer.

Key words: BMP-4, goosecoid, homeobox, mesoderm, organizer, *Xenopus*, *Xvent-2*

INTRODUCTION

In the amphibian embryo mesodermal tissues arise from a ring of cells located between the animal and vegetal pole, called the marginal zone, by an inductive process between cells located in the animal and vegetal hemisphere (for reviews, see Gurdon, 1992; Tiedemann et al., 1995). The dorsal marginal zone or Spemann organizer plays an important role in patterning the embryo. Grafting experiments have shown that during gastrulation signals emanating from the Spemann organizer dorsalize the ventral marginal zone to form intermediate types of mesoderm (Smith and Slack, 1983; Dale and Slack, 1987a; for reviews, see Slack 1993; Kimelman et al., 1992). For a long time ventral mesoderm was therefore considered to be ground-state mesodermal tissue, which serves as the passive substrate upon which the organizer acts.

Recently, a number of studies have suggested that the ventral marginal zone not only requires active signals for the specification of the ventral state, but also signals to antagonize the organizer. First, the ventral marginal zone expresses two peptide growth factors, *Xwnt-8* and bone morphogenetic protein 4 (*BMP-4*), which are able to override dorsal mesodermal specification (Koster et al., 1991; Dale et al., 1992; Jones et al., 1992; Christian and Moon, 1993; Fainsod et al., 1994; Schmidt et al., 1995). Second, microinjection of mRNA coding for dominant-negative *BMP-4* receptors leads to dorsalization of ventral mesoderm (Graff et al., 1994; Suzuki et al., 1994). These results suggested that marginal zone patterning may be the result of antagonizing dorsal and ventral signals (reviewed in Sive, 1993; Harland, 1994).

Little is known about genes that may function in ventral signalling pathways. Homeobox genes are excellent candidates for providing positional specification. We have recently identified a homeobox gene, *Xvent-1*, which is differentially expressed in the ventral marginal zone, and provided evidence for a function of this gene in antagonizing the organizer and in maintenance of ventral type mesoderm (Gawantka et al., 1995). Here we describe a novel homeobox gene, *Xvent-2*, which is related to *Xvent-1* and is expressed in the marginal zone of the early *Xenopus* gastrula, excluding the organizer region. We present evidence to suggest that this gene is part of a signalling pathway downstream of *BMP-4*, involved in specifying ventral mesodermal fate and in antagonizing organizer function.

MATERIALS AND METHODS

Embryos and explants

In vitro fertilization, embryo culture, staging, microinjection and culture of marginal zone explants and animal caps were carried out as described (Niehrs and De Robertis, 1991). LiCl treatment was performed at the 32-cell stage by incubating embryos for 40 minutes in 0.12 M LiCl and subsequent washing. UV treatment was carried out as previously described (Fainsod et al., 1994).

cDNA library screening

The original Xvent-2 clone (pXvent-2) was isolated by screening a

neurula stage plasmid library (cloned in pBSII-KS$^+$) by in situ whole-mount hybridisation as described (Gawantka et al., 1995). Double-stranded plasmid DNA of pXvent-2 was sequenced according to Sanger et al. (1977) using T7 DNA polymerase. Accession no. X98849.

Whole-mount in situ hybridisation

Whole-mount in situ hybridisation was performed according to the protocol of Harland (1991) with modifications (Gawantka et al., 1995).

Northern blotting

0.2 μg of stage-13 poly(A)$^+$ RNA and 0.2 ng of in vitro transcribed *Xvent-2* RNA were separated on a 1.0% glyoxal gel (Sambrook et al., 1989). A ^{32}P random-primed 0.9 kb *Kpn*I fragment of pXvent-2 was used as a probe and hybridization was carried out as described (Gawantka et al., 1995).

Constructs

pRNXvent-2 was constructed by cloning a *Sal*I (blunt)-*Not*I fragment of pXvent-2, containing full-length *Xvent-2*, into *Eco*RI (blunt)-*Not*I cut pRN3 (Lemaire et al., 1995). pΔXvent-2 was constructed by excision of a 5′ 614 bp *Sal*I-*Bgl*II fragment from pXvent-2 and cloning into *Sal*I-*Bam*HI-cut pBSII-KS$^+$. *ΔXvent-2* was excized from pΔXvent-2 and subcloned into pRN3 like full-length *Xvent-2* to create pRNΔXvent-2. *ΔXvent-2* lacks the 133 carboxyterminal amino acids, including most of the homeobox.

Microinjection experiments

pRNXvent-2 and pRNΔXvent-2 DNA were linearized with *Pst*I and transcribed with T3 RNA polymerase using the Megascript kit (Ambion) and a cap:GTP ratio of 5:1. pSPgsc (Niehrs et al., 1994) was linearized with *Eco*RI and transcribed with SP6 RNA polymerase. pBMP-4 (Fainsod et al., 1994) was linearized with *Xho*I and transcribed with T3 RNA polymerase. Radial injection refers to microinjection of all four blastomeres of 4-cell stage embryos into the equatorial region.

RT-PCR

RT-PCR assays were carried out as described previously (Gawantka et al., 1995). Although not shown in all cases, parallel control samples in which reverse transcriptase had been omitted were analyzed in all PCR assays. In these control samples gene-specific products were absent. The gene-specific primers used were as described previously (Gawantka et al., 1995). The primers used for *Xvent-2* were (upstream: 5′-TGA-GACTTGGGCACTGTCTG; downstream 5′-CCTCT-GTTGAATGGCTTGCT; 175 bp).

RESULTS

Cloning of *Xvent-2*

We are carrying out a large-scale screen by whole-mount in situ hybridisation of randomly picked cDNA clones from a neurula cDNA plasmid library. In this screen, we have identified a 1.3 kb cDNA that is expressed in gastrula mesoderm. The DNA sequence of this cDNA, which we have named *Xvent-2*, contains an open reading frame coding for 328 amino acids and includes a homeobox. The deduced amino acid sequence is shown in Fig. 1A. Fig. 1B shows that the homeodomain of *Xvent-2* has 73% sequence identity most closely related to *Xvent-1*, a ventral-expressed homeobox gene that we have recently identified by the same strategy (Gawantka et al., 1995). No significant homology of *Xvent-2* with *Xvent-1* or any other gene was found in the amino acid sequence coded by the DNA sequence outside the homeodomain. A 73% sequence identity in the homeodomain between *Xvent-1* and *Xvent-2* indicates that they are related genes. The closest relative found in the database is the *Drosophila ladybird-early* gene (*Lbe*), which shows 55% sequence identity with *Xvent-2*, a degree of homology characteristic for genes belonging to different homeobox gene classes (Duboule, 1994). These results indicate that *Xvent-1* and *Xvent-2* are members of a novel class of homeobox genes.

