Requirement for Xvent-1 and Xvent-2 gene function in dorsoventral patterning of Xenopus mesoderm

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SUMMARY

Xvent-1 and Xvent-2 are members of a novel homeobox subfamily that have been implicated in dorsoventral patterning in Xenopus mesoderm and are thought to function in BMP signalling. Here we investigate the requirement for Xvent function by employing two dominant-negative strategies. Loss of Xvent function dorsalizes ventral mesoderm, induces secondary embryonic axes and directly neuralizes ectoderm. We further find that (1) Xvents act as transcriptional repressors, (2) Xvents function in an additive fashion and (3) a surprising number of genes are able to rescue dominant-negative Xvent phenotypes including Bmp-4, Smad-1 and wild-type Xvents and Xhox3, but not Xvent-8. The results show that Xvent-1 and Xvent-2 are essential for ventral mesoderm formation and for preventing neural differentiation. A model is suggested to explain how Bmp-4 positional information is converted into distinct cellular responses.

Key words: BMP-4, Homeobox, Mesoderm, Organizer, Xenopus, Xvent-1, Xvent-2

INTRODUCTION

In an emerging view, mesodermal patterning after midblastula transition (MBT) is the result of interaction between ventralizing instructive growth factors and dorsalizing signal antagonists. Bone morphogenetic protein 4 (Bmp-4) is expressed in ventral mesoderm and is antagonized by noggin, chordin, follistatin secreted from dorsal mesoderm or Spemann organizer while Xwnt-8 is antagonized by frzb (De Robertis and Sasai, 1996; Hogan, 1996; Lemaire and Kodjabachian, 1996; Hemmati-Brivanlou and Melton, 1997; Leyns et al., 1997; Wang et al., 1997).

Recently, Bmp-4 was shown to act as a ventral morphogen in Xenopus mesoderm in a direct, long-range fashion. It is both necessary and sufficient for specifying three dorsoventral mesodermal cell fates at different concentrations (Dosch et al., 1997). The notion of Bmp-4 acting as a morphogen is supported by the observation of a graded response to Bmp-4 of zebrafish ectoderm and mesoderm (Neave et al., 1997), as well as Xenopus ectoderm (Knecht and Harland, 1997). In addition, Bmp-4 induces a graded response in mediolateral patterning of somitic and lateral mesoderm in chicken (Tonegawa et al., 1997). Furthermore, in Drosophila, the Bmp-4 homolog decapentaplegic (dpp) acts as a morphogen to pattern the blastoderm in dorsoventral (Ferguson and Anderson, 1992; Wharton et al., 1993), the wing in anteroposterior (Lecuit et al., 1996; Nellen et al., 1996) and together with wingless the leg in proximodistal direction (Lecuit and Cohen, 1997). Thus, Bmp-4/dpp may act as a morphogen in a variety of tissues and organisms to provide positional information.

An important question is how the quantitative differences in Bmp-4 signalling are converted into qualitative discrete cellular responses. Following receptor activation, BMP signalling is transduced by members of Smad family of DNA-binding proteins, which themselves are able to act in a dose-dependent fashion (Massague et al., 1997). Thus, the conversion of positional information into distinct responses is likely to occur at the level of induced target genes which mediate BMP signalling. In the Drosophila wing, the transcription factors spalt, spalt-related and optomotor blind (omb) are expressed in nested domains whose boundaries of expression are a function of the distance from a local dpp source (Lecuit et al., 1996; Nellen et al., 1996). These genes have important roles in mediating the transcriptional effects at distinct concentration thresholds downstream of the dpp morphogen (De Celis et al., 1996; Grimm and Pflugfelder, 1996; Sturtevant et al., 1997).

In Xenopus, candidate transcriptional targets that mediate the effects of Bmp-4 are the Xvent homeobox genes Xvent-1 (Gawantka et al., 1995; also called PV.1; Tidman-Ault et al., 1996) and Xvent-2 (Onichtchouk et al., 1996; also called Xbr1; Papalopulu and Kintner, 1996; Vox, Schmidt et al., 1996; Xom, Ladher et al., 1996); see Lemaire (1996) for review. The expression of both genes overlaps in the gastrula marginal zone but they show distinct dorsal boundaries of expression (Onichtchouk et al., 1996). Similar to the situation in Drosophila where the anterior expression boundaries of omb and spalt are regulated by the distance from the dpp source, the different dorsal boundaries of Xvent-1 and Xvent-2 expression are regulated by the local activity of Bmp-4 in the marginal zone (Dosch et al., 1997). Consistent with Xvent
genes acting in mediating BMP signalling, overexpression on the dorsal side of both Xvent genes leads to ventralization of embryos (Gawantka et al., 1995; Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996; Tidman-Ault et al., 1996). These results raise the possibility that Xvent genes play a role in mediating the transcriptional response of the Bmp-4 morphogen.

