Fish and frog embryos are patterned along the dorsal–ventral axis during the gastrula stage by opposing gradients of Bmps and Bmp inhibitory proteins. Three transcriptional repressors with partially overlapping expression domains have been proposed to be important mediators of Bmp function in Xenopus. We find that two related factors are expressed in the early zebrafish embryo. Although these factors are considerably divergent from the related Xenopus genes, they are expressed in domains similar to those of their Xenopus relatives throughout embryogenesis. Both of the zebrafish genes, which we have named vox and vent, are potent ventralizing factors in both zebrafish and Xenopus embryos. Using mutants in the Bmp pathway, we find that there are Bmp-dependent and Bmp-independent domains of vox expression, whereas vent is mostly dependent upon Bmp signaling. We show that ectopic vox or vent negatively regulates expression of the early dorsal gene bozozok (boz) and that ectopic boz eliminates vox and vent expression. Moreover, the normal exclusion of vox and vent from the organizer region is lost in boz mutant embryos. Our results show that boz and vox/vent are mutually antagonistic and indicate that the early establishment of the size of the organizer domain is dependent on an interplay between these early expressed transcriptional repressors.

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Onichtchouk et al., 1998; Trindade et al., 1999). These results indicate that Vox and Xvent-1 work principally to limit the extent of the dorsal organizer and axial mesoderm (reviewed in Kimelman, 1999). The second class of Bmp-dependent genes involved in patterning the early embryo has only a single member thus far, Xmsx-1 (Maea et al., 1997; Suzuki et al., 1997). While ectopic expression of Xmsx-1 can ventralize mesoderm and inhibit neuralization, its targets are not yet known.

One of the key early genes in dorsal–ventral patterning in zebrafish is a negative regulator of the bmps, bozozok (boz), initially identified as nieuwkoid/dharma (Fekany et al., 1999; Koos and Ho, 1998, 1999; Yamanaka et al., 1998), is one of the earliest expressed dorsal-specific genes, with expression beginning immediately after the start of zygotic expression (Koos and Ho, 1998; Yamanaka et al., 1998). boz is expressed in both the dorsal extraembryonic yolk syncytial layer and the dorsal blastomeres and is activated by the Wnt pathway (Yamanaka et al., 1998). Loss of boz produces severe dorsal defects (Fekany et al., 1999; Koos and Ho, 1999; Solnica-Krezel et al., 1996), whereas ectopic expression of boz induces ectodermal dorsal structures (Koos and Ho, 1998; Yamanaka et al., 1998). Ectopic boz expression inhibits bmp2b expression (Koos and Ho, 1999), but it is not yet clear if this is the principal mechanism by which boz regulates dorsal–ventral patterning. All of these results indicate that boz acts very early in embryogenesis to specify the zebrafish dorsal–ventral axis.

We were interested to know whether regulatory mechanisms similar to those characterized in Xenopus were used downstream of Bmp in zebrafish. The zebrafish system also presents some significant advantages because mutants in the Bmp pathway have been identified (reviewed in Kodjabachian et al., 1999). We report the identification of two genes belonging to the Vox/Xvent-1/PV.1 family of genes in gastrula stage zebrafish embryos. While neither gene is specifically identified as orthologous to the frog genes based on amino acid sequence, their expression patterns clearly mirror either Vox or Xvent-1/PV.1 and we have therefore named them vox and vent. We show that expression of both genes is diminished in fish defective in the Bmp pathway, and both act as potent ventralizing factors in both fish and frog embryos, indicating a conserved function for these genes. The expression of vox and vent is shown to be regulated by bozozok, and both genes act as negative regulators of bozozok in overexpression studies. Our results indicate that an interplay between bozozok and the Bmp-regulated vox and vent genes is used to establish the size of the organizer in the early zebrafish embryo.