Expression of *Xvent-2*

Northern-blot analysis shows that in vitro transcribed *Xvent-2* mRNA comigrates with a 1.3 kb mRNA detected in poly(A$^+$) mRNA, indicating that the *Xvent-2* clone is full length (Fig. 2A). The slightly higher molecular mass of the in vitro transcribed RNA is probably due to an additional 80 bp of remaining polylinker. RT-PCR analysis (Fig. 2B) shows that *Xvent-2* begins to be expressed at midblastula transition, shows maximal expression during late neurula and is still detectable by the tadpole stage.

In situ hybridisation on whole-mount and sagittally cut embryos (Fig. 3) shows *Xvent-2* transcripts in the marginal zone and animal cap region of gastrulae, specifically excluding the organizer region (Fig. 3A,B). This expression is clearly different from that of *Xvent-1*, whose expression boundary in the marginal zone is in a more lateral position (Fig. 3C). The difference in dorsal boundaries was highly reproducible and independent of the staining time. Even in embryos deliberately overstained, the expression boundary of *Xvent-1* was more lateral than that of *Xvent-2*.

In neurula embryos *Xvent-2* is expressed in two longitudi-

A

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  1  MTKAFSSVEW LAQSSRRSHR EQPSKVDQRY SPYPRPSLPS WNSDVSPSSW 50

 51  NSQLSPDPDS AQVSPCPVSA QVSPYSSDSE ISLYSHEEEA SFYGMDFNTS 100

101  SSPGDNGLLH RDTTSYSRGM EAMSASTPAT SPVKGAQPVD SAYSTSTDSG 150

151  YESETSRSNS TAPEGDASVS LSPNDTSDEE GKMGRRLRTA FTSDQISTLE 200

201  KTFQKHRYLG ASERRKLAAK LQLSEVQIKT WFQNRRMKYK REIQDGRPDS 250

251  YHPAQFFGVY GYAQQPTPVF QHAVQHPYPG YNPLMETLPG TMPYTMHPPA 300

301  MDSLTPFNSQ PFQMLYLPQQ HLGQPLAY   328
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B

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Xvent-2   GRRLRTAFTSDQISTLEKTFQKHRYLGASERRKLAAKLQLSEVQIKTWFQNRRMKYKREI
Xvent-1   Q--------PQ---K--QA-N-Q-----P--K---TS-----I-V----------L--Q- 73%

Lbe       K-KS-----NH--FE---R-LYQK--SPAD-DEI--S-G--NA-VI---------Q--D- 55%
HBox-2.8  S------Y-NT-LLE---E-HFNK--CRPR-VEI--L-D-T-R-V-V--------H--QT 53%
pS6       PG-----Y-NT-LLE---E-HFNK--CRPR-VEI--L-D---R-V-L--------H--QT 53%
Mox-1     A-KE-----KE-LRE--AE-AH-N--TRLR-YEI-VN-D---R-V-V--------W--VK 52%
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Fig. 1. *Xvent-2* and *Xvent-1* are members of a new class of homeobox genes. (A) Deduced amino acid sequence of Xvent-2 protein. The homeodomain is underlined. (B) Sequence alignment of the Xvent-2 homeodomain with other homeoprotein sequences. Amino acids identical to those of Xvent-2 are indicated by bars, and are expressed as % homology. Xvent-1 (Gawantka et al., 1995); Lbe (Jagla et al., 1994); HBox-2.8 (Belleville et al., 1992); pS6 (Fjose et al., 1988); Mox-1 (Candia et al., 1992).

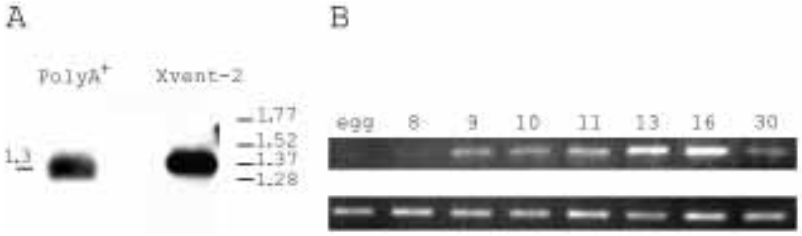

Fig. 2. Northern blot and expression profile of *Xvent-2*. (A) A northern blot of stage 13 poly(A^+) mRNA (PolyA^+) and *in vitro* transcribed *Xvent-2* mRNA was probed with *Xvent-2*. Sizes of markers and the band observed in PolyA^+ are indicated in kb on the right and left, respectively. (B) RT-PCR analysis of *Xvent-2* expression at the embryonic stages indicated. Top, *Xvent-2*; bottom, Histone H4, for normalization.

