Xvent-1 mediates BMP-4-induced suppression of the dorsal-lip-specific early response gene *XFD-1'* in *Xenopus* embryos

Henner Friedle, Sepand Rastegar, Hubert Paul, Eckhard Kaufmann and Walter Knöchel¹

Abteilung Biochemie, Universität Ulm, Albert-Einstein-Allee 11, D-89081 Ulm, Germany

¹Corresponding author
e-mail: walter.knoechel@medizin.uni-ulm.de

S.Rastegar and H.Paul contributed equally to this work

Ectopic expression of the ventralizing morphogen BMP-4 (bone morphogenetic protein-4) in the dorsal lip (Spemann organizer) of *Xenopus* embryos blocks transcription of dorsal-lip-specific early response genes. We investigated the molecular mechanism underlying the BMP-4-induced inhibition of the fork head gene *XFD-1'*. The promoter of this gene contains a BMP-triggered inhibitory element (BIE) which prevents activation of this gene at the ventral/vegetal side of the embryo *in vivo*. In the present study, we show that BMP-4-induced inhibition is not direct but indirect, and is mediated by Xvent homeobox proteins. Micro-injections of Xvent-1 RNA and *XFD-1'* promoter deletion mutants demonstrate that Xvent-1 mimics the effect of BMP-4 signalling not only by suppression of the *XFD-1'* gene, but also by utilizing the BIE. Suppression could be reverted using a dominant-negative Xvent-1 mutant. The repressor domain was localized to the N-terminal region of the protein. Gel-shift and footprint analyses prove that Xvent-1 binds to the BIE. Moreover, PCR-based target-site selection for the Xvent-1 homeodomain confirms distinct motifs within the BIE as preferential binding sites. Thus, biological and molecular data suggest that Xvent-1 acts as direct repressor for *XFD-1'* transcription and mediates BMP-4-induced inhibition.

Keywords: BMP-4 signalling/mesoderm/Spemann organizer/transcription factors

Introduction

Mesoderm formation and dorsal/ventral patterning are crucial events in the embryogenesis of all vertebrate organisms. Numerous studies, mainly performed with amphibian embryos, have shown that different parts of this germ layer are induced by distinct members of different growth factor families, like the FGF, the TGF- β or the Wnt families (for recent reviews, see Kessler and Melton, 1994; Dawid, 1994; Slack, 1994; Smith, 1995; Tiedemann *et al.*, 1996). For example, members of the TGF- β family have been identified in the animal cap assay and/or *in vivo* to be potent inducers of dorsal or ventral mesoderm formation. The most dorsal part, the dorsal

blastopore lip, acquires organizing properties. Spemann and Mangold discovered in the early 1920s that transplantation of this structure into the ventral/vegetal region of a host embryo recruits the neighbouring cells in the organization of a complete secondary body axis (Spemann and Mangold, 1924). Thus, the Spemann organizer had been thought to be a local source of activating signals which led to dorsalization and neural induction. However, in the light of recent findings, this model has been revised (for review, see Graff, 1997). In fact, one of the primary functions of the organizer is to antagonize ventralization. BMP-4 as a ventralizing morphogen is bound and sequestered by organizer-secreted molecules, such as chordin (Piccolo *et al.*, 1996), noggin (Zimmerman *et al.*, 1996) or follistatin (Fainsod *et al.*, 1997); dorsal and neural structures only develop in the absence of BMP-4 signalling. This concept is supported by previous findings that inhibition of BMP signalling by a dominant-negative receptor leads to dorsalized embryos often having a second axis (Graff *et al.*, 1994; Suzuki *et al.*, 1994), whereas ectopic expression of BMP-4 on the dorsal side yields completely ventralized embryos (Dale *et al.*, 1992; Jones *et al.*, 1992). While the lack of dorsal structures implies, and is known to correlate with, a repression of dorsal-lip-specific early response genes, like *gooseoid* (Fainsod *et al.*, 1994) or *XFD-1/XFD-1'* (Clement *et al.*, 1995; Re'em-Kalma *et al.*, 1995; Jones *et al.*, 1996; Kaufmann *et al.*, 1996), the underlying mechanism is still unknown.

An important aspect in understanding the organizer's role is to gain insight into the regulation of genes and genetic cascades which are activated in this specific embryonic structure. Of particular interest are those genes which can be activated in the animal cap assay by the dorsal inducer activin A in the presence of cycloheximide, like *gooseoid* (Cho *et al.*, 1991) or *XFD-1'*. *XFD-1'/XFD-1* (*XFKH1/pintallavis*) (Dirksen and Jamrich, 1992; Knöchel *et al.*, 1992; Ruiz i Altaba and Jessell, 1992) are pseudo-allelic fork head/winged helix transcription factors that are specifically activated within the organizer after midblastula transition, the onset of zygotic transcription. As in the case of another activin A-induced gene, the *Xmix.2* gene (Huang *et al.*, 1995), the promoters of *gooseoid* (Watabe *et al.*, 1995) and *XFD-1'* (Kaufmann *et al.*, 1996; Howell and Hill, 1997) genes contain distinct activin responsive elements mediating activin signalling. An important finding in these studies was the discovery that particular promoter deletion mutants enabled transcription of reporter genes not only in the dorsal, but also in the ventral/vegetal region of the embryo, which provided an argument for an endogenous inducer being present throughout the vegetal half. However, an analysis of the *XFD-1'* promoter has demonstrated the existence of an additional BMP inhibitory element (BIE) located further upstream, which responds to BMP-2/4 signalling and

which might be responsible for the suppression of this gene in the ventral/vegetal region *in vivo* (Kaufmann *et al.*, 1996). The inhibition mediated by the BIE is much more effective than the activation induced by activin A or an activin homologue. This observation is fully compatible with earlier results obtained from animal cap assays suggesting that BMP-4 signalling overrides the inducing activity of activin A (Dale *et al.*, 1992; Jones *et al.*, 1992). Therefore, the local expression of the dorsal-lip-specific *XFD-1'* gene is not achieved by direct local activation, but rather results as a consequence of the lack of inhibition by BMP-4.

In the present study, we have analysed the underlying mechanism for the inhibition caused by BMP-4. By investigating the regulation of the *XFD-1'* gene, we have found that repression is not based upon a direct inhibition by BMP signalling but involves expression of another transcriptional repressor as a mediator. This repressor was found to be Xvent-1 (Gawantka *et al.*, 1995), a member of a recently discovered group of *Xenopus* homeobox proteins expressed by BMP signalling (Ladher *et al.*, 1996; Onichtchouk *et al.*, 1996; Papalopulu and Kintner 1996; Schmidt *et al.*, 1996; Tidman-Ault *et al.*, 1996). Based upon a common characteristic feature, an isoleucine to threonine exchange within the third helix of the homeo-domain, it has been suggested that these proteins are related to the Bar family of homeobox proteins (Papalopulu and Kintner, 1996).