METHODS

Isolation of vox and vent

Zebrafish vox was amplified from cDNA produced from zebrafish shield-stage RNA with the primers CGCGGACGCATCAAR-CANMGNTAYTTNGG and CGCGGATCCKRTTYYTGRAAC-CANGT. Zebrafish vent was amplified from the same cDNA with the primers CGCGGACGCATCAAR-CANMGNTAYTTNGG and CGCGGACGCATCAAR-CANMGNTAYTTNGG. The italicized regions are the primers CGCGGACGCATCAAR-CANMGNTAYTTNGG and CGCGGACGCATCAAR-CANMGNTAYTTNGG. The amplified regions show the SacI and BamHI restriction sites used for cloning the PCR products. The amplification conditions were 1 cycle at 94°C for 3 min; 2 cycles at 94°C for 1 min, 37°C for 1 min, 72°C for 30 s; 35 cycles at 94°C for 1 min, 65°C for 1 min, 72°C for 30 s; and 1 cycle at 72°C for 10 min. The cloned PCR products were used to screen a Lambda ZAP zebrafish gastrula-stage cDNA library (gift from T. Lepage) and full-length clones were identified. Plasmids were rescued from the phage using the Rapid Excision Kit (Stratagene). The accession number for vox is AF255045 and for vent is AF255044.

In Situ Hybridization

In situ hybridization was done using standard methods (Westfield, 1994). Digoxigenin-labeled probes were hybridized to embryos fixed overnight at 4°C in 4% paraformaldehyde. Hybridized probes were detected using anti-digoxigenin antibodies conjugated to alkaline phosphatase (Boehringer). Labeled embryos were dehydrated in methanol, cleared in methyl salicylate, and mounted in Permount on glass slides between coverslip bridges. Flat-mounted embryos were cleared in 70% glycerol, the yolk was dissected away, and then the embryos were mounted between two coverslips using vacuum grease as a spacer (C. Moens, personal communication).

Embryo Stocks

In the case of swirl and snailhouse mutant embryos, heterozygous fish were crossed, and the embryos were collected and fixed at various stages. We used the alleles swirl265 and sbn166. Mutants were identified on the basis of their altered patterns of gene expression, which were present in 25% of the embryos, as predicted for recessive effects. We obtained somitabun mutants by crossing females heterozygous for sbn166 with sbn166 males. Because of the maternal dominant effect of sbn166, 75% of the embryos from these crosses were class 4 dorsalized mutants and 25% were class 5 mutants (Hild et al., 1999; Mullins et al., 1996). bozozok mutant embryos were obtained by crossing homozygous boz166 mutant parents. Because of variable penetrance and expressivity, viable homozygous boz mutants can be raised to adulthood and crossed; all of their progeny are boz mutants (Fekany et al., 1999).

Embryo Injection

RNA for injection was prepared using the mMessage mMachine kit (Ambion). The coding regions of vent and vox were subcloned into the CS2+ expression vector (Turner and Weintraub, 1994) to produce the plasmids ZV86 and ZV100, respectively. The boz expression plasmid was kindly provided by D. Koos and R. Ho (Koos and Ho, 1999). Injection needles were pulled with a Kopf vertical pipette puller and back-filled with RNA, and then their tips were broken slightly by touching the end of the needle to a pair of forceps. A Pico-Spritzer (General Valve Corp.) was used for pressure injection of RNA. Pressure was adjusted so that a bolus of RNA of a volume of about 0.2 nl was injected. Calibration was by eye, comparing the size of the bolus to the size of embryonic blastomeres. Embryos were injected at the 1- to 4-cell stage and left to develop inside their chorions. Injected embryos were either fixed at the appropriate stage or allowed to develop overnight and then scored for phenotypes. Treatment with LiCl was similar to the protocol used previously (Stachel et al., 1993). At the 64-cell stage,
embryos still in their chorions were transferred into 0.3 M LiCl for 10 min and then washed thoroughly to remove residual LiCl.