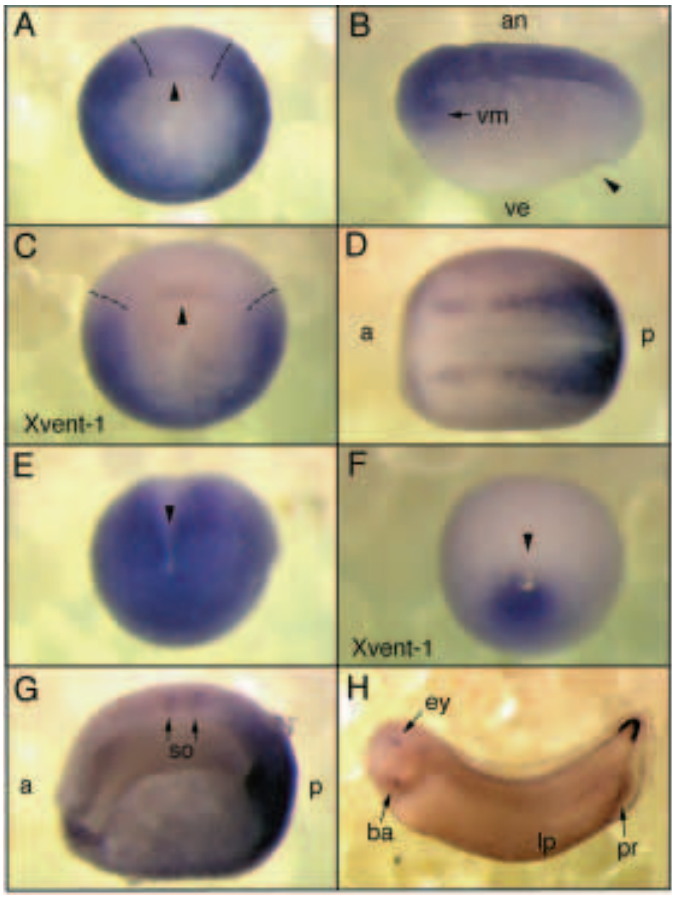

Fig. 3. Spatial expression of *Xvent-2*. *Xvent-2* expression was analyzed by in situ hybridisation of whole-mount (A,D,E,H) and sagittally cut (B,G) *Xenopus* embryos. For comparison, whole-mount in situ hybridisations of *Xvent-1* are shown (C,F). The dorsal blastopore lip is indicated by the arrowhead. (A) Stage 10 (early gastrula) embryo shown in vegetal view. The dorsal expression boundary of *Xvent-2* is indicated by dashed lines. (B) Stage 10 (early gastrula) embryo shown in lateral view. (C) Stage 10 (early gastrula) embryo shown in vegetal view. Note that the dorsal expression boundary of *Xvent-1* (dashed lines) is more lateral than that of *Xvent-2* (A). (D-F) Stage 13 (early neurula) embryos shown in dorsal (D) and posterior view (E,F). Note that the expression of *Xvent-2* comprises the whole circumblastoporal colar (E) while the expression domain of *Xvent-1* (F) is more ventral. (G) Stage 13 (early neurula) embryo para-sagittally cut and shown in lateral view. Note two staining somites (arrows). (H) Stage 30 (tailbud) embryo in lateral view. a, anterior; an, animal pole; ba, branchial arches; ey, eye; lp, lateral plate mesoderm; p, posterior; pr, proctodeum; so, somites; ve, vegetal pole.

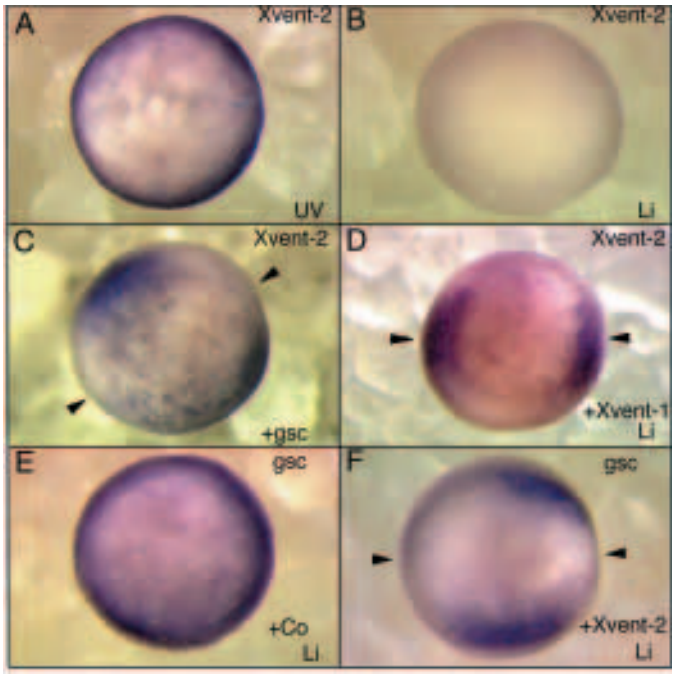

Fig. 4. Interaction of *Xvent-2* with *Xvent-1* and *gsc*. All embryos were analyzed at the gastrula stage (stage 10-11) by whole-mount in situ hybridisation and are shown in vegetal view. (A) UV-ventralized embryo probed for *Xvent-2* expression. (B) LiCl-dorsalized embryo probed for *Xvent-2* expression. (C) Embryo microinjected into two opposite blastomeres at the 4-cell stage with 50 pg *gsc* mRNA each and probed for *Xvent-2* expression. Note the two domains lacking *Xvent-2* expression (arrowheads). (D) Embryo microinjected with 0.1 ng *Xvent-1* mRNA each into two opposite blastomeres at the 4-cell stage, treated with LiCl and probed for *Xvent-2* expression. Note the two opposite domains of induced expression (arrowheads). (E) Embryo microinjected with 1.5 ng *ΔXvent-2* mRNA (Co) each into two opposite blastomeres at the 4-cell stage, treated with LiCl and probed for *gsc* expression. *gsc* is radially expressed. (F) Embryo microinjected with 1.5 ng *Xvent-2* mRNA each into two opposite blastomeres at the 4-cell stage, treated with LiCl and probed for *gsc* expression. Note the two domains lacking *gsc* expression (arrowheads).

nal stripes, corresponding to prospective dorsal neural tube, which broaden in the posterior and converge with the circumblastoporal collar (Fig. 3D). The strongest expression of *Xvent-2* is found in the posterior of the embryo in all three germ layers, including the entire circumblastoporal and ventral region of the embryo (Fig. 3D,E,G). Again, this expression pattern is distinct from that of *Xvent-1*, which is not expressed in the neural plate but only in the ventral side of the circumblastoporal collar (Fig. 3F). In neurula embryos para-sagittally cut before staining, two transverse stripes of expression of *Xvent-2* are found in somitic mesoderm (Fig. 3G).

In tadpole embryos, *Xvent-2* expression is maintained in the

tail and proctodeum, the dorsal part of the eye, the ventral tip of the branchial arches and in the lateral plate mesoderm (Fig. 3H). In comparison, *Xvent-1* only shows very weak expression in the proctodeum at tadpole stages (not shown). Transverse sections of tadpole stage embryos showed no expression of *Xvent-2* in the neural tube (not shown).