Ectopic expression studies by Xvent-1 RNA injections, in combination with the investigation of *XFD-1'* promoter deletion mutants, showed the previously identified BIE to be responsible for down-regulation of *XFD-1'* transcription in the ventral/vegetal region of the embryo *in vivo*. Since gel-shift assays and footprint analyses provide direct proof of the binding of Xvent-1 to the BIE, we conclude that BMP-4-induced inhibition of the dorsal-lip-specific early response gene *XFD-1'* in the ventral/vegetal region of the embryo is directly caused by the transcriptional repressor Xvent-1.

Results

BMP-4 signalling does not directly inhibit transcription of *XFD-1'*

By testing various combinations of activin A, BMP-4 and cycloheximide in the animal cap assay, we could demonstrate that the inhibitory effect of BMP-4 signalling on *XFD-1'* gene expression is not direct but indirect and requires *de novo* protein synthesis. It is known that transcriptional activation of *XFD-1'* by activin A is independent of cycloheximide (Dirksen and Jamrich, 1992; Ruiz i Altaba and Jessell, 1992), but is strongly reduced in the presence of BMP-4 (Clement *et al.*, 1995; Re'em-Kalma *et al.*, 1995; Jones *et al.*, 1996; Kaufmann *et al.*, 1996). As shown in Figure 1, in the presence of cycloheximide, BMP-4 is no longer able to override activin A. Interestingly, treatment of caps with cycloheximide alone results in a slight autoinduction; moreover, incubation with activin A and cycloheximide leads to a superinduction of *XFD-1'* transcription. Similar results have previously been achieved with the *goosecoid* (Tadano *et al.*, 1993) and the *Xnot* gene (von Dassow *et al.*, 1993) which might reflect a common feature for certain dorsal-lip-specific

Fig. 1. RT-PCR of *XFD-1'* transcripts in animal caps. RNA was extracted from animal caps, which had been treated with growth factors in the absence or presence of cycloheximide, and assayed for *XFD-1'* transcripts by RT-PCR. Transcription of the ubiquitous histone H4 gene served as an internal reference. From left to right: P, products of the RT-PCR in the presence of primers but in the absence of template RNA; C, RT-PCR of RNA from untreated caps; the remaining lanes show transcription of *XFD-1'* and histone H4 in the presence of activin A, BMP and cycloheximide in the indicated combinations.

genes. However, this effect does not interfere with our finding that cycloheximide prevents the BMP-4-induced inhibition of *XFD-1'* transcription. Therefore, we conclude that BMP signalling *in vivo* leads to the expression of another factor which, as a transcriptional suppressor, subsequently down-regulates the *XFD-1'* gene. Such intermediate synthesis gives rise to the question of whether there might be a temporal delay for the inhibitory mechanism *in vivo*, allowing an initial transcriptional activation of the *XFD-1'* gene. Transient activation of the *goosecoid* gene after midblastula transition in BMP-4 RNA injected embryos has recently been described (Jones *et al.*, 1996). However, whole mount *in situ* hybridizations for *XFD-1'* transcripts in wild-type and BMP-4 RNA injected embryos from blastula stage until the end of gastrulation (Figure 2A) revealed no evidence for such a delay in the inhibition of *XFD-1'* transcription. From blastula stage until the end of gastrulation, we were unable to detect *XFD-1'* transcripts in BMP-4 RNA injected embryos (the staining observed in some embryos most probably reflects inefficient translation of BMP-4 RNA). This suggests that the inhibitor is rapidly expressed after midblastula transition and is present in sufficient amounts to repress *XFD-1'* gene activation.

Smad1 and Xvent-1 mimic BMP-4-induced inhibition of the *XFD-1'* gene

In search of candidate factors acting as repressors, we decided to investigate whether Xvent homeobox proteins could serve as such mediators for the following reasons: (i) the previously identified BIE sequence contains nucleotide motifs similar to homeobox binding sites already described, it contains a canonical Oct-1 target site (Kaufmann *et al.*, 1996); (ii) Xvents are downstream of BMP-4, Xvent proteins mimic BMP-4 signalling and can rescue the phenotype created by dominant-negative BMP-4 receptor; (iii) *Xvent* genes are directly activated by BMP-4, transcripts co-localize on the ventral side of the embryo; and (iv) ectopic Xvent expression leads to a down-regulation of *goosecoid* (for review, see Lemaire, 1996). Thus, this newly described subclass of homeobox proteins fulfils all requirements with respect to their spatial

Fig. 2. Transcription of the XFD-1' gene. (A) BMP-4 inhibits transcriptional activation of XFD-1'. 1 ng BMP-4 mRNA was injected into both dorsal blastomeres of four-cell-stage *Xenopus* embryos. Injected embryos (lower) and uninjected controls (upper) were fixed at various developmental stages (from left to right: early gastrula, middle/late gastrula, early neurula) and submitted to whole mount *in situ* hybridization for XFD-1' transcripts. Note that injected embryos show a slightly retarded development as compared with uninjected controls. (B) XFD-1' transcription is blocked by BMP-4, Smad1 and Xvent-1. Four-cell-stage embryos were injected into the marginal zone of both dorsal blastomeres with 1 ng BMP-4, Smad1 or Xvent-1 mRNA. Whole mount *in situ* hybridizations for Xvent-1 (top) and XFD-1' (middle) were carried out at gastrula stage. The resulting phenotype (bottom) is shown for embryos at stage 28.

expression and biological function to serve as mediators of BMP-4 action. Indeed, micro-injection experiments performed with BMP-4, Smad1 as a transducer of BMP signalling (Graff *et al.*, 1996; Thomsen, 1996) or Xvent-1 RNA revealed that ectopic overexpression of BMP-4 or Smad1 activates *Xvent-1*, and that all injections, including that of Xvent-1 mRNA, lead to an inhibition of *XFD-1'* transcription (Figure 2B). The results suggest that suppression of the dorsal-lip-specific *XFD-1'* gene in the ventral/vegetal region *in vivo* is mediated by the following cascade: BMP-4 signalling including the signal transducer

Smad1 activates *Xvent-1* which subsequently represses *XFD-1'*.