**RESULTS**

**Isolation of vox and vent**

To identify the fish orthologs of the Xvent-1/Vox/PV.1 genes, degenerate primers were designed within the homeobox. DNA fragments were amplified from gastrula-stage zebrafish RNA using the reverse transcriptase-polymerase chain reaction and resulting clones were individually sequenced. The two unique sequences identified by this method were used to screen a gastrula-stage cDNA library (Lepage et al., submitted for publication), and full-length cDNAs corresponding to the fragments were identified. The encoded fish proteins are smaller than the frog counterparts and have little homology outside of the homeobox region (Fig. 1A). Based on conservation of amino acid sequences over the entire protein or within the homeobox, it was not possible to definitively assign either protein to one of the frog proteins (Fig. 1B). Indeed, the two fish proteins were more closely related to each other than to either Vox or Xvent-1. However, we named one gene vox and the other vent to indicate their similarities to Vox and Xvent-1 based on their expression patterns (see below).

### Expression of vox and vent during Development

The gene we named vox is first expressed at the sphere stage in a mottled pattern (not shown), but by 30% epiboly vox is expressed uniformly throughout the embryo except for a small region of clearing on the dorsal side (Fig. 2A). vent is first expressed at the dome stage (not shown), starting as a faint half-ring of expression at the blastoderm margin, which becomes stronger by 30% epiboly (Fig. 2B). In contrast to vox, early expression of vent is mostly confined to the margin, the presumptive mesodermal region (Kimmel et al., 1990). In addition, the expression of vent does not extend as far dorsally as vox (Figs. 2, compare 2A to 2B).

By the shield stage, vox expression is heavy at the margin, but also still found in the animal blastomeres (Figs. 2C and 2E). vox expression is cleared from a region on the dorsal side, corresponding to the organizer and neurectoderm (Kimmel et al., 1990; Melby et al., 1996). vent expression at the shield stage is confined to the margin, extending less far dorsally than vox (Figs. 2D and 2F). These expression patterns were consistent with the function of each gene in the differentiation of corresponding cell types.
In the late gastrula, vox expression flanks the axis and the presumptive neural plate (Fig. 2K), while vent expression retreats ventrally (Fig. 3B). Vox is also expressed in deep cells that resemble the fkd2-expressing endodermal cells (Warga and Nusslein-Volhard, 1999). From the bud stage through somitogenesis, vox and vent are expressed posteriorly in the tailbud, similar to other genes involved in the specification of ventral tissues (Connors et al., 1999; Joly et al., 1993), vent is mainly expressed posteriorly while vox is expressed more extensively, extending farther dorsoanteriorly and flanking the posterior dorsal axis (Figs. 2G and 2H). During the somite stages, vox shows complex transient expression patterns in addition to the strong expression in the tail region (Figs. 2I, 2L, and 2M). Vox is expressed in two stripes of expression in the neural tube (Fig. 2L), similar to that of the neural crest marker fkd6 (Odenthal and Nusslein-Volhard, 1998). Unlike fkd6, however, the vox expression regions in the neural tube are confined to the head, whereas fkd6 is expressed throughout the neural crest. At later stages, vox is expressed in the eye (Fig. 2M), as is the Xenopus Vox gene (Papalopulu and Kintner, 1996), and in the posterior notochord (not shown). Vent, in contrast, remains restricted to the tail region of the somitostage embryo (Fig. 2J).

**Regulation of vox and vent by Bmps**

Since the Xenopus genes Vox, Xvent-1, and PV.1 are regulated by Bmps in ectopic expression experiments (Ault et al., 1996; Gawantka et al., 1995; Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996), we asked if vox and vent are similarly regulated by Bmps in zebrafish, taking advantage of mutants that alter this pathway. We investigated the expression of vox and vent in the bmp2b mutant swirl (swr; Hammerschmidt et al., 1996; Kishimoto et al., 1997; Mullins et al., 1996; Nguyen et al., 1998), the bmp7 mutant snailhouse (snh; Dick et al., 2000; Mullins et al., 1996; Schmid et al., 2000), and the smad5 mutant somitabun (sbn; Hild et al., 1999; Mullins et al., 1996). In zebrafish, bmp2b, bmp4, and bmp7 are initially expressed in a broad ventrolateral domain that becomes progressively restricted to the more ventral regions (Dick et al., 2000; Hammerschmidt et al., 1996; Kishimoto et al., 1997; Schmid et al., 2000), similar to the expression of vox (Fig. 2). Unlike vox, however, the bmps are expressed in a subset of the dorsal cells, although whether this is of functional significance has not yet been established. Analysis of mutant embryos has shown that the maintenance, although not the initiation, of ventral expression of all three bmps depends on Bmp2b signaling (Hild et al., 1999; Kishimoto et al., 1997; Schmid et al., 2000). Smad5 is required to transduce the Bmp signals (Hild et al., 1999).