Here we investigate the requirement for Xvent-1 and Xvent-2 by using dominant-negative approaches. The results show that Xvents are essential for ventral mesoderm formation and that they act in an additive fashion. This suggests that Bmp-4 positional information regionalizes the marginal zone by the transcriptional activation of Xvent genes in distinct territories that act additively to specify mesodermal cell fates.

MATERIALS AND METHODS

Embryos and explants

In vitro fertilisation, embryo culture, staging, microinjection and culture of marginal zone explants and animal caps were carried out as described (Niehrs et al., 1993).

Constructs

To construct VPXvent-1 and VPXvent-2, we fused the VP16 activation domain sequence and 3' portions of Xvent genes (3'Xvent-1, 3'Xvent-2; see also Fig. 1). The VP16 activation domain (VP16, 261 bp; Friedman et al., 1988) was PCR amplified from pCMV-GLVP2(H) (Wang et al., 1994) as template with the primers, f: GGGGAATTCCGTAGGACTCCACGACG; (EcoRI site and Met codon are in bold) and r: GTCATTCCAAGGGCATG-GTAAC. The amplified product was phosphorylated, blunted, EcoRI cut and gel purified. VP16 was fused with 3'Xvent-1 or 3'Xvent-2, starting 16 amino acids upstream of the homeodomain (nucleotide position 362 for Xvent-1 and 531 for Xvent-2). 3'Xvent-1 was PCR amplified from pXvent-1 (Gawantka et al., 1995) using f: AATGACACTGAGAAGGAGG; r: T7. 3'Xvent-2 was PCR amplified from pXvent-2 (Onichtchouk et al., 1996) using f: TCCGTATCTCTGAGCCTCAG; r: T7. The amplified products were phosphorylated, blunted, NotI cut and gel purified. Finally, VP16 and 3'Xvent-1 or 3'Xvent-2 were coligated with EcoRI-NotI-cut pRN3 vector (Lemaire et al., 1995) to obtain VPXvent-1 and VPXvent-2, respectively.

To construct EveXvent-1 and EveXvent-2, we fused the even-skipped repression domain sequence (Han and Manley, 1993) and 3' portions of Xvent genes (3'Xvent-1, 3'Xvent-2; see also Fig. 1). The even-skipped repression domain sequence (Eve, 777 bp) was PCR amplified using eve-BSK plasmid (gift of G. Ryeffel) as a template, primers were f: GGGGAATTCCGTAGGACTCCACGACG; (EveRI site and Met codon are shown in bold) r: GCCTCACTG-CTGTAGGGG. The amplified products were phosphorylated, blunted, EcoRI cut and gel purified. Finally, Eve was coligated with EcoRI-NotI-cut pRN3 vector and either 3'Xvent-1 or 3'Xvent-2 to obtain EveXvent-1 and EveXvent-2, respectively. In the control constructs VPXvent-1(fs) and EveXvent-1(fs), the VP16 or Eve are followed by frame-shifted 3'Xvent-1 created by nucleotide deletions.

Xvent-2(40) and Xvent-1(40) were constructed by PCR-mediated mutagenesis, changing Leu to Pro in the position 40 of Xvent-1 and Xvent-2 homeodomains (Fig. 1). Cloning was performed in two steps. First, each gene was amplified as two overlapping fragments (5'Xvent-1(2P) and 3'Xvent-1(2P)), carrying the desired mutation. An AvalI site was inserted only into 5'Xvent-2(40) and 3'Xvent-2(40), without changing the amino acid sequence. Fragments were PCR amplified from pXvent-1 (Gawantka et al., 1995) or pXvent-2 (Onichtchouk et al., 1996), respectively using f: T3, r: CCGTATACGACGCTGAGG; for 3'Xvent-1(40) f: T3, r: CCGTATACGACGCTGAGG; for 3'Xvent-2(40) f: T3, r: CCGTATACGACGCTGAGG; for 3'Xvent-2(40) f: T3, r: CCGTATACGACGCTGAGG.

Fragments were PCR amplified from pXvent-1 (Gawantka et al., 1995) or pXvent-2 (Onichtchouk et al., 1996), respectively using f: T3, r: CCGTATACGACGCTGAGG; for 3'Xvent-1(40) f: T3, r: CCGTATACGACGCTGAGG; for 3'Xvent-2(40) f: T3, r: CCGTATACGACGCTGAGG; for 3'Xvent-2(40) f: T3, r: CCGTATACGACGCTGAGG; for 3'Xvent-2(40) f: T3, r: CCGTATACGACGCTGAGG.