The pattern of expression of *Xvent-2* closely parallels the pattern of expression of *BMP-4* in early ventrolateral mesoderm and animal cap, tailbud, proctodeum and lateral plate mesoderm, as well as the dorsal eye and the tip of the branchial arches. An exception to this congruence is the lack of *BMP-4* expression in the two transverse somitic stripes and the two longitudinal stripes in prospective dorsal neural tube at the neurula stage, though *BMP-4* is expressed specifically in the dorsal neural tube at the tailbud stage (Fainsod et al., 1994; V. Gawantka and C. Niehrs, unpublished results).

These expression data show that (1) *Xvent-2* and *Xvent-1* are distinctly expressed during all stages analyzed and in particular, have different dorsal boundaries of expression in the marginal zone of the gastrula; and that (2) *Xvent-2* is expressed in most regions that also express *BMP-4*, suggesting a functional relationship between the two genes.

Expression of *Xvent-2* closely follows dorsoventral specification of gastrula mesoderm. In embryos ventralized by UV-irradiation (Scharf and Gerhart, 1983) *Xvent-2* is expressed in the entire circumference of the marginal zone (Fig. 4A). In embryos dorsalized by LiCl treatment (Kao and Elinson, 1988) *Xvent-2* expression is repressed (Fig. 4B).

Interaction of *Xvent-2* with genes affecting dorsoventral pattern of mesoderm

The expression profile of *Xvent-2* suggested that it may be regulated by and interacting with genes involved in dorsoventral patterning.

The homeobox gene *gsc* has been implicated in organizer function (Cho et al., 1991; Niehrs et al., 1993, 1994). Fig. 4C shows that microinjection of synthetic *gsc* mRNA into two opposite blastomeres of 4-cell embryos suppresses *Xvent-2* expression. To address whether *Xvent-2* can regulate *gsc* expression, in the inverse experiment 4-cell embryos were injected into two opposite blastomeres with mRNA of *Xvent-2* or of a control construct *ΔXvent-2* lacking part of the homeodomain, and subsequently treated with LiCl to dorsalize them. Fig. 4E shows embryos injected with control mRNA, in which *gsc* is, as expected for Li-treated embryos, expressed in the entire marginal zone. In contrast, in *Xvent-2*-injected embryos, *gsc* expression is inhibited on the sides of injection (Fig. 4F). Thus, *gsc* and *Xvent-2* can repress each other.

To investigate the interaction of *Xvent-1* and *Xvent-2*, embryos were dorsalized with LiCl to inhibit expression of both genes and injected into two opposite blastomeres with *Xvent-1*. Fig. 4D shows that microinjection of *Xvent-1* mRNA can rescue the expression of *Xvent-2*.

Signalling by *BMP-4* is important for the maintenance of ventral mesoderm (Koster et al., 1991; Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Graff et al., 1994; Suzuki et al., 1994; Schmidt et al., 1995). To study the effect of *BMP-4* on *Xvent-2* and *Xvent-1* expression, synthetic *BMP-4* mRNA was microinjected into two blastomeres of 4-cell embryos. These embryos were subsequently dorsalized by LiCl treatment to repress normal *Xvent* expression. Fig. 5B,E shows that *BMP-4* injection induces *Xvent-1* as well as *Xvent-2*.

To test whether *BMP-4* is also required for *Xvent-2* expression, we microinjected mRNA encoding a dominant-negative *BMP-4* receptor (Suzuki et al., 1994) radially in all blastomeres of 4-cell stage embryos. Fig. 5C,F shows that this treatment abolishes *Xvent-1* and *Xvent-2* expression.

The results demonstrate that *BMP-4* is both necessary and sufficient for expression of *Xvent-2* and *Xvent-1*.

Xvent-2 microinjection ventralizes embryos

To test whether *Xvent-2* might play a regulatory role in dorsoventral patterning of the mesoderm, we conducted a series of microinjection experiments.

Fig. 6 shows the phenotypes of embryos radially microinjected into the equatorial region with *Xvent-2* mRNA at the 4-cell stage. The embryonic phenotypes observed were dose-dependent. At a low concentration *Xvent-2* caused mild microcephaly, reducing the development of the forebrain region. At an intermediate concentration, loss of head structures and axial defects were evident. At a high concentration, embryos exhibited a *Bauchstück* phenotype, showing complete loss of axial structures, characteristic of maximally ventralized embryos with a dorsoanterior index of 0 (Kao and Elinson, 1988). Control microinjections using *ΔXvent-2* mRNA never produced this phenotype, but gastrulation defects were occasionally observed. The results are summarized in Table 1.

Ventralization of cell fate was further studied by analysis of marker gene expression. Dorsal marginal zone explants from embryos microinjected either with *Xvent-2* or control (*ΔXvent-2*) mRNA were analyzed by RT-PCR for the expression levels of various mesodermal maker genes at the tailbud stage.

As shown in Fig. 7, the expression of *gsc*, a marker for head mesoderm in tailbud embryos, is strongly repressed by *Xvent-2*, confirming the results obtained by in situ hybridisation. Likewise, the expression of *Xnot*, a marker for notochord (von Dassow et al., 1993; Gont et al., 1993) is repressed, but is signifiantly less sensitive than *gsc*, being turned off completely only at the highest concentration. This indicates that the formation of both head mesoderm and notochord is affected by *Xvent-2*. The loss of dorsal mesoderm marker gene expression is paralleled by a gain in expression of intermediate and ventroposterior mesoderm markers. Cardiac actin, a marker for dorsolateral mesoderm, is induced at low concentrations of *Xvent-2*. Induction of ventral marker genes varies for the different markers with the concentration of *Xvent-2*. While *Xwnt-8* is steadily induced, *BMP-4*, *Xvent-1* and *Xhox3* exhibit a more

Table 1. Ectopic expression of *Xvent-2* ventralizes embryos in a dose-dependent manner

mRNA injected	ng/blastomere	*n*	Microcephalic (%)	*Bauchstück* (%)
ΔXvent-2	1.5	49	16	0
Xvent-2	0.4	62	37	0
Xvent-2	0.8	34	79	3
Xvent-2	1.0	97	88	1
Xvent-2	1.6	108	48	51

4-cell stage embryos were microinjected into the equatorial region of the four blastomeres. The amount of mRNA injected and number of embryos (*n*) are indicated.