***Xvent-1* as *XFD-1'* repressor requires the BMP-triggered inhibitory element (BIE)**

To investigate whether this inhibition correlates with an interaction with the previously identified BIE, which has been mapped between positions -289 and -188 upstream of the transcription start site of the *XFD-1'* gene (Kaufmann *et al.*, 1996), we tested various promoter deletion mutants fused to the luciferase reporter gene for

Fig. 3. Xvent-1 down-regulates the XFD-1' promoter *in vivo*. (A) Four-cell-stage embryos were injected into the marginal zone of both dorsal blastomeres with 20 pg of indicated XFD-1' promoter deletion/luciferase constructs, or co-injected with 0.5 ng Xvent-1 mRNA. Luciferase activities were determined at stage 11. Results obtained from DNA injections in the absence of Xvent-1 mRNA were set as 100%. (B) 0.3 ng of Xvent-1 RNA or indicated Xvent-1 RNA mutants (ΔC : truncated at the 3' end; ΔN : truncated at the 5' end; homeobox: truncated at the 5' and the 3' ends) were co-injected with 20 pg of the -257 promoter deletion mutant/luciferase construct (control) into both dorsal blastomeres of four-cell-stage embryos. The luciferase activity was determined at stage 11. (C) 20 pg of -257 promoter deletion/luciferase DNA (control) were co-injected with 0.2 ng ΔN Xvent-1/myc, 0.3 ng Xvent-1 or indicated amounts of VP16/Xvent-1 RNAs, respectively, into both dorsal blastomeres of four-cell-stage embryos. Note that reversal of promoter activity, as shown in the last column, was achieved by co-injection with 5 pg VP16/Xvent-1 RNA. The luciferase activity was determined at stage 11.

their ability to be repressed upon coinjection with Xvent-1 RNA into the dorsal blastomeres of four-cell-stage embryos. Figure 3A demonstrates that reporter gene activities of all 5'-deletions down to position -233 are strongly inhibited by Xvent-1 injection, whereas more extensive deletions become refractory and the -180 deletion mutant regains full activity. This implies that Xvent proteins act as repressors on the BIE which, with consideration of our previous findings (see above), can now be narrowed to the region between -233 and -188. We have also investigated the localization of the repressor domain within the Xvent-1 protein. Co-injections of the -257 promoter/luciferase construct with Xvent-1 RNA mutants containing the DNA binding domain, but deleted at either the N- or at the C-terminal coding regions, revealed that the repressor function requires the N-terminus of the protein (Figure 3B). In line with this finding, injection of the ΔC mutant containing the coding region of the N-terminus led to ventralized embryos, whereas injection of the ΔN mutant did not alter the normal phenotype (data not shown). It may be argued that failure of the ΔN mutant to inhibit reporter gene activity does not result from the lack of a repressor domain but is due to inefficient translation or removal of a nuclear localization signal (NLS). Therefore, we have fused the ΔN mutant to a myc-tag and an NLS-containing vector (Rupp *et al.*, 1994). Following injection of this RNA, the corresponding protein could be visualized on Western blots of embryonic protein extracts by immunostaining with anti-myc antibodies (data not shown). Also, co-injection of the mutant RNA with the -257 promoter/

luciferase construct did not lead to a significant reduction of reporter gene activity (Figure 3C). We conclude, therefore, that the N-terminal domain is required for repressor function.

XFD-1' activity is rescued by a dominant-negative Xvent-1 mutant

Initial loss-of-function studies, i.e. to rescue for normal phenotypes after ventralization and to revert inhibition of *XFD-1'* activity, were carried out with the myc-tagged ΔN mutant. While this mutant led to neither ventralization nor to a significant inhibition of the -257 promoter/reporter construct, it was rather inefficient in competing for the ventralizing and inhibitory effect of the wild-type protein. Upon co-injection of Xvent-1 and ΔN mutant RNAs, we observed neither the rescue of ventralized phenotypes, nor a reversal of promoter activity of the *XFD-1'* gene (Figure 3C). Also, at higher ratios of mutant to wild type Xvent-1, we did not observe a significant increase of reporter gene activity (data not shown). We therefore used a recently described dominant-negative Xvent-1 mutant in which the N-terminal domain had been replaced by the VP16 activating domain (Onichtchouk *et al.*, 1998). This VP16/Xvent-1 mutant exhibits dorsaling activity when injected at the ventral side and is able to rescue for normal phenotypes after ventralization by Xvent-1. We show here that this mutant is also able to revert the inhibitory effect of wild-type Xvent-1 on the BIE and to restore reporter gene activity to normal values (Figure 3C). Additionally, upon injection of this mutant we observe a significant enhancement of *XFD-1'* promoter

Fig. 4. Xvent-1 homeodomain binds to the BIE. (A) Mobility shift assays were performed with bacterially expressed Xvent-1 homeodomain and labelled -256/-188 BIE fragment of the XFD-1' promoter. Lane a shows free DNA, while the amounts of protein added to the other reactions were 50 ng (lane b), 100 ng (lane c), 200 ng (lane d) and 400 ng (lanes e-i) (denoted by the black bar at the top of the figure). White triangles denote addition of unlabelled, unspecific competitor DNA (lanes f and g) and unlabelled BIE as specific competitor (lanes h and i) at 10- or 100-fold excess, respectively. (B) The binding site of Xvent-1 homeodomain protein is located between -233 and -188 of the XFD-1' promoter. The scheme shows the results from four mobility shift assays carried out with promoter fragments as shown; (+) indicates a shift with Xvent-1 homeodomain, (-) no shift detected.

activity above normal levels in a dose-dependant manner, which can readily be explained by replacement of the repressor to a strong activating domain. Thus, results obtained with the dominant-negative VP16/Xvent-1 mutant support our notion that Xvent-1 acts on the BIE and regulates *XFD-1'* activity.

Xvent-1/BIE complex formation

All results presented so far do not exclude the possibility that Xvent-1 might only act as an intermediate factor, being required for the activation of another gene whose product would bind to the BIE. Therefore, we have checked the BIE-binding properties of Xvent-1 by gel-shift and footprint analyses. First, gel-shift analysis shows that bacterially expressed Xvent-1 homeodomain binds to the labelled BIE fragment in a concentration-dependent manner, with higher protein concentrations leading to the formation of oligomers (Figure 4A). This binding is independent of the presence of an unspecific competitor DNA, but is significantly reduced by the addition of unlabelled BIE as specific competitor. Further fragmentation of the BIE localized the binding site between positions -233 and -188 (Figure 4B).

This result is strongly supported by DNase I footprint experiments which were performed on both strands (Figure

Fig. 5. DNase I footprinting specifies DNA-binding sites of Xvent-1. Both strands of the BIE (-256/-188) were used for DNase I protection assays. 3'-end labelled templates were incubated with increasing amounts (10, 20 and 40 ng; see triangle) of Xvent-1 homeodomain and subsequently digested with DNase I. G/A: Maxam-Gilbert reaction specific for dG and dA residues. Sequences and protected regions of the sense and antisense strands are shown on the left or, as a double strand, at the bottom. Protected regions (PR-1 and PR-2) are denoted by lines.

5). For each strand, we can demonstrate two protected regions of different lengths (PR-1 and PR-2). The positions of these regions overlap for both strands, and are located between -232 and -211 (PR-1), and -207 and -200 (PR-2). Nucleotide sequence comparison between PR-1 and PR-2 revealed a common 5'-CTATT(T/C)G-3' consensus motif (reverse complementary in PR-2). Interestingly, this motif is part of a canonical Oct-1 target site (5'-ATTTGCAT-3'; Singh *et al.*, 1986) present within PR-1, and displays a distant relationship to homeobox target sites (Locker, 1996) described previously. However, as noted above, Xvent proteins are related to the Bar family of homeobox proteins, which are characterized by an isoleucine to threonine exchange within the third helix. Therefore, they might exhibit different target site specificity and bind to related, but not to identical, sequence motifs as has been found for other classes of homeobox proteins.