Constructs for Xvent-1 and Xvent-2 were made in similar fashion and were referred to as Xvent-1/2. (Xvent-1/2) Wild-type genes, homeodomain is shown as a black box. (VPXvent-1/2) The N-terminus is replaced by the VP16 transcriptional activator domain, indicated by a light-dashed box. (VPXvent-1/2(fs)) VP16 activation domain followed by frame-shifted Xvent-1 was used as a control for VPXvent-1/2 injections. (EveXvent-1/2) The N-terminus is replaced by the transcriptional repressor domain of even-skipped indicated by a dark-dashed box. (EveXvent-1/2(fs)) even-skipped repression domain followed by frame-shifted Xvent-1 was used as a control for EveXvent-1/2 injections. (Xvent-1/2P(40)) Leu-Pro mutation in the position 40 of the homeodomain (indicated by white vertical line).

Microinjection experiments

Plasmids were linearized and capped mRNA was transcribed using the Megascript kit (Ambion) and a cap:GTP ratio of 5:1 as follows: Xvent-2(40), Xvent-1(40), VPXvent-1, VPXvent-1(fs), VPXvent-2, EveXvent-1, EveXvent-1(fs), EveXvent-2 and pRNAmix1 (Rosa, 1989; Sfl, T3 RNA polymerase); pRNXvent-2 (PsrI, blunted, T3 RNA polymerase); pXvent-1 (Gawantka et al., 1995; NotI, T3 RNA polymerase); pBMP-4 (Fainsod et al., 1994; XhoI, T3 RNA polymerase); pchXos3 (Ruiz i Altua and Melton, 1989a; HindIII, T7 polymerase); pCSXSmad1 (Dosch et al., 1997; Asp718, Sp6 polymerase); tBR (Suzuki et al., 1994; EcoRI, Sp6 polymerase); dXvent (Hoppler et al., 1996; EcoRI, Sp6 polymerase); pSP64-T-GATA2a (Walmsley et al., 1994) and pSP64TNSGal (lacZ; Lemaire et al., 1995; XhoI, Sp6 polymerase) pActivin (Thomsen et al., 1990;
Embryonic axes in case of both mRNAs results in the formation of incomplete secondary effect (see Fig. 2 legend).

Followed by a frame-shifted repressor domain control construct containing the even-skipped genes leads to ventralization as judged by the loss of mRNAs develop normally. Furthermore, radial mRNA injection leads to ventralization as judged by the loss of muscle actin, marker. The gene-specific primers used were Bmp-4, gsc, Histone H4, muscle actin, Xbra, Xhox3, Knot, Xvent-1, Xvent-8 as in Gawantka et al. (1995), otx2, XAG1 as in Glinka et al. (1997), NCAM as in Blitz and Cho (1995), XmyoD as in Rupp and Weintraub (1991), Xvent-2 as in Onichtchouk et al. (1996) and Xmyf-5 (C CAGAATGGAGATGGTAGATAGC r. AGCGTGTTCACCTTTTGTGC), engraviled-2 (Hemmati-Brivanlou and Melton, 1994).

RESULTS

Xvent genes are required for ventral mesoderm formation and act as transcriptional repressors

Gain-of-function studies have shown that ectopic expression of Xvent genes leads to ventralization of dorsal mesoderm (Gawantka et al., 1995; Ladher et al., 1996; Schmidt et al., 1996; Tidman-Ault et al., 1996). If Xvent genes were to mediate Bmp-4 signalling then one might expect that loss of Xvent gene function leads to dorsализation of ventral mesoderm and formation of secondary embryonic axes. To abolish Xvent gene function, we chose two dominant-negative strategies.

One strategy to create dominant-negative transcription factors is to convert transcriptional activators into repressors and vice versa by creating fusion constructs between the DNA-binding domain of interest with strong repressor or activator domains, respectively e.g. (Jaynes and O’Farrell, 1991; Bellefroid et al., 1996; Conlon et al., 1996). The amino acid sequences of Xvent genes have no features that would allow us to predict whether they act as activators or repressors. We therefore replaced the aminoterminal domains preceding Xvent homeodomains either by the VP16 activator domain (Friedman et al., 1988; VPXvent-1/2) or the even-skipped repressor domain (Han and Manley, 1993; EveXvent-1/2; Fig. 1).