The observed phenotypes are shown as % of total number.

abrupt response at the highest concentration of injected *Xvent-2*. Control injections with *ΔXvent-2* mRNA (Co) show marker gene expression profiles similar to those of uninjected dorsal marginal zones (DMZ).

Xvent-2 mRNA microinjection does not lead to mesoderm induction in explanted animal caps, as judged by RT-PCR assays using *Xbra* and cardiac actin as markers, and also by histology (not shown).

The results show that overexpression of *Xvent-2* mRNA in explanted dorsal marginal zones leads to a downregulation of dorsal, and a parallel upregulation of ventral, marker genes in a dose-dependent manner.

Xvent-2 rescues dorsalization by a dominant-negative BMP-4 receptor

The congruence of expression patterns between *BMP-4* and *Xvent-2*, the common ventralization exerted by both genes as well as their ability mutually to induce each other's expression, suggested that they may act in a common pathway. To test this possibility further and to analyze the hierarchy of the two genes, 4-cell stage embryos were microinjected into the ventral side with mRNA encoding a dominant-negative *BMP-4* receptor. As described (Graff et al., 1994; Suzuki et al., 1994) this leads to dorsalization of embryonic mesoderm and to the formation of secondary embryonic axes (Fig. 8A, Table 2). Fig. 8B and Table 2 show that the formation of secondary axes by a dominant-negative *BMP-4* receptor can be efficiently reversed by coinjection with *Xvent-2* mRNA, but not with control (*ΔXvent-2*) mRNA. These results support the hypothesis that *Xvent-2* is part of the *BMP-4* pathway and suggest that *Xvent-2* acts downstream of *BMP-4*.

Table 2. Microinjection of *Xvent-2* mRNA rescues dorsalization by a dominant-negative BMP-4 receptor

mRNA injected	*n*	Normal (%)	Secondary axis (%)
ΔmTFR11	29	10	55
ΔmTFR11 + *ΔXvent-2*	24	25	67
ΔmTFR11 + *Xvent-2*	21	81	5

4-cell stage embryos were microinjected into two ventral blastomeres with 0.2 ng mRNA encoding a dominant-negative BMP-4 receptor (ΔmTFR11) or a mixture of 0.2 ng ΔmTFR11 and either 1.5 ng *ΔXvent-2* or *Xvent-2* mRNA.

The numbers of normal embryos and embryos displaying secondary embryonic axes were analyzed and are shown as % of total. Sum of values of normal and secondary-axis embryos is less than 100% because abnormal embryos showing other malformations like *spina bifida*, formation of edema or kinked axes are included in the total number of embryos but were not scored separately.

Overexpression of *Xvent-2* leads to fate change of notochord and head mesoderm cells

The cell fate of individual blastomeres microinjected with *Xvent-2* mRNA was analyzed by coinjection with the lineage tracer colloidal gold into 32-cell stage embryos. In this mosaic analysis, changes of cell fate can be analyzed in the context of an otherwise normal embryo (Niehrs and De Robertis, 1991). Three different blastomeres were selected for analysis, which either give rise to ventral mesodermal tissue (C4) or to dorsal mesodermal tissues derived from the organizer such as notochord (B1) and head mesoderm (C1). Blastomeres were injected with an intermediate dose of *Xvent-2* mRNA, that yields headless embryos in 4-cell injections or control mRNA (*ΔXvent-2*).

Fig. 9A,B shows that targeting of the C4 blastomere (ventral) does not affect cell fate. In control- and *Xvent-2*-injected embryos, labeled cells give rise to somitic and lateral plate mesoderm as expected from the fate map (Dale and Slack, 1987b; Moody, 1987).

In contrast, targeting of the B1 blastomere (dorsal) with *Xvent-2* mRNA changes cell fate. Descendants of B1 blastomeres normally give rise to the notochord as well as to paraxial somites and the central nervous system (CNS) (Dale and Slack, 1987b; Moody, 1987), as shown in the control-injected embryos (Fig. 9C). In *Xvent-2*-injected embryos notochord cells were always unlabeled, unlike somitic muscle, which continued to be populated. Instead, in *Xvent-2*-injected embryos labeled cells were reproducibly found in a position just ventral to the notochord (closed arrowheads in Fig. 9D). These cells may represent immature notochord or cells of the hypochord.

While elimination of notochordal fate was also observed in *Xvent-1*-injected embryos, we found that *Xvent-2* expression interferes with neuronal differentiation, unlike *Xvent-1*-injected embryos, in which CNS development appeared normal (Gawantka et al., 1995). Labeled cells in the brain of *Xvent-2*-injected embryos had an abnormal morphology, never showing the typical comma-like shape of a fully differentiated neurons. Unlike in normal embryos, labeled cells were rarely located in a ventral position (Fig. 9C,D). Some labeled cells were found in head epidermis, which is not a normal B1 fate.

Targeting of C1 blastomeres (dorsal), which give rise to head mesoderm, also leads to cell fate changes. Descendants of C1 blastomeres predominantly give rise to head endoderm and head mesoderm (Dale and Slack, 1987b; Moody 1987), as is shown in control-injected embryos (Fig. 9E). As with *Xvent-1*-injected embryos, embryos showed more labeled somites (Fig. 9F), which is in accord with induction of actin expression observed in RT-PCR assays (Fig. 7). This increase may occur at the expense of head mesoderm, which tended to house fewer labeled cells, with an abnormal morphology, not lining the anatomical features of the branchial arches as seen in control-injected embryos, but clustering (Fig. 9F).

These results show that expression of *Xvent-2* is not compatible with dorsal mesodermal cell fate (notochord, head mesoderm) as well as neuronal cell fate in the brain, and corroborate the ventralizing effect of *Xvent-2*.

DISCUSSION

In this study, we describe a new homeobox gene *Xvent-2*, define a novel homeobox gene class and provide evidence that *Xvent-2* is part of a *BMP-4* signalling pathway, functioning in the specification of ventral mesoderm.