Xvent-1 target site selection

To gain additional information on these binding motifs, we have used the bacterially expressed Xvent-1 homeo-

Fig. 6. PCR-based target site selection. A comparison of five consecutive matches between protected regions from the footprint (PR-1 and PR-2) and 15 sequences (from a total of 70) obtained by target site selection after seven rounds of amplification is shown. Based upon their homology to the PR-1 sequence, the targets are subdivided into three categories. 60% of all 70 sequences fall into the first category (consensus: 5'-CTATTTG-3'), 25% into the second (consensus: 5'-TGCATTTG-3') and 10% into the third category (consensus: 5'-TTGATC-3'). Note that the PR-2 motif matches the first category.

domain to perform a PCR-based target site selection from a mixture of 18-fold degenerated oligonucleotides. After seven rounds of selection/amplification, the targets were cloned. A computer-aided comparison of 70 resulting sequences with the PR-1 and PR-2 sequences led to the finding that 95% of all target sequences can be aligned to three distinct types of motifs, all being present within PR-1. Figure 6 shows a selection of 15 sequences. A major group (~60%) is characterized by the consensus 5'-CTATTTG-3', which had already been detected in footprint studies (see above), a second group (25%) by 5'-TGCATTTG-3' and a minor group (10%) by 5'-TTGATC-3'. Although we are aware that these sites share an overlap of three or four nucleotides, we cannot yet explain why these different types of targets are selected. Gel-shift assays with randomly selected targets from each of the three groups revealed highest affinities for targets of the major group (data not shown). A more detailed characterization of individual sequences for their binding properties, including determination of K_D values, and mutational analyses are currently under investigation. However, the fact that the major group of selected sequences shares extensive homologies with the PR-1, as well as with the PR-2 motifs, strongly suggests that these sites represent a preferential target site for the Xvent-1 homeodomain and thereby also supports our findings from the footprint experiments.

Discussion

The ventralizing morphogen BMP-4 is regarded as a key molecule in patterning the mesoderm of vertebrate embryos. The molecular basis for the dorsalizing and neuralizing activities of the organizer are secreted proteins,

such as chordin (Piccolo *et al.*, 1996), noggin (Zimmerman *et al.*, 1996) and follistatin (Fainsod *et al.*, 1997), which bind and sequester the BMP-4 protein. Expression of these antagonizing proteins leads to an elimination of BMP-4 signalling at the dorsal side of the gastrula stage embryo which, in turn, is a pre-requisite for formation of dorsal and neural structures. A primary step in organizer formation is the activation of early response genes, which are induced by activin or activin-like signalling even in the absence of *de novo* protein synthesis. Distinct but different activin responsive elements have been described for the *Xmix.2*, *gooseoid*, *XFD-1'* and *Xlim-1* genes (Huang *et al.*, 1995; Watabe *et al.*, 1995; Kaufmann *et al.*, 1996; Howell and Hill, 1997; Rebbert and Dawid, 1997). Remarkably, certain promoter deletions of *gooseoid* and *XFD-1'* genes render expression of reporter genes also in the ventral/vegetal region of the embryo, which provides an argument for an ubiquitous distribution of dorsalizing activity within the vegetal half. Thus, there is demand for a mechanism which prevents transcription of dorsal-lip-specific genes at the ventral side of the embryo *in vivo*. Since BMP-2/4 are known to inhibit transcription of *XFD-1/XFD-1'* (Clement *et al.*, 1995; Re'em-Kalma *et al.*, 1995; Jones *et al.*, 1996), we have focused our interest on the molecular mechanism underlying the inhibitory action of BMPs on dorsal-lip-specific genes. Our hypothesis of BMPs serving as natural inhibitors is also based upon the distribution of BMP-4 transcripts in the ventral/lateral mesoderm and their absence from the dorsal lip (Fainsod *et al.*, 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt *et al.*, 1995). In a previous study, we have shown that the *XFD-1'* promoter contains a BMP-4-triggered inhibitory element (BIE) leading to a ventral inactivation of reporter genes in whole embryos (Kaufmann *et al.*, 1996), but it was uncertain whether this element directly responded to BMP signalling. From the results of our present study, it is now clear that inhibition by BMP-4 signalling is not direct but indirect, and that Xvent homeobox proteins serve as mediators in the BMP-4-induced inhibition of the dorsal-lip-specific *XFD-1'* gene. Based upon co-injection of Xvent-1 mRNA and *XFD-1'* promoter deletion mutants, we can show that the BIE is required for down-regulation of reporter gene activity. Furthermore, gel-shift and footprint experiments performed *in vitro* provide evidence of the physical interaction between the Xvent-1 homeodomain and distinct sequence motifs within the BIE. These findings are further supported by results from PCR-based target site selection. Although it is known that homeodomains bind DNA *in vitro* with relatively low sequence specificity, and this approach in the case of the Xvent-1 homeodomain also did not reveal a strictly conserved target motif, a major group of binding sequences can be aligned to the motif 5'-CTATT(CT)G-3', also present within the BIE (PR-1 and PR-2). Thus, all the experimental evidence suggests that Xvent-1 serves as an intermediate and as a transcriptional repressor in BMP-4-induced inhibition of *XFD-1'* gene activation.

The repressor function of Xvent-1 resides in the N-terminal part of the protein. While a mutant lacking the N-terminus did not suppress -257 promoter/reporter activity, it was not, on the other hand, sufficient by itself to compensate for the effects obtained with wild-type Xvent-1. However, ventralized phenotypes have recently

been reported to be rescued using a dominant-negative mutant in which the N-terminus had been replaced by the VP16 activating domain (Onichtchouk *et al.*, 1998). We demonstrate here that this VP16/Xvent-1 mutant also restores promoter activity of the *XFD-1'* gene when co-injected wild-type Xvent-1. The observation that the *XFD-1'* promoter can even be stimulated by this mutant in a dose-dependent manner above normal levels, additionally argues for an interaction of Xvent-1 with the BIE. The failure of the ΔN mutant in rescue experiments remains to be elucidated. It may be speculated that the VP16 mutant, as well as the wild-type protein, interact with additional proteins, thereby stabilizing the DNA-protein complex. Increased binding specificity and affinity through interactions with other transcription factors has been reported for other homeodomain proteins (for reviews, see Gehring *et al.*, 1994; Vershon, 1996). The ΔN mutant binds to DNA *in vitro* (data not shown), but due to the loss of the N-terminus, which might be required for additional protein interactions, this complex may not be very stable and may be displaced *in vivo* by the full-length protein.