Embryos microinjected ventrally with EveXvent-1/2 mRNAs develop normally. Furthermore, radial mRNA injection leads to ventralization as judged by the loss of anterior head structures and embryos resemble those injected with wild-type Xvent genes (Fig. 2A-D). Microinjection of a control construct containing the even-skipped repressor domain followed by a frame-shifted Xvent-1 (EveXvent-1(fs)) had no effect (see Fig. 2 legend).

In contrast, ventral injection of VPXvent-1 and VPXvent-2 mRNAs results in the formation of incomplete secondary embryonic axes in case of both Xvent genes (Fig. 2E,F; Table 1, I-1, II-1).

The VP16 activator domain alone is not responsible for this dorsализing effect since (1) microinjection of a control construct containing the VP16 domain followed by a frameshifted Xvent-1 (VPXvent-1(fs)) had no effect (Fig. 2K, Table 1-3) and (2) a VP16 fusion with the dorsaling homeobox gene goosecoid (gsc) has ventralizing activity (B. Ferreiro et al., 1998). The secondary embryonic axes induced by VPXvent-1 and VPXvent-2 mRNAs can be rescued by coinjection of the respective wild-type Xvent mRNAs (Fig. 2G,H; Table 1, I-2, II-2).

An alternative way to generate a dominant-negative...
construct for the homodimerizing homeobox gene Mix.1 is to point-mutate Val 40 preceding helix III of the homeodomain to Pro. The mutated protein is thought to sequester wild-type Mix.1 and prevent its binding to target sequences (Mead et al., 1996). In using the same approach for Xvent genes, Leu 40 was exchanged for Pro (Xvent-1/2P(40), Fig. 1).

Microinjection of Xvent-1P(40) mRNA had no effect and yielded apparently normal embryos (Fig. 2I; Table 1, III-1). However, ventral microinjection of Xvent-2P(40) resulted in the formation of incomplete secondary embryonic axes (Fig. 2J; Table 1, III-2). This raises the interesting possibility that Xvent-2 but not Xvent-1 is able to homodimerize. Secondary axis formation by Xvent-2P(40), again, can be rescued by wild-type Xvent-2 mRNA coinjection (Fig. 2L; Table 1, III-3).

To rule out the possibility that dominant-negative Xvents have an effect on Bmp-4 unrelated induction processes, we tested their influence on activin-mediated mesoderm induction. Activin mRNA microinjected animal cap elongate and express Xbra (Fig. 3A,B). Elongation is unaffected by coinjection of either VPXvent gene. At low activin dose, which is sufficient to induce Xbra but not dorsal mesodermal markers, VPXvent mRNA coinjections elicit muscle actin expression and hence dorsalize the activin-induced mesoderm (Fig. 3B). At high activin dose, no differences are observed in the presence VPXvent mRNAs (Fig. 3A,B). These results are consistent with Xvent genes specifically interfering with BMP but not activin signalling.

We conclude (1) that the function of both Xvent genes is required for ventral mesoderm formation and (2) that Xvent genes act as transcriptional repressors, since repressor and activator domain fusions lead to wild-type and dominant-negative phenotypes, respectively.

Table 1. Secondary axis formation by microinjection of a dominant-negative Xvent-1 and Xvent-2 mRNA

<table>
<thead>
<tr>
<th>RNA(ng/blastomere)</th>
<th>n</th>
<th>% s.a.</th>
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<tbody>
<tr>
<td>I-1 VPXvent-1 (0.1)</td>
<td>250</td>
<td>32</td>
</tr>
<tr>
<td>I-2 VPXvent-1 (0.1)</td>
<td>110</td>
<td>28</td>
</tr>
<tr>
<td>VPXvent-1 (0.1) + Xvent-1 (0.15)</td>
<td>81</td>
<td>0</td>
</tr>
<tr>
<td>I-3 VPXvent-1P(40)</td>
<td>55</td>
<td>0</td>
</tr>
<tr>
<td>II-1 VPXvent-2 (0.5)</td>
<td>208</td>
<td>35</td>
</tr>
<tr>
<td>II-2 VPXvent-2 (0.5)</td>
<td>65</td>
<td>24</td>
</tr>
<tr>
<td>V      VPXvent-2 (0.5) + Xvent-2 (1)</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td>III-1 Xvent-1P(40) (0.5-2.5)</td>
<td>74</td>
<td>0</td>
</tr>
<tr>
<td>III-2 Xvent-2P(40) (2.5)</td>
<td>219</td>
<td>46</td>
</tr>
<tr>
<td>III-3 Xvent-2P(40) (2.5) + Xvent-2 (1)</td>
<td>57</td>
<td>0</td>
</tr>
</tbody>
</table>

4- to 8-cell-stage embryos were microinjected ventrally with the mRNAs indicated and scored for secondary axis (s.a.) phenotype.