Vent genes constitute a new homeobox gene class

The amino acid sequences of the homeodomains of *Xvent-1* and *Xvent-2* are closely related and distinct from known homeobox gene classes (Duboule, 1994). In addition, *Xvent-1* and *Xvent-2* are related by their common early embryonic expression in non-organizer mesoderm, as well as their ventralizing phenotype after microinjection of mRNA. These features lead us to propose that vent genes constitute a novel

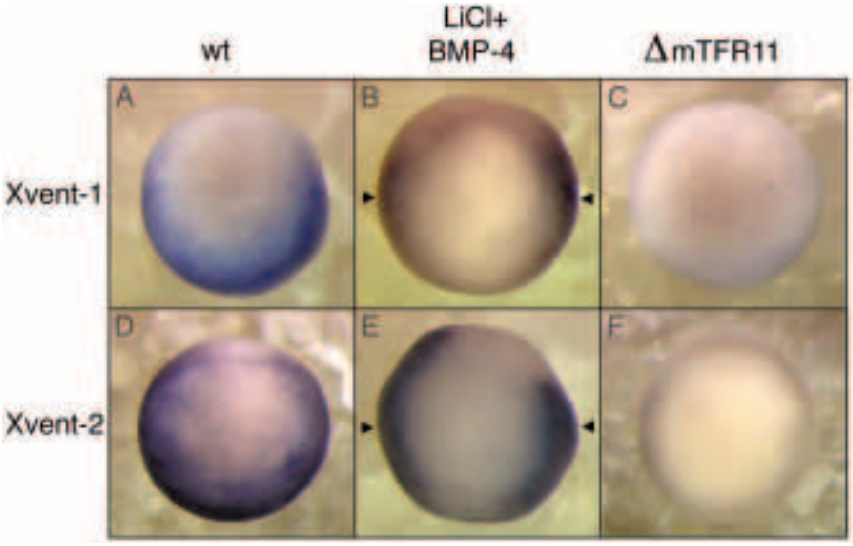

Fig. 5. *BMP-4* is necessary and sufficient for expression of *Xvent-2* and *Xvent-1*. Wild-type embryos or treated embryos (as indicated on the top) were probed at the early gastrula stage for *Xvent-1* or *Xvent-2* expression as indicated on the left. (A,D) Wild-type (wt) embryos. (B,E) Embryos were microinjected with 0.6 ng *BMP-4* mRNA each into two opposite blastomeres at the 4-cell stage and treated with LiCl. Note the two opposite domains of induced expression (arrowheads). (C,F) Embryos were microinjected with 1 ng each of dominant-negative *BMP-4* receptor mRNA (ΔmTFR11) into four blastomeres of 4-cell stage embryos. Expression of both *Xvent-1* and *Xvent-2* is repressed.

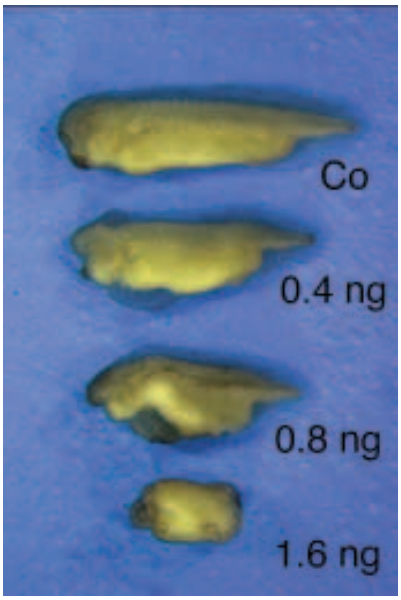

Fig. 6. *Xvent-2* mRNA microinjection causes axial defects in a dose-dependent manner. Phenotype of embryos microinjected at the 4-cell stage into four blastomeres with 1.6 ng per blastomere *ΔXvent-2* mRNA (Co, top embryo) or with the indicated amount of *Xvent-2* mRNA (bottom three embryos).

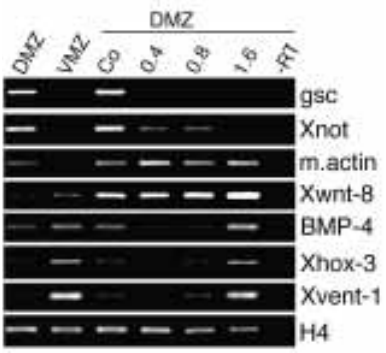

Fig. 7. *Xvent-2* mRNA microinjection ventralizes dorsal mesoderm. Embryos were either uninjected (DMZ, VMZ, –RT), microinjected with 1.6 ng *ΔXvent-2* (Co), or microinjected with increasing amounts of *Xvent-2* mRNA (indicated on top in ng mRNA per blastomere) into the equatorial region of four blastomeres at the 4-cell stage. Dorsal (DMZ) or ventral marginal zones (VMZ) as indicated on top were explanted at the early gastrula stage and incubated until sibling embryos reached stage 19. Total RNA was isolated and analyzed by RT-PCR assays for expression of *gsc* (Cho et al., 1991), *Xnot* (von Dassow et al., 1993; Gont et al., 1993), cardiac actin (m.actin) (Mohun et al., 1984), *Xwnt-8* (Christian and Moon, 1993), *BMP-4* (Dale et al., 1992; Jones et al., 1992), *Xhox3* (Ruiz i Altaba and Melton, 1989), *Xvent-1* (Gawantka et al., 1995) and Histone H4, as indicated. –RT, uninjected control DMZ sample without reverse transcription.

homeobox gene class. Both *Xvent-1* and *Xvent-2* have a Thr at position 47 instead of Ile as found in the Antennapedia class (Duboule, 1994). This rare substitution in the recognition helix has been shown to alter the DNA binding specificity of *HOX11* (Dear et al., 1993) and *Lbe* (Jagla et al., 1994) for the TAAT motif. This suggests that vent homeobox genes also recognize divergent binding elements. Whether there are other vent class members and whether they are located in a common chromosome cluster is currently being investigated.

The expression patterns of *Xvent-1* and *Xvent-2*, though related, are clearly distinct. At the neurula and tadpole stages, *Xvent-2* shows a more complex expression pattern than *Xvent-1*, suggestive of a more widespread role of the former. At the gastrula stage, the dorsoventral boundary of expression of *Xvent-1* is significantly more lateral than the boundary of *Xvent-2*. This staggered expression is reminiscent of the expression of Hox genes. Hox genes are characterized by their expression along the anteroposterior (A-P) axis of the embryo in the nervous system and the mesoderm. They show sharp anterior boundaries, proceeding in an A-P order colinear with their chromosomal location. Hox genes are thought to act in a combinatorial fashion, corresponding to a Hox code, to define different A-P levels in the embryo (reviewed in McGinnis and Krumlauf, 1992). It is an intriguing possibility that vent genes may act similarly to specify different dorsoventral levels of the gastrula mesoderm.