In view of the important role of Xvent-1 for regulation of *XFD-1'*, the question arises, how is *Xvent-1* expression activated by BMP signalling? Accumulating evidence suggests that *Xvent* genes are directly activated by the BMP signalling pathway. Here, we demonstrate by whole mount *in situ* hybridization (Figure 2B) that Smad1 RNA injection activates *Xvent-1* transcription, thereby confirming previous results obtained by RT-PCR (Meersseman *et al.*, 1996). Furthermore, BMP-4 induces *Xom* (*Xvent-2*) and *Xvent-1* transcription in dispersed cells even in the presence of cycloheximide (Ladher *et al.*, 1996; our unpublished data). Thus, it is apparent that no other *de novo*-synthesized intermediates are necessary, and that *Xvent-1* is a direct target of BMP signalling. This signalling pathway, in addition to type II and type I receptors, involves distinct members of the Smad family as intracellular signal transducers (for recent reviews, see Whitman, 1997; Wrana and Pawson, 1997). BMP signalling is mediated by Smad1 or Smad5, which are phosphorylated by the activated type I receptor and form heteromeric complexes with Smad 4. When translocated to the nucleus, the Smad proteins act as transcription activators/transactivators. While *Drosophila* Mad has recently been shown to bind directly to DNA (Kim *et al.*, 1997), results obtained with the activin signalling pathway on the *Xmix.2* promoter in *Xenopus* suggest that an additional transcription factor, FAST-1, interacts with Smad2/Smad4 proteins and mediates DNA binding (Chen *et al.*, 1996). In the case of BMP signalling to the *Xvent-1* promoter, it will be interesting to see whether *Xenopus* homologues of the *Drosophila* protein, schnurri, which acts in dpp signalling (Arora *et al.*, 1995; Grieder *et al.*, 1995), will participate in the formation of such a complex. The regulation of the dorsal-lip-specific *XFD-1'* gene is schematically shown in Figure 7. The activating pathway induced by activin A has recently been investigated in more detail (Howell and Hill, 1997). One activin response element (ARE) is located within the first intron, and responds to activin A also in the presence of cycloheximide, whereas the previously identified ARE within the 5' region (Kaufmann *et al.*, 1996) responds in an indirect

Fig. 7. Regulation of the *XFD-1'* gene. Expression of the *XFD-1'* gene is established by an activating signalling pathway which might be triggered by activin or an activin-like ligand and finally acts on a direct activin response element (ARE) within the intron and, indirectly, on a previously identified element within the 5' proximal region (Kaufmann *et al.*, 1996; Howell and Hill, 1997), which we now refer to as activin-triggered activating element (AAE). While at least this latter pathway operates pan-mesodermally, the inhibitory pathway is triggered in ventral and lateral mesoderm by BMP and leads to the expression of Xvent-1, which binds as a repressor to the BIE and overrides the action of the activator. While Smad1/Smad4 and Smad2/Smad4 are defined components of the BMP and activin signalling pathways, respectively, additional factors will probably be required in the formation of corresponding transcription complexes. Also, the transcription activator mediating the indirect activin pathway remains to be determined. Exons I and II are shown as black boxes, and the transcription start site by an arrow.

manner and requires synthesis of an as yet unknown factor. We therefore suggest that this 5' located element should be referred to as activin-triggered activating element (AAE) instead of ARE. While direct activation by activin A is probably mediated by the Smad2/Smad4 complex and additional transcription factors, we demonstrate here the hierarchy of events leading to repression of this gene. Since BMP-4 as well as Xvent-1 expression does not overlap with the organizer, the inhibitory pathway, as shown in Figure 7, may presumably be regarded as the regulatory key that prevents transcription of this dorsal-lip-specific gene in ventral mesoderm *in vivo*. However, additional mechanisms cannot be excluded. Two other homeobox proteins, Mix.1 and XIPOU2, have recently been observed to transform dorsal mesoderm to a ventral fate and to suppress a number of dorsal mesoderm-specific genes (Mead *et al.*, 1996; Witta and Sato, 1997). Whereas XIPOU2 has been positioned downstream of GSK-3 β of the Wnt pathway and upstream of chordin, Mix.1 is also induced by BMP-4 and acts in a signalling cascade that participates in ventral patterning. Moreover, the observation of heterodimer formation between Mix.1 and siamois (Mead *et al.*, 1996) gives rise to speculation that Xvent-1 may interact with other homeobox proteins, especially with other members of the same family. In this context, it is tempting to investigate whether Xvent-2, another closely related homeobox protein [73% identity within the homeodomain (Onichtchouk *et al.*, 1996)], exerts similar effects or co-operates with Xvent-1. *Xvent-2* is also known as a direct target of BMP signalling, it ventralizes dorsal mesoderm, and preliminary footprint data suggest that it also interacts with the BIE (our unpublished data). Accordingly, the inhibition of the *XFD-1'* gene in ventral mesoderm *in vivo* could include additional factors which will either co-operate with Xvent-1 at the BIE motif or

act on other, as yet unidentified targets. Also, the recently described antagonism between BMP and activin signalling pathways mediated by Smads (Candia *et al.*, 1997) has to be considered as an additional component in our model. A limited pool of Smad4 is competed for by Smad1 as well as Smad2, thereby leading to an intracellular antagonism between both pathways. Whereas these findings do not account for separate DNA-binding motifs for activin and BMP signalling or an involvement of the transcriptional repressor Xvent-1, it should be anticipated that *Xvent-1* and *XFD-1'* activation is modulated by an analogous intracellular competition for Smad4 as has been shown for *Xvent-2* and *goosecoid* activation. It is evident that all regulatory events of Xvent-1 expression also affect the subsequent inactivation of *XFD-1'*; we have therefore started to analyse the promoter of the *Xvent-1* gene for an interaction with transcription activators associated with Smad1. The results of these studies will give further insight into the inhibitory pathway that controls the spatial expression of dorsal-lip-specific genes and prevents their activation in ventral mesoderm. The present work demonstrates that as a transcriptional repressor, Xvent-1 mediates the final step within this cascade. Binding of Xvent-1 to distinct sequence motifs within the BIE promotes a suppressory mechanism that overrides the activin-directed activating pathway and inhibits *XFD-1'* transcription.

Materials and methods

Embryos and animal cap assays

In vitro fertilization, embryo culture and preparation of animal caps were carried out as described (Kaufmann *et al.*, 1996). Animal pole explants were dissected from *Xenopus laevis* embryos at stage 8 (stages according to Nieuwkoop and Faber, 1975), incubated for 45 min with 66 ng/ml activin A and/or BMP-4 extracts from Baculovirus-infected Sf9 cells in 0.1% albumin/Barth solution [44 mM NaCl, 0.5 mM KCl, 0.41 mM MgSO₄, 0.17 mM Ca(NO₃)₂, 0.2 mM CaCl₂, 0.6 mM NaHCO₃, 0.05 mM Na₂HPO₄, 0.07 mM KH₂PO₄] and 5 mM HEPES pH 7.3, and subsequently cultured for 3 h in Barth solution at room temperature. Cycloheximide (5 µM, Sigma) was applied to whole embryos 1 h before dissecting the animal caps, which were subsequently incubated in the absence or presence of growth factors and 17.8 µM cycloheximide.