To further characterize the phenotypes elicited by Xvent-1 and Xvent-2 loss-of-function, we analyzed embryos with secondary embryonic axes by immune whole-mount staining and histological analysis. In these and the following experiments, we used VPXvent constructs for injections, since of the XventP(40) constructs only Xvent-2P(40) gave a distinctive phenotype.

Immune whole-mount staining and histology show that, similar to secondary embryonic axes induced by microinjection of dominant-negative Bmp receptor (tBR) mRNA (Fig. 4C,F; Suzuki et al., 1994), those induced by injection of dominant-negative Xvent RNAs contained muscle

Fig. 3. VPXvents do not interfere with mesoderm induction by activin in animal caps. Embryos were microinjected with lacZ (0.5 ng/blastomere), VPXvent-1(0.1 ng/blastomere) or VPXvent-2 (0.5 ng/blastomere) with or without activin RNA as indicated. Animal caps were cut at stage 8-9 and cultivated until sibling embryos reached stage 20. In this experiment, ventral control injections with both VPXvent-1 and VPXvent-2 induced more than 50% secondary axes. (A) Coinjection of VPXvent constructs does not inhibit elongation of animal caps, induced by activin (0.1 ng/blastomere; top; 100% elongation activin alone (n=17), 100% elongation VPXvent-1 + activin (n=15), 89% elongation VPXvent-2 + activin (n=18)). VPXvent constructs alone do not induce elongation of animal caps (bottom; lacZ, n=13 ; VPXvent-1, n=16; VPXvent-2, n=16). (B) VPXvent constructs do not inhibit activin-induced mesodermal marker expression in animal caps. Total RNA was isolated from animal caps at stage 20 and analysed by RT-PCR for expression of marker genes indicated. Low and high activin correspond to 0.07 and 0.1 ng/blastomere.
In ectoderm, Bmp-4 has antineuralizing effects and induces epidermal differentiation (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). Both Xvent genes are also expressed in the gastrula animal cap (Gawantka et al., 1995; Onichtchouk et al., 1996), suggesting that they may function in mediating Bmp-4 signalling there. As shown in Fig. 6A, ectodermal overexpression of VPXvents leads to induction of neural marker genes, as does injection of tBR mRNA. This neural induction is direct as it occurs in the absence of
mesoderm formation as judged by muscle actin (Mohun et al., 1984; Fig. 6A) and Xbra (Smith et al., 1991; Fig. 6B) expression. Interestingly, typical organizer genes such as gsc and chordin as well as the dorsolateral marker Xmyf-5 (Dosch et al., 1997) are also induced by VPXvent genes in animal caps (Fig. 6A,B).

We conclude that Xvent genes are required to maintain ventral mesodermal as well as non-neural ectodermal cell fates.

Rescue of Xvent loss-of-function

There is strong evidence for Xvent genes acting in the Bmp-4 pathway. Bmp-4 is necessary (Ladher et al., 1996; Onichtchouk et al., 1996) and sufficient for Xvent gene expression (Gawantka et al., 1995; Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996; Tidman-Ault et al., 1996), and Xvent genes are expressed in a similar fashion as Bmp-4. Both Xvent-1 (Tidman-Ault et al., 1996) and Xvent-2 (Onichtchouk et al., 1996) are able to rescue secondary axes induced by tBR suggesting that they act downstream of Bmp-4. Other genes implicated in mediating Bmp-4 signalling are Smad-1 (Massague et al., 1997) and possibly Xho3 (Ruiz i Altaba and Melton, 1989b).

To address the epistatic relationship of genes acting in the Bmp-4 pathway, rescue experiments were performed. VPXvent mRNAs were coinjected with wild-type mRNA of various genes into the ventral side and rescue to normal embryos was scored. In these experiments, wild-type Xvent-2 is able to rescue VPXvent-1 and wild-type Xvent-1 is able to rescue VPXvent-2 phenotypes (Fig. 7C,D; Table 2, I,II). Likewise Xho3 is able to rescue secondary axes induced by both VPXvent mRNAs (Fig. 7E,F; Table 2, III,IV). Even Bmp-4 is able to rescue VPXvent-1 and VPXvent-2 (Fig. 7G,H; Table 2, V,VI), which is surprising since previously the reverse was found, i.e. Xvent-1 and Xvent-2 are able to rescue loss of Bmp function caused by tBR mRNA microinjection (Onichtchouk et al., 1996; Tidman-Ault et al., 1996). Furthermore, Bmp-4 could even rescue secondary axes induced by coinjection of mRNA of both VPXvent-1 and VPXvent-2, as could Smad-1 mRNA injection (Table 2, VII).