The effect of microinjection of both genes is ventralization of mesoderm leading to changes in dorsal mesodermal cell fate, but the phenotype observed for the highest dose of *Xvent-1* mRNA that stops short of causing severe gastrulation defects, is microcephaly (Gawantka et al., 1995). In contrast, *Xvent-2* mRNA microinjection can result in maximal ventralization, i.e. *Bauchstück* embryos. A further difference between *Xvent-1* and *Xvent-2* is the ability of the latter to induce *BMP-4* in dorsal marginal zones. This may also explain the abnormal brain cell fate observed in *Xvent-2* but not *Xvent-1* lineage-

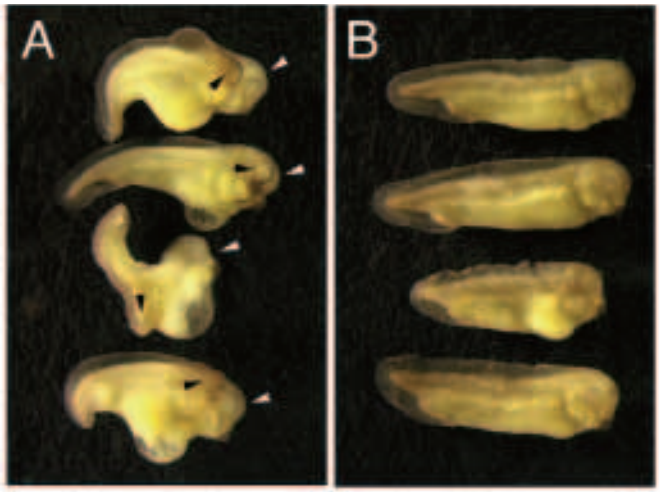

Fig. 8. Microinjection of *Xvent-2* mRNA rescues dorsalization by a dominant-negative BMP-4 receptor. 4-cell stage embryos were microinjected into two ventral blastomeres with (A) 0.2 ng mRNA encoding a dominant-negative BMP-4 receptor (ΔmTFR11; Suzuki et al., 1994), or (B) a mixture of 0.2 ng ΔmTFR11 and 1.5 ng *Xvent-2* mRNA. White and black arrowheads in (A) point to primary and secondary embryonic axes, respectively.

traced embryos (Gawantka et al., 1995). It has been shown that *BMP-4* can inhibit neuralization and promote epidermal cell fate (Wilson and Hemmati-Brivanlou, 1995). The abnormal neural brain fate could therefore be the consequence of *BMP-4* induction by *Xvent-2*. Alternatively, *Xvent-2* may have a direct function in neural patterning, possibly dorsal neural tube specification, as the gene is expressed in the precursors of the corresponding cells at the neurula.

Xvent-2 participates in the *BMP-4* signalling pathway

Signalling pathways involving *BMP-4*-like genes are implicated in many different kinds of developmental processes, in both invertebrates and vertebrates (Kingsley, 1994). Some constituents of a *BMP-4* signalling pathway are beginning to emerge. In *Xenopus*, *BMP-4* signalling appears to involve a ras pathway (Xu et al., 1996). In *Drosophila* signalling by the *BMP-4* homolog decapentaplegic (*dpp*) requires *tolloid*, a metalloprotease homologous to *BMP-1*, acting in concert with *dpp* (Shimell et al., 1991) as well as the zinc finger protein *schnurri* (Grieder et al., 1995; Arora et al., 1995) acting downstream. In addition, the gene *mothers against dpp* (*Mad*) and related members of the dwarfin gene family in *C. elegans* appear to be dowstream of *dpp* and *C. elegans* TGF-β signalling