RT-PCR

Total RNA derived from animal caps was extracted by employing the RNeasy extraction kit (Qiagen). Following its isolation, RNA was treated with DNase I (Boehringer Mannheim) to remove contaminating DNA. RT-PCR was performed in a volume of 50 µl including 0.5 µg of RNA. Reverse transcription and PCR amplification was performed in a single buffer system due to the unique capacity of the rTth DNA polymerase (Perkin Elmer) to act both as a reverse transcriptase and a thermostable DNA polymerase. The amplification of a 190 bp DNA fragment from the ubiquitous histone H4 RNA using two primers (5'-CGGG-ATAACATTCAGGGTATCACT-3' and 5'-ATCCATGGCGGTAAGTGTCTTCCT-3') served as an internal reference for the amount of RNA. Two other primers (5'-AGGAGATGAACTGGAGGGAGCTTAA-3' and 5'-GCCAAGGTAGCCATCATTAGAGAGAC-3') resulted in the generation of a 232 bp DNA fragment from *XFD-1'* RNA. For quantitative RT-PCR, the linear range of amplification was within 29 cycles for histone H4 and for 37 cycles for *XFD-1'* RNA. The annealing temperature for histone H4 was 50°C and for *XFD-1'* it was 56°C.

Reporter gene constructs, synthesis and micro-injection of mRNAs

Various deletions of the 5'-flanking region of a *Xenopus XFD-1'* genomic clone (Kaufmann *et al.*, 1996) were generated by PCR, employing appropriate primers containing restriction sites for *Bam*HI at the 5'-end and *Hind*III at the 3'-end. These promoter fragments were cloned into the *Bgl*III and *Hind*III sites of the pGL3-basic vector (Promega).

cDNAs of BMP-4 (Köster *et al.*, 1991), Smad1 (Graff *et al.*, 1996; Thomsen, 1996) and Xvent-1 (Gawantka *et al.*, 1995) were cloned into pSP64T3 transcription vector. RNAs for micro-injections were prepared by *in vitro* transcription using SP6 cap scribe kit (Boehringer Mannheim). Indicated amounts of RNA were injected or co-injected with 20 µg of $\Delta 5'$ -*XFD-1'* promoter deletion/pGL3-basic vector constructs into the marginal zone of dorsal blastomeres of *Xenopus* embryos at the four-cell-stage.

The DNA fragments coding for Xvent-1 mutants were created by PCR and subsequently cloned into the *Xho*I-*Bgl*III sites of the pSP64T3. The mutant Δ C Xvent-1 encodes amino acids 1–188 (primers: 5'-CCGCTCGAGATGGTTCAACAGGGATTCTC-3' and 5'-GGAAGATCTTCATATCTGCCTCTTCAGTTTCAT-3'); the mutant Δ N Xvent-1 besides an artificial ATG encodes amino acids from 129 to 264 (primers: 5'-CCGCTCGAGATGCAGCGCCGGCTGAGAACGGCAT-3' and 5'-GGAAGATCTTCATTACATATACTGAGCCCCAA-3'); the homeobox is located between amino acids 129 and 188 (Gawantka *et al.*, 1995). The Δ N Xvent-1/myc-tagged mutant containing a NLS was created using the upstream primer as described above and a downstream primer (5'-GCTCTAGATATCTGCCTCTTCAGTTTCAT-3'). The PCR product was cloned into the *Xho*I-*Xba*I sites of the CS2+NLS MT vector (Rupp *et al.*, 1994).

Whole mount *in situ* hybridization

XFD-1' and Xvent-1 transcripts in *Xenopus* embryos were detected by using the whole mount technique (Harland, 1991) with some modifications as described (Kaufmann *et al.*, 1996). *In situ* hybridization was performed with digoxigenin-labelled antisense RNA transcribed from *XFD-1'* or Xvent-1 cDNAs, respectively.

Preparation of embryo extracts and luciferase assay

Whole cell extracts were made on ice by homogenization with 30 µl embryo lysis buffer {2 mM CDTA (1,2-cyclohexanediaminetetraacetic acid), 2 mM DTT, 10% glycerol, 1% Triton-X100, 25 mM Tricine [N-tris(hydroxymethyl)methyl-glycine], pH 7.8}. After 10 min on ice and centrifugation for 15 min at 20 000 g at 4°C, the resulting supernatant was taken for luciferase assay. Luciferase assays were performed according to Joore *et al.* (1996) with several modifications. 90 µl of embryo extract and 30 µl of luciferase assay substrate [270 µM coenzyme A, 470 µM luciferin, 530 µM ATP-Na₂, 33.3 mM DTT, 0.1 mM EDTA, 2.67 mM MgSO₄, 1.07 mM (MgCO₃)₄×Mg(OH)₂×5 H₂O, 20 mM Tricine] were used for each reaction. Analysis of luciferase activity was carried out with Lumat LB 9507 (EG and G Berthold) for 2 s at room temperature.

Mobility shift assays and DNase I footprinting

The Xvent-1 homeodomain was bacterially expressed in the pRSET expression system (Invitrogen). The protein was purified by affinity chromatography on Ni-NTA agarose (Qiagen). For mobility shift assays and DNase I footprinting studies, the desired DNA fragments were excised from promoter deletion/pGL3 vectors containing either the previously identified BIE (Kaufmann *et al.*, 1996) or the nucleotides –256 to –188 and 3'-labelled on one strand by a fill-in reaction with [α -³²P]dCTP and Klenow DNA polymerase. Binding reactions for all mobility shift assays were carried out on ice for 25 min in 30 µl binding buffer (1 mM DTT, 1 mM MgCl₂, 30 mM KCl, 13.2% glycerol, 30 mM HEPES pH 6.8) containing 1 µg poly(dI-dC) and 1 ng of the gel-purified probe. After 5 min of pre-incubation the protein was added. The samples were separated on native 7% polyacrylamide gels at 4°C for 2–3 h at 160 V in 0.5×TB (89 mM boric acid, 89 mM Tris pH 8.7). Gels were pre-electrophoresed at 250 V for 1 h. DNA target site selection was performed under the same conditions as described (Kaufmann *et al.*, 1995).

Formation of the protein–DNA complex for DNase I footprinting was achieved under the same conditions as described above. The concentration of MgCl₂ was subsequently raised to 5 mM, and 0.065 U (free DNA) or 0.195 U (DNA + protein) of DNase I were added at room temperature for 30 s (free DNA) or 90 s (DNA + protein). Hydrolysis was stopped by adding an equal volume of sample buffer (20 mM EDTA, 880 mM sucrose, 14 M urea). Sequencing reactions were performed according to Maxam and Gilbert (1980). After pre-electrophoresis for 2 h at 70 W, samples were separated on 8 M urea–11% polyacrylamide gels at 60 W.