The early expression of vox and vent was unaffected in the bmp mutant fish; no differences could be detected between the wild-type and the mutant embryos at the shield stage (not shown). By 70% epiboly, both the swr/
bmp2b and the sbn/smads5 mutations had a strong effect on vox and vent expression. The overall level of expression was lower in the mutants and the pattern of expression had shifted such that the more dorsal expression of vox and vent was eliminated, although vox expression was maintained at the margin (Figs. 3C and 3E), demonstrating that this expression of vox is not dependent on Bmp signaling. In some swr mutant embryos, vent expression was completely eliminated (Fig. 3D), showing that vent expression is almost completely dependent upon Bmp signaling. In crosses of heterozygous sbn/smads5 females to heterozygous sbn/smads5 males, 21% (7/33) of the embryos showed a strong effect on vox expression, whereas 79% (26/33) of the embryos were less strongly affected. Similarly, in crosses of sbn/smads5 heterozygotes, 82% of the embryos had residual vent expression (Fig. 3E; 26/33) whereas 18% had no apparent vent expression (not shown; 5/28). These numbers are in agreement with the previous observations that crosses of sbn/smads5 heterozygotes produce approximately 25% of the embryos with a very strong (class 5) phenotype and 75% with a less severe (class 4) effect (Mullins et al., 1996). We examined swr/bmp2b embryos at later stages to see if the expression of vox was ever eliminated and found that vox expression persisted in the tailbud, albeit at reduced levels relative to wild type (data not shown). snh/bmp7 mutants also had reduced vox and vent expression, but this effect was not seen until the end of gastrulation (Figs. 3G–3J). The weaker effects of snh compared to swr and sbn are likely to be due to the fact that the allele of snh we used is hypomorphic (Schmid et al., 2000). Our results indicate that while the initial expression of vox and vent is independent of Bmp signals, they become dependent on Bmp signaling during the gastrula stages. Moreover, the marginal expression of vox is independent of Bmp signaling.

**vox and vent Act to Ventralize Zebrafish Embryos**

In Xenopus, ectopic expression of Vox and Xvent-1 ventralizes embryos, mimicking the effects of Bmp overexpression (Ault et al., 1996; Gawantka et al., 1995; Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996). To determine whether vox and vent had similar properties, we ectopically expressed these proteins in zebrafish and Xenopus embryos. In zebrafish, ectopic expression of 10–20 pg of vox and vent RNA led to the formation of ventralized embryos, characterized by head defects, lack of notochord, and an excess of blood (Fig. 4; Table 1). These effects were less severe than those seen with ectopic expression of bmp4 (Kishimoto et al., 1997). Higher doses of vox and vent RNA resulted in a high proportion of nonspecific effects and so these embryos could not be reliably scored.

Both vox and vent were potent ventralizing agents in Xenopus embryos. Injection of either vox or vent RNA in the two dorsal blastomeres at the four- to eight-cell stage resulted in embryos with an average dorsoanterior index (Kao and Elinson, 1988) of approximately 2.0 with 0.25 ng and approximately 1.0 with 0.5 ng (Fig. 5). When the same amount of RNA was injected, vox and vent were 10-fold more effective at ventralizing Xenopus embryos than Xenopus Vox (not shown). These results demonstrate that vox and vent are able to ventralize fish and frog embryos and

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**FIG. 3.** Expression of vox and vent in dorsalized zebrafish mutants. (A–F) Expression of vox and vent in 70% epiboly embryos, lateral view, dorsal is to the right. (A, B) Wild-type (wt) embryos. (C, D) swirl (swr) mutants. (E, F) somitabun (sbn) mutants. Note that the marginal expression of vox is retained in swr (C) and sbn (E) embryos. (G–J) Expression of vox and vent in 100% epiboly embryos, vegetal view, dorsal is to the top. (G, H) Wild-type embryos. (I, J) snailhouse (shn) embryos. Note that the expression level is reduced in shn embryos but the pattern of expression is unchanged.
have a function conserved with that of their Xenopus counterparts.