The only gene that was found not to rescue secondary embryonic axes was Xwnt-8 (Christian and Moon, 1993),

Table 2. Rescue of secondary embryonic axis phenotypes of VPXvent-1 and VPXvent-2

<table>
<thead>
<tr>
<th>RNA (ng/blastomere)</th>
<th>n</th>
<th>% s.a.</th>
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<tbody>
<tr>
<td>I VPXvent-1 (0.1)</td>
<td>110</td>
<td>28</td>
</tr>
<tr>
<td>II VPXvent-1 (0.1) + Xvent-2 (1)</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>III VPXvent-2 (0.5)</td>
<td>65</td>
<td>24</td>
</tr>
<tr>
<td>IV VPXvent-2 (0.5) + Xvent-1 (0.15)</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>V VPXvent-1 (0.1)</td>
<td>55</td>
<td>34</td>
</tr>
<tr>
<td>VI VPXvent-1 (0.1) + Xho3 (0.2)</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>VII VPXvent-2 (0.5)</td>
<td>66</td>
<td>28</td>
</tr>
<tr>
<td>VIII VPXvent-2 (0.5) + Xho3 (0.2)</td>
<td>71</td>
<td>4</td>
</tr>
<tr>
<td>IX VPXvent-1 (0.1)</td>
<td>76</td>
<td>20</td>
</tr>
<tr>
<td>X VPXvent-1 (0.1) + Bmp-4 (0.3)</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>XI VPXvent-2 (0.5)</td>
<td>58</td>
<td>31</td>
</tr>
<tr>
<td>XII VPXvent-2 (0.5) + Bmp-4 (0.3)</td>
<td>61</td>
<td>0</td>
</tr>
<tr>
<td>XIII VPXvent-1 (0.1) + VPXvent-2 (0.5)</td>
<td>76</td>
<td>31</td>
</tr>
<tr>
<td>XIV VPXvent-1 (0.1) + VPXvent-2 (0.5) + Bmp-4 (0.5)</td>
<td>84</td>
<td>2</td>
</tr>
<tr>
<td>XV VPXvent-1 (0.1) + VPXvent-2 (0.5) + Smad-1 (1)</td>
<td>86</td>
<td>3</td>
</tr>
<tr>
<td>XVI VPXvent-1 (0.05)</td>
<td>93</td>
<td>10</td>
</tr>
<tr>
<td>XVII VPXvent-1 (0.05) + CSKAXwnt-8 DNA (0.05)</td>
<td>141</td>
<td>38</td>
</tr>
<tr>
<td>XVIII VPXvent-1 (0.1)</td>
<td>42</td>
<td>33</td>
</tr>
<tr>
<td>XIX VPXvent-1 (0.1) + CSKAXwnt-8 DNA (0.05)</td>
<td>17</td>
<td>58</td>
</tr>
<tr>
<td>XX VPXvent-2 (0.5)</td>
<td>113</td>
<td>26</td>
</tr>
<tr>
<td>XXI VPXvent-2 (0.5) + CSKAXwnt-8 DNA (0.05)</td>
<td>146</td>
<td>31</td>
</tr>
</tbody>
</table>

4- to 8-cell-stage embryos were microinjected ventrally with VPXvent-1 or VPXvent-2 mRNA either alone or in combination with the mRNAs or DNA indicated. s.a., embryos with secondary axes.
another ventralizing gene whose relationship with the Bmp-4 pathway is unclear. Xvent-8 was injected as plasmid DNA pCSKAXvent-8, where the gene is expressed after the midblastula stage under control of the cytoskeletal actin promoter. In fact, Xvent-8 injection enhances the frequency of secondary embryonic axis formation by VPXvent-1 and to a lower degree by VPXvent-2 (Fig. 7L; Table 2, VIII-X). This suggests that, while Xvent-8 may interact with Xvent genes, it is part of a separate pathway. To test this further, we coinjected VPXvent mRNA with dominant-negative Xvent-8 (dnXvent-8; Hoppler et al., 1996). While VPXvent mRNAs alone always induce incomplete secondary embryonic axes, coinjection with dnXvent-8 mRNA induces formation of complete secondary embryonic axes with cement gland and one eye in case VPXvent-1 (50%, n=34; Fig. 7K) and VPXvent-2 (24%, n=49; Fig. 7L). dnXvent-8 alone does not induce secondary embryonic axes (Hoppler et al., 1996; Glinka et al., 1997). This phenotype is consistent with the observation that simultaneous inhibition of Wnt and BMP signalling is required for the formation of complete secondary embryonic axes (Glinka et al., 1997). Thus, Xvent-8 and Xvent genes can interact genetically in a manner that indicates that they belong to separate signalling pathways.