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References

- Arora,K., Dai,H., Kazuko,S.G., Jamal,J., O'Connor,M.B., Letsou,A. and Warrior,R. (1995) The *Drosophila schnurri* gene acts in the Dpp/TGF β signaling pathway and encodes a transcription factor homologous to the human MBP family. *Cell*, **81**, 781–790.
- Candia,A.F., Watabe,T., Hawley,S.H.B., Onichtchouk,D., Zhang,Y., Derynck,R., Niehrs,C. and Cho,K.W.Y. (1997) Cellular interpretation of multiple TGF- β signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads. *Development*, **124**, 4467–4480.
- Chen,X., Rubock,M.J. and Whitman,M. (1996) A transcriptional partner for MAD proteins in TGF- β signalling. *Nature*, **383**, 691–696.
- Cho,K.W.Y., Blumberg,B., Steinbeisser,H. and De Robertis,E.M. (1991) Molecular nature of Spemann's organizer: The role of the *Xenopus* homeobox gene *gooseoid*. *Cell*, **67**, 1111–1120.
- Clement,J.H., Fettes,P., Knöchel,S., Lef,J. and Knöchel,W. (1995) Bone morphogenetic protein 2 in the early development of *Xenopus laevis*. *Mech. Dev.*, **52**, 357–370.
- Dale,L., Howes,G., Price,B.M.J. and Smith,J.C. (1992) Bone morphogenetic protein 4: a ventralizing factor in *Xenopus* development. *Development*, **115**, 573–585.
- Dawid,I.B. (1994) Intercellular signalling and gene regulation during early embryogenesis of *Xenopus laevis*. *J. Biol. Chem.*, **269**, 6259–6262.
- Dirksen,M.L. and Jamrich,M. (1992) A novel, activin-inducible, blastopore lip-specific gene of *Xenopus laevis* contains a fork head DNA-binding domain. *Genes Dev.*, **6**, 599–608.
- Fainsod,A., Steinbeisser,H. and De Robertis,E.M. (1994) On the function of BMP-4 in patterning the marginal zone of the *Xenopus* embryo. *EMBO J.*, **13**, 5015–5025.
- Fainsod,A., Deißler,K., Yelin,R., Marom,K., Epstein,M., Pillemer,G., Steinbeisser,H. and Blum,M. (1997) The dorsalizing and neural inducing gene *folistatin* is an antagonist of BMP-4. *Mech. Dev.*, **63**, 39–50.
- Gawantka,V., Delius,H., Hirschfeld,K., Blumenstock,C. and Niehrs,C. (1995) Antagonizing the Spemann organizer: role of the homeobox gene *Xvent-1*. *EMBO J.*, **14**, 6268–6279.
- Gehring,W.J., Affolter,M. and Bürglin,T. (1994) Homeodomain proteins. *Annu. Rev. Biochem.*, **63**, 487–526.
- Graff,J.M., Thies,R.S., Song,J.J., Celeste,A.J. and Melton,D.A. (1994) Studies with a *Xenopus* BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals *in vivo*. *Cell*, **79**, 169–179.
- Graff,J.M., Bansal,A. and Melton,D.A. (1996) *Xenopus* Mad proteins transduce distinct subsets of signals for the TGF- β superfamily. *Cell*, **85**, 479–487.
- Graff,J.M. (1997) Embryonic patterning: to BMP or not to BMP, that is the question. *Cell*, **89**, 171–174.
- Grieder,N.C., Nellen,D., Burke,R., Basler,K. and Affolter,M. (1995) *schnurri* is required for *Drosophila* Dpp signaling and encodes a zinc finger protein similar to the mammalian transcription factor PRDII-BF1. *Cell*, **81**, 791–800.
- Harland,R.M. (1991) *In situ* hybridization: an improved whole-mount method for *Xenopus* embryos. In Kay,B.K. and Peng,H.B. (eds), *Methods in Cell Biology*. Academic Press, San Diego, CA, pp. 685–695.
- Hemmati-Brivanlou,A. and Thomsen,G.H. (1995) Ventral mesodermal patterning in *Xenopus* embryos: expression patterns and activities of BMP-2 and BMP-4. *Dev. Genet.*, **17**, 78–89.
- Howell,M. and Hill,C.S. (1997) Xsmad2 directly activates the activin-inducible, dorsal mesoderm gene *XFKH1* in *Xenopus* embryos. *EMBO J.*, **16**, 7411–7421.
- Huang,H.C., Murtaugh,L.C., Vize,P.D. and Whitman,M. (1995) Identification of a potential regulator of early transcriptional responses to mesoderm inducers in the frog embryo. *EMBO J.*, **14**, 5965–5973.
- Jones,C.M., Lyons,K.M., Lapan,P.M., Wright,C.V.E. and Hogan,B.L.M. (1992) DVR-4 (bone morphogenetic protein-4) as a postero-ventralizing factor in *Xenopus* mesoderm induction. *Development*, **115**, 639–647.
- Jones,C.M., Dale,L., Hogan,B.L., Wright,C.V. and Smith,J.C. (1996) Bone morphogenetic protein-4 (BMP-4) acts during gastrula stages to cause ventralization of *Xenopus* embryos. *Development*, **122**, 1545–1554.
- Joore,J., Fascinia,C., Speksnijder,J.E., Kruijer,W., Destrée,O.H.J., van de Eijnden-van Raaij,A.J.M., de Laat,S.W. and Zivkovic,D. (1996) Regulation of the zebrafish *gooseoid* promoter by mesoderm inducing factors and Xwnt1. *Mech. Dev.*, **55**, 3–18.
- Kaufmann,E., Müller,D. and Knöchel,W. (1995) DNA recognition site analysis of *Xenopus* winged helix proteins. *J. Mol. Biol.*, **248**, 239–254.
- Kaufmann,E., Paul,H., Friedle,H., Metz,A., Scheucher,M., Clement,J.H. and Knöchel,W. (1996) Antagonistic actions of activin A and BMP-2/4 control dorsal-lip-specific activation of the early response gene *XFD-1'* in *Xenopus laevis* embryos. *EMBO J.*, **15**, 6739–6749.
- Kessler,D.S. and Melton,D.A. (1994) Vertebrate embryonic induction: mesodermal and neural patterning. *Science*, **266**, 596–604.
- Kim,J., Johnson,K., Chen,H.J., Carroll,S. and Laughon,A. (1997) *Drosophila* MAD binds to DNA and directly mediates activation of *vestigial* by decapentaplegic. *Nature*, **388**, 304–308.
- Knöchel,S., Lef,J., Clement,J., Klocke,B., Hille,S., Köster,M. and Knöchel,W. (1992) Activin A induced expression of a fork head related gene in posterior chordamesoderm (notochord) of *Xenopus laevis* embryos. *Mech. Dev.*, **38**, 157–165.
- Köster,M., Plessow,S., Clement,J.H., Lorenz,A., Tiedemann,H. and Knöchel,W. (1991) Bone morphogenetic protein 4 (BMP-4), a member of the TGF- β family, in early embryos of *Xenopus laevis*: analysis of mesoderm inducing activity. *Mech. Dev.*, **33**, 191–200.
- Ladher,R., Mohun,T.J., Smith,J.C. and Snape,A.M. (1996) *Xom*: a *Xenopus* homeobox gene that mediates the early effects of BMP-4. *Development*, **122**, 2385–2394.
- Lemaire,P. (1996) The coming of age of ventralising homeobox genes in amphibian development. *BioEssays*, **18**, 701–704.
- Locker,J. (1996) *Transcription Factors—Essential Data*. 1st edn, John Wiley and Sons, Chichester, UK.
- Maxam,A. and Gilbert,W. (1980) Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.*, **65**, 499–525.
- Mead,P.E., Brivanlou,I.H., Kelley,C.M. and Zon,L.I. (1996) BMP-4-responsive regulation of dorsal-ventral patterning by the homeobox protein Mix.1. *Nature*, **382**, 357–360.
- Meersseman,G. et al. (1997) The C-terminal domain of Mad-like signal transducers is sufficient for biological activity in the *Xenopus* embryo and transcriptional activation. *Mech. Dev.*, **61**, 127–140.
- Nieuwkoop,P.D. and Faber,J. (1975) *Normal Table of Xenopus laevis*. (Daudin), 2nd edn, Elsevier/North-Holland Publishing Co., Amsterdam.
- Onichtchouk,D., Gawantka,V., Dosch,R., Delius,H., Hirschfeld,K., Blumenstock,C. and Niehrs,C. (1996) The *Xvent-2* homeobox gene is part of the BMP-4 signaling pathway controlling dorsoventral patterning of *Xenopus* mesoderm. *Development*, **122**, 3045–3053.
- Onichtchouk,D., Glinka,A. and Niehrs,C. (1998) Requirement for *Xvent-1* and *Xvent-2* gene function in dorsoventral patterning of *Xenopus* mesoderm. *Development*, **125**, 1447–1456.
- Papalopulu,N. and Kintner,C.A. (1996) A *Xenopus* gene, *Xbr-1*, defines a novel class of homeobox genes and is expressed in the dorsal ciliary margin of the eye. *Dev. Biol.*, **174**, 104–114.
- Piccolo,S., Sasai,Y., Lu,B. and De Robertis,E.M. (1996) Dorsoventral patterning in *Xenopus*: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell*, **86**, 589–598.
- Rebbert,M.L. and Dawid,I.B. (1997) Transcriptional regulation of the *Xlim-1* gene by activin is mediated by an element in intron 1. *Proc. Natl Acad. Sci. USA*, **94**, 9717–9722.
- Re'em-Kalma,Y., Lamb,T. and Frank,D. (1995) Competition between noggin and bone morphogenetic protein 4 activities may regulate dorsalization during *Xenopus* development. *Proc. Natl Acad. Sci. USA*, **92**, 12141–12145.
- Ruiz i Altaba,A. and Jessell,T.M. (1992) *Pintallavis*, a gene expressed in the organizer and midline cells of frog embryos: involvement in the development of the neural axis. *Development*, **116**, 81–93.
- Rupp,R.A., Snider,L. and Weintraub,H. (1994) *Xenopus* embryos regulate the nuclear localization of XMyoD. *Genes Dev.*, **8**, 1311–1323.
- Schmidt,J.E., Suzuki,A., Ueno,N. and Kimelman,D. (1995) Localized BMP-4 mediates dorsal/ventral patterning in the early *Xenopus* embryo. *Dev. Biol.*, **169**, 37–50.
- Schmidt,J.E., von Dassow,G. and Kimelman,D. (1996) Regulation of dorsal-ventral patterning: the ventralizing effects of the novel *Xenopus* homeobox gene *Vox*. *Development*, **122**, 1711–1721.
- Singh,H., Sen,R., Baltimore,D. and Sharp,P.A. (1986) A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. *Nature*, **319**, 154–158.
- Slack,J.M.W. (1994) Inducing factors in *Xenopus* early embryos. *Curr. Biol.*, **4**, 116–126.