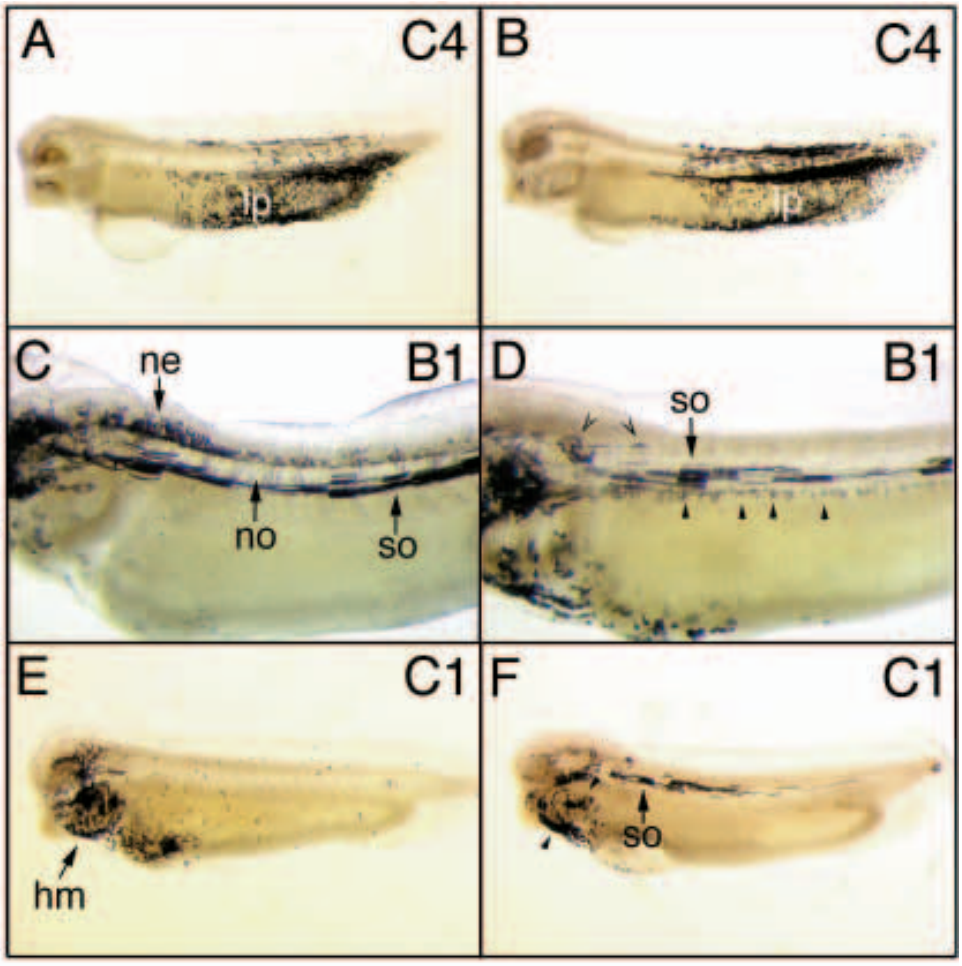

Fig. 9. Cell fate changes induced by *Xvent-2* mRNA injection. 32-cell stage embryos were coinjected into individual blastomeres (indicated at the upper right in each part) with colloidal gold-BSA and either 0.8 ng *ΔXvent-2* mRNA (control, left column) or 0.8 ng *Xvent-2* mRNA (right column). Embryos were fixed at the tadpole stage and processed for silver staining to visualize microinjected cells. (A,B) Embryos were injected into C4 blastomeres. Embryos injected with *Xvent-2* mRNA show the normal lateral plate and somitic fate of descendants. (C,D) Embryos were injected into B1 blastomeres. Embryos injected with *Xvent-2* mRNA specifically fail to populate the notochord. 0% (n=31) of embryos injected with *Xvent-2* showed more than ten labeled notochord cells, in contrast to 83% (n=12) of control-injected embryos. Notochord (no) in the control is the column of labeled thin vertical lines. Note labeled cells in a position ventral to the notochord (closed arrowheads in D), possibly corresponding to immature notochord cells. Note also the absence of well-differentiated neurons in the brain (open arrowheads in D). (E,F) Embryos were injected into C1 blastomeres. In embryos injected with *Xvent-2* mRNA more labeled descendants populate somitic mesoderm. Head mesodermal cells were generally populated to a lesser degree than in control embryos and labeled cells did not line up with the anatomical feature of the arches, appearing more clustered instead (arrowheads in F). hm, head mesoderm; lp, lateral plate; ne, neural tissue; no, notochord; so, somites.

pathways, respectively (Sekelsky et al., 1995; Savage et al., 1996).

For dorsoventral patterning of frog mesoderm, active ventral signalling by *BMP-4* is required to maintain the ventral state and to repress dorsal mesoderm formation (Dale et al., 1992; Jones et al., 1992; Graff et al., 1994; Suzuki et al., 1994; Steinbeisser et al., 1995). *BMP-4* and *chordin* may define antagonizing signalling pathways whose interactions control the dorsoventral pattern of *Xenopus* mesoderm (Sasai et al., 1994; Holley et al., 1995; Jones and Smith, 1995). In the absence of negative regulation either ventral or dorsal cell fate become dominant, as seen in mRNA microinjection experiments with the dominant negative *BMP-4* receptor (Graff et al., 1994; Suzuki et al., 1994), as well as with antisense *gsc* (Steinbeisser et al., 1995).

We provide strong evidence that *Xvent-2* is part of a vertebrate *BMP-4* pathway. First, *Xvent-2* is expressed in most regions that also express *BMP-4*. Second, *BMP-4* induces expression of *Xvent-2* and vice versa. Third, both genes are able to ventralize dorsal mesoderm in a dose-dependent manner, resulting in phenotypes ranging from microcephaly to *Bauchstück* pieces. Fourth, like *BMP-4*, *Xvent-2* and *gsc* are able to interact in a cross-regulatory loop to suppress each other, consistent with their mutually exclusive expression in the early embryo. Fifth, microinjection of *Xvent-2* mRNA can rescue dorsalization by a dominant negative BMP-4 receptor.

With respect to the hierarchy, the rescue by *Xvent-2* of dorsalization by a dominant negative BMP-4 receptor would place *Xvent-2* downstream of *BMP-4*. Yet the induction by *Xvent-2* of *BMP-4* raises the possibility that *Xvent-2* is required for the maintenance of *BMP-4* expression. The hierarchical relationship between *Xvent-1* and *Xvent-2* in the *BMP-4* signalling pathway is unclear. That *Xvent-2* may be upstream of *Xvent-1* is suggested by the larger expression domain of the former as well as the observation that *Xvent-1* causes more limited phenotypic effects compared to *Xvent-2*. However, the capacity of both genes to mutually induce each other's expression after microinjection (Figs 4D, 7) appears inconsistent with this possibility. The negative crossregulatory loops exerted between both *Xvent-1* and *gsc*, as well as *Xvent-2* and *gsc*, make it interesting to test if vent genes are direct targets of *gsc* and vice versa.

In *Drosophila*, *dpp* is functioning as a morphogen that controls dorsoventral cell fates in a dosage-dependent manner. Evidence is mounting that the same may be true in *Xenopus.* Ventralization by *BMP-4* is dose-dependent, ranging from mild microcephaly to *Bauchstück* embryos (Dale et al., 1992; Jones et al., 1992; Schmidt et al., 1995; R. Dosch and C. Niehrs, unpublished results). It is intriguing that *Xvent-2* also ventralizes dorsal mesoderm in a dose-dependent manner, suggesting that it may act to translate positional information provided by *BMP-4* into positional specification. However, at least by in situ hybridisation we have not found any evidence for a dorsoventral graded expression of *BMP-4* or *Xvent-2* mRNA that would support this hypothesis. Possibly, their protein products may be regulated posttranslationally in a graded fashion. Nevertheless, the fact that *Xvent-2* injections are able to elicit the full range of *BMP-4* phenotypes suggests that it is a main nuclear target of *BMP-4*.

More experiments are needed to test the hypothesis of *BMP-4* acting as a morphogen in *Xenopus*, and it needs to be investigated whether *Xvent-2* and *BMP-4* proteins are expressed in a graded fashion. Finally, loss of function experiments will be necessary to address the specific roles and hierarchy of *Xvent-1* and *Xvent-2* in the *BMP-4* pathway.

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Xvent-1 was also isolated as Vox and Xbr-1 in screens for homeobox genes.

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