We also tested whether VPXvent phenotypes could be rescued by Mix.1 or GATA-2, two genes implicated in ventral mesoderm formation (Walisney et al., 1994; Mead et al., 1996), but we always observed gastrulation defects in coinjections (data not shown).

In summary, Bmp-4, Smad-1 and XhoX3 are able to rescue loss of Xvent gene function and thus act in a common pathway with Xvent genes.

### Cooperation of Xvent genes

Xvent genes seem to function in a very similar fashion. Microinjection of both wild-type Xvent genes is ventralizing and both genes have very similar amino acid sequences in the DNA-binding helix III of the homeodomain, suggesting that they bind similar target sequences. Moreover, wild-type Xvents can mutually rescue their loss-of-function, indicating that they have similar activities.

While Xvent genes act similarly they show a significant difference in expression. In the gastrula marginal zone, Xvent-1 and Xvent-2 are coexpressed in all of the lateroventral marginal zone, but Xvent-2 expression extends further to the dorsolateral region (Onichtchouk et al., 1996). Thus, at the gastrula stage, the two genes are coexpressed in the region specified as blood and mesenchyme (lateroventral) while only Xvent-2 is expressed in the region specified as muscle (dorsolateral). This suggests that high levels of Bmp-4 may be translated into lateroventral positional values by the coexpression of both Xvent genes while dorsolateral positional values are specified by the expression of Xvent-2 only. This scenario predicts that (1) coexpression of both Xvent genes should be more ventralizing than expression of an individual Xvent gene and (2) coexpression of both dominant-negative Xvent genes should be more dorsalizing than expression of any individual dominant-negative Xvent.
To test the first prediction, we microinjected wild-type Xvent mRNAs either individually or in combination and analysed the resulting phenotypes as well as expression of marker genes in dorsal marginal zone (DMZ) explants. Fig. 8A shows that when coinjected at suboptimal doses, both Xvent mRNAs lead to a stronger ventralized phenotype than injection of the individual mRNAs. Similarly, RT-PCR analysis shows that coinjection of Xvent genes leads to an additive effect in downregulation of gsc and chordin and upregulation of Xwnt-8 (Fig. 8B).

In the reverse experiment, VPXvent mRNAs were either injected individually or in combination at suboptimal doses and marker gene expression was analysed in gastrula-stage VMZs. Fig. 8C shows the expected dorsalization following individual mRNA injection. Coinjection of both VPXvent genes resulted in an additive dorsalization indicated by upregulation of gsc, chordin and Xmyf-5 expression. Interestingly, ventral marker genes Bmp-4 and Xwnt-8, as well as Xvent genes themselves, were not significantly affected by VPXvent mRNA injection at the gastrula stage.

We conclude that Xvent-1 and Xvent-2 function in an additive fashion.

DISCUSSION

We set out to test if Xvent-1 and Xvent-2 have an essential role in mesoderm development. We find that the function of both Xvent genes is required for ventral mesoderm formation and for antagonizing the organizer.

Epistatic relationships in the Bmp-4 pathway

The similarity between Xvent loss-of-function phenotypes obtained in this study and the phenotype elicited by tBR adds to the evidence that these homeobox genes function in mediating BMP signalling. An important question is at which position they act within the signalling pathway. The observation that Xvent-1 and Xvent-2 can rescue secondary axes induced by tBR suggested that the genes act downstream of Bmp-4. Yet, the results show that loss of Xvent function can be likewise rescued by Bmp-4. The most likely explanation for this is the existence of multiple, parallel and redundant pathways that are activated by this cytokine. This is supported by the finding that even simultaneous block of both Xvent genes is rescued by Bmp-4 and its mediator Smad-1.