- Smith,J.C. (1995) Mesoderm inducing factors and mesodermal patterning. *Curr. Biol.* **7**, 856–861.
- Spemann,H. and Mangold,H. (1924) Über die Induktion von Embryoanlagen durch Implantation artfremder Organisatoren. *Roux Arch. Entwicklungsmech.*, **100**, 599–638.
- Suzuki,A., Thies,R.S., Yamaji,N., Song,J.J., Wozney,J.M., Murakami,K. and Ueno,N. (1994) A truncated bone morphogenetic protein receptor affects dorsal–ventral patterning in the early *Xenopus* embryo. *Proc. Natl Acad. Sci. USA*, **91**, 10255–10259.
- Tadano,T., Otani,H., Taira,M. and Dawid,I.B. (1993) Differential induction of regulatory genes during mesoderm formation in *Xenopus laevis* embryos. *Dev. Genet.*, **14**, 204–211.
- Thomsen,G.H. (1996) *Xenopus* mothers against decapentaplegic is an embryonic ventralizing agent that acts downstream of the BMP-2/4 receptor. *Development*, **122**, 2359–2366.
- Tidman-Ault,C., Dirksen,M.L. and Jamrich,M. (1996) A novel homeobox gene *PV.I* mediates induction of ventral mesoderm in *Xenopus* embryos. *Proc. Natl Acad. Sci. USA*, **93**, 6415–6420.
- Tiedemann,H., Asashima,M., Born,J., Grunz,H., Knöchel,W. and Tiedemann,H. (1996) Determination, induction and pattern formation in early amphibian embryos. *Dev. Growth Differ.*, **38**, 233–246.
- Vershon,A.K. (1996) Protein interactions of homeodomain proteins. *Curr. Opin. Biotech.*, **7**, 392–396.
- von Dassow,G., Schmidt,J.E. and Kimelman,D. (1993) Induction of the *Xenopus* organizer: expression and regulation of *Xnot*, a novel FGF and activin-regulated homeobox gene. *Genes Dev.*, **7**, 355–366.
- Watabe,T., Kim,S., Candia,A., Rothbacher,U., Hashimoto,C., Inoue,K. and Cho,K.W.Y. (1995) Molecular mechanisms of Spemann's organizer formation: conserved growth factor synergy between *Xenopus* and mouse. *Genes Dev.*, **9**, 3038–3050.
- Whitman,M. (1997) Feedback from inhibitory SMADs. *Nature*, **389**, 549–551.
- Witta,S.E. and Sato,S.M. (1997) *XIPOU 2* is a potential regulator of Spemann's organizer. *Development*, **124**, 1179–1189.
- Wrana,J. and Pawson,T. (1997) Mad about SMADs. *Nature*, **388**, 28–29.
- Zimmerman,L.B., De Jesús-Escobar,J.-E. and Harland,R.M. (1996) The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein-4. *Cell*, **86**, 599–606.

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