**vox and vent Are Negative Regulators of Dorsal Gene Expression**

In Xenopus, Vox and Xvent-1 act as transcriptional repressors (Melby et al., 1999; Onichtchouk et al., 1998; Trindade et al., 1999) which inhibit the expression of early dorsal genes (Ault et al., 1996; Gawantka et al., 1995; Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996). To determine if the same was true in zebrafish, we examined the expression of goosecoid (gsc) in shield-stage embryos that were injected at the one- to four-cell stage. Both vox and vent inhibited gsc, causing a range of effects from weak expression to a complete absence of expression (Table 2). Some embryos were not affected, but this was likely due to the fact that the site of injection was not targeted to the dorsal side.

The formation of the organizer in zebrafish depends on the activity of bozozok (Fekany et al., 1999; Koos and Ho, 1999), which has a pregastrula expression pattern complementary to that of vox. We asked if vox and/or vent might function as a negative regulator of bozozok. Because the expression of boz is very limited, and since it is not possible to target RNA injections to the dorsal side in fish, we were concerned that it would be difficult to reliably ectopically express vox and vent in the same regions that boz is normally expressed. boz is activated by the Wnt pathway (Yamanaka et al., 1998), which can be induced by the addition of the GSK-3 inhibitor LiCl (Klein and Melton, 1996; Stambolic et al., 1996). We treated zebrafish embryos with LiCl to upregulate boz expression in most of the marginal zone (Fig. 6). We found that ectopic expression of either vox or vent blocked the expression of boz, in many cases entirely abolishing boz expression (Fig. 6; Table 3). Even in the absence of LiCl, we observed that ectopic vox and vent were potent inhibitors of boz expression (Table 3). Of the two genes, vox was more effective at inhibiting boz than was vent, both in the presence and in the absence of LiCl. These results suggest that vox and vent function to regulate the expression domain of boz.

**vox and vent Are Regulated by boz**

Ectopically expressed boz can induce a secondary axis non-cell autonomously (Fekany et al., 1999; Koos and Ho, 1998, 1999; Yamanaka et al., 1998). Surprisingly, Bozozok contains an N-terminal amino acid motif that provides a transcriptional repressing function in the Drosophila and vertebrate Goosecoid proteins (Ferreiro et al., 1998; Mailhos

**TABLE 1**

<table>
<thead>
<tr>
<th>RNA</th>
<th>n</th>
<th>WT (%)</th>
<th>V1a (%)</th>
<th>V2a (%)</th>
<th>V3a (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vox</td>
<td>95</td>
<td>31</td>
<td>32</td>
<td>27</td>
<td>11</td>
</tr>
<tr>
<td>vent</td>
<td>89</td>
<td>19</td>
<td>21</td>
<td>38</td>
<td>21</td>
</tr>
</tbody>
</table>

* Scoring according to Kishimoto et al. (1997).
et al., 1998). Since vox and vent are not expressed in the region where boz is present, we wondered whether boz might inhibit the transcription of these genes. We found that the expression of both vox and vent was expanded in boz mutants. vox transcripts were present uniformly throughout boz mutants (compare Fig. 7B to 7A; 94%, n = 33), whereas vent transcripts were present on the dorsal side in boz, but the expression was not uniform (compare Fig. 7D to 7C; 86%, n = 43). These results suggest that the dorsal side may have another repressor that keeps vent expression asymmetric. To more directly examine the effects of boz on vox and vent, 5 pg of boz RNA was injected into zebrafish embryos. Ectopic boz eliminated the expression of vent (Fig. 7H; 85%, n = 27) and eliminated (25%, n = 28) or reduced (29%, partial reduction, 43%, very weak expression; n = 28) the expression of vox (Fig. 7F). We conclude that the lack of vox and vent expression on the dorsal side of wild-type embryos is due to boz function.

DISCUSSION

Our results demonstrate that the zebrafish, like Xenopus (Ault et al., 1996; Gawantka et al., 1995; Ladher et al., 1996; Onichtchouk et al., 1996; Papalopulu and Kintner, 1996; Schmidt et al., 1996), has multiple members of the Vox/ Xvent-1 family. As in frogs, one of the genes (vent) is more restricted to the ventral side, whereas the other (vox) extends over a broader region of the embryo. In frogs, the different expression patterns of Xvent-1 and Vox have been attributed to a Bmp morphogen gradient (Dosch et al., 1997), with higher Bmp levels required for Xvent-1 expression, based on overexpression studies. Similarly, we find that vox extends over a much broader region of the embryo than does vent. As there is strong support for a dorsal–ventral Bmp morphogen gradient in zebrafish as well (Kodjabachian et al., 1999; Nguyen et al., 1998), vox and vent are likely to be similarly regulated. Since both fish and frog embryos are increasingly ventralized by higher doses of these genes (our results; Ault et al., 1996; Gawantka et al., 1995; Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996), the combination of vox and vent on the most ventral side of the embryo could have a stronger ventralizing effect than the lateral regions which express only vox. Rigorously testing these ideas awaits the identification of mutants in vox and vent.

Regulation of vox and vent by Bmps

One major advantage to working in zebrafish is the availability of mutants in specific signaling pathways, which permits the possibility of uncovering subtleties of regulatory interactions that may be lost in ectopic expression studies. Our results using mutants in the Bmp pathway demonstrate that while vox and vent are regulated by Bmps as seen in frogs, Bmp2b and Bmp7 signaling is needed for the maintenance but not the initiation of vox and vent transcription. The initial expression might be due to a maternal Bmp or activation of vox and vent through a Bmp-independent mechanism. Moreover, we find that the equatorial expression of vox is maintained in even the most severe bmp mutants while equatorial vent is lost. This region of expression, which corresponds to the nascent mesoderm, is potentially under the control of wnt8, which is expressed throughout the ventral and lateral mesoderm during the early gastrula stages (Kelly et al., 1995). In support of this, ectopic expression of a dominant-negative Xwnt8 mutant in Xenopus eliminated Vox expression within the presumptive mesoderm (Hoppler and Moon, 1998).

A Conserved Ventralizing Function

The zebrafish genes, vox and vent, are functionally similar to their Xenopus orthologs, Vox and Xvent-1. All of

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**TABLE 2**

<table>
<thead>
<tr>
<th>RNA</th>
<th>pg injected</th>
<th>n</th>
<th>Wild-type gsc (%)</th>
<th>Disrupted gsc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vox</td>
<td>50</td>
<td>25</td>
<td>64</td>
<td>36</td>
</tr>
<tr>
<td>vox</td>
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<td>48</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>vent</td>
<td>50</td>
<td>40</td>
<td>48</td>
<td>53</td>
</tr>
<tr>
<td>vent</td>
<td>10</td>
<td>27</td>
<td>48</td>
<td>52</td>
</tr>
</tbody>
</table>

*Disrupted goosecoid (gsc) includes lack of expression, reduced expression, and expression in two small domains.

---

**TABLE 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Radial boz expression (%)</th>
<th>&gt;50% boz expression (%)</th>
<th>Slightly expanded boz (%)</th>
<th>Normal boz (%)</th>
<th>No boz expression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>76</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>91</td>
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<tr>
<td>LiCl</td>
<td>107</td>
<td>34</td>
<td>14</td>
<td>36</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>LiCl + vox</td>
<td>59</td>
<td>7</td>
<td>8</td>
<td>19</td>
<td>37</td>
<td>29</td>
</tr>
<tr>
<td>LiCl + vent</td>
<td>34</td>
<td>12</td>
<td>24</td>
<td>24</td>
<td>35</td>
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<tr>
<td>vent</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>72</td>
<td>28</td>
</tr>
</tbody>
</table>
**FIG. 6.** Inhibition of boz expression by vox and vent. (A) Untreated embryo, dorsal to the right. boz is expressed in a small group of dorsal cells and the dorsal YSL. (B) LiCl-treated embryo. boz expression is expanded throughout the marginal blastomeres and YSL. (C, D) vox (C) or vent (D) RNA-injected embryos treated with LiCl. Most of the boz expression has been eliminated in these embryos. In some embryos, expression of boz was completely eliminated. Embryos are at sphere stage. Side views with dorsal to the right.

**FIG. 7.** bozozok regulates the expression of vox and vent. (A–D) Expression of vox (A, B) and vent (B, D) in wild-type (+; A, C) and boz mutant (B, D) embryos at 30% epiboly. Note that vox and vent are expressed in the dorsal region in boz mutants. (E–H) Expression of vox (E, F) and vent (G, H) in uninjected (control; E, G) and boz-injected (G, H) embryos at shield stage. Dorsal is to the right in all.
these genes can morphologically ventralize embryos when ectopically expressed, and they can all inhibit the expression of dorsal organizer genes (Ault et al., 1996; Gawantka et al., 1995; Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996). This function is conserved between the two species since vox and vent are potent ventralizing factors when expressed in Xenopus. Vox and Xvent-1 have been shown to function as transcriptional repressors in Xenopus (Melby et al., 1999; Onichtchouk et al., 1998), and as vox and vent act identically when expressed in Xenopus, they are also likely to be repressors. Interestingly, zebrafish Vox and Vent, as well as Xvent-1, have an N-terminal region that is conserved in a number of transcriptional repressors (Smith and Jaynes, 1996), including Goosecoid (Ferreiro et al., 1998; Mailhos et al., 1998). This region is also partially conserved in Xenopus Vox. However, a mutation in a key conserved phenylalanine, which was shown to eliminate activity in Drosophila Engrailed (Smith and Jaynes, 1996; Tolknova et al., 1998), failed to abolish ventralizing activity or gsc repression by either vox or vent (our unpublished results). While it is possible that this domain is not functional in zebrafish Vox or Vent, a study on Xenopus Vox suggests that there are binding sites for additional corepressors elsewhere in the protein. Trindade et al. (1999) showed that deletion of either the N- or the C-terminal domain of Xenopus Vox does not abolish its repressive activity, demonstrating that it contains two repressing regions. We have previously observed that the Xenopus transcription factor XTcf-3 binds multiple corepressors (Brannon et al., 1999), as does the Drosophila Hairy protein (Poortinga et al., 1998; Zhang and Levine, 1999), and this may be a fairly common theme among transcriptional repressors.

The Role of vox and vent in Early Zebrafish Development

Our previous studies in Xenopus had shown that Vox is a direct repressor of chordin and goosecoid (Melby et al., 1999), which has been confirmed by functional analysis of the goosecoid promoter (Trindade et al., 1999). The results presented here indicate that in zebrafish, vox and perhaps vent function at earlier times since they interact with bozozok. boz is the earliest expressed dorsal-specific gene, and studies of boz embryos and the effects of ectopic boz expression indicate that it functions at the top of a hierarchy to establish the dorsal organizer, which then expresses a battery of dorsal genes including goosecoid and chordin (Fekany et al., 1999; Koos and Ho, 1998, 1999; Yamanaka et al., 1998). vox and vent expression is present on the dorsal side in boz mutants, in contrast to wild-type embryos, and ectopic bozozok eliminates vox and vent transcripts from the embryo. As Bozozok is a potential transcriptional repressor (Koos and Ho, 1998, 1999), containing the same N-terminal motif found in Vox and Vent, these results suggest that Boz might function to directly inhibit the transcription of vox and vent. Boz is also likely to regulate vox and vent non-cell autonomously by inhibiting bmp transcription (Koos and Ho, 1999), since the boz expression domain on the dorsal side of the embryo is smaller than the region from which vox and vent are excluded. However