Since Xhox3 is expressed later than Xvent-1 and Xvent-2 (Ruiz i Altaba and Melton, 1989a), its ability to rescue VPXvent phenotypes is consistent with it acting downstream of Xvents. In contrast, Xwnt-8 is not able to rescue VPXvent phenotypes but curiously seems to enhance secondary axis formation. Since the mesoderm of secondary axes induced by VPXvents is mostly of muscle origin, this is compatible with the proposed role of Xwnt-8 to promote muscle formation (Hoppler et al., 1996). While this shows that Xvents are interacting with Xwnt-8, this gene is clearly not downstream of Xvents. Yet, Bmp-4 is necessary and sufficient for Xwnt-8 expression (Graff et al., 1994) and late overexpressed Xwnt-8 is mildly ventralizing, suggesting that it interacts with the Bmp-4 pathway.

An interesting possibility is that Xvents can act upstream of Bmp-4. For example, maternally expressed Bmp-2 (Clement et al., 1995) could induce Bmp-4 via Xvents, and Bmp-4 in turn maintains later Xvent expression. This is consistent with the Bmp-4 rescue of VPXvents and the observation that Xvent-2 is able to induce Bmp-4 expression (Onichtchouk et al., 1996; Schmidt et al., 1996). However, such an autoregulatory loop is in conflict with the short-range action of the BMP mediator Smad-1, which induces Xvents in a cell-autonomous fashion (Dosch et al., 1997). Moreover, loss of Xvent function does not lead to significant reduction of Bmp-4 mRNA levels in gastrula VMZ (Fig. 8C) and animal caps (data not shown).

In conclusion, Bmp-4 signalling appears to activate multiple, redundant pathways that may interact with each other. Understanding of this pathway requires identification of more downstream components and investigation of combined loss-of-function in epistasis analysis.

Xvent genes may act additively in transducing Bmp-4 positional information

Both Xvent genes are necessary and sufficient for ventral mesoderm development and both seem to act as repressors, suggesting that their major function is in antagonizing dorsal and dorsolateral mesodermal genes. What distinguishes Xvents is their effect following wild-type overexpression, where Xvent-2 is able to ventralize embryos in a dose-dependent manner, while Xvent-1 causes milder ventralization and lethality at higher dose. In addition, unlike VPXvent-2, VPXvent-1 induces secondary embryonic axes that frequently contain notochords. Finally, while the Leu (40) to Pro mutation in the homeodomain leads to a dominant-negative Xvent-2, the same mutation inactivated Xvent-1. Possibly, homodimerization of Xvent-2 but not Xvent-1 accounts for these functional differences, e.g. by altering the DNA-binding affinity.

Since Xvents have very similar DNA-binding domains and in both gain-and loss-of-function experiments affect expression of a similar set of marker genes, they may share the same targets. Thus, the functional differences between both genes may be more quantitative than qualitative, e.g. differences in DNA-binding affinity or strength of repressor domains rather than DNA-binding specificity.

The functional similarities taken together with the distinct dorsoventral expression domains of Xvent-1 and Xvent-2 support a model (Fig. 9) in which the sum of Xvent genes determines the specification of mesodermal cells. The dorsolateral boundaries of Xvent gene expression are determined by the local activity of Bmp-4 (Dosch et al., 1997). In ventrolateral mesoderm, both Xvents are expressed and the sum of Xvent activity is high, suppressing muscle and notochord and allowing mesenchyme and blood differentiation. In dorsolateral mesoderm, only Xvent-2 is expressed and Xvent activity is low, which suppresses notochord but allows muscle differentiation. This model is supported by the findings (1) that Xvents act in an additive fashion both in gain- and loss-of-function experiments and (2) that a high dose injection of Bmp-4 mRNA induces both Xvent genes and represses muscle and notochord differentiation while a low dose of Bmp-4 induces Xvent-2 and muscle and represses notochord differentiation (Dosch et al., 1997). This very simplified model does not take into account the action of other ventralizing genes, such as Xwnt-8, that also repress notochord.

Recent evidence suggests that Bmp-4 may also act as a
morphein in ectodermal patterning, where low doses induce neural crest and high doses epidermis (Liem et al., 1995; Knecht and Harland, 1997; Neave et al., 1997). Intriguingly, Xvent-1 and Xvent-2 are also expressed in a nested fashion in gastrula ectoderm, with Xvent-2 reaching further dorsal, overlapping with prospective neural crest (Mayor, 1995), while Xvents are coexpressed in prospective epidermis and absent from prospective neural plate (Gawantka et al., 1995; Onichtchouk et al., 1996). Our observation that Xvent gene function is required for repressing neural marker gene expression in ectoderm raises the possibility that they may also function in Bmp-4-mediated patterning of embryonic ectoderm in addition to mesoderm.

In conclusion, we propose that Bmp-4 positional information regionalizes the marginal zone by the transcriptional activation of Xvent genes in distinct territories that specify mesodermal cell fates in an additive fashion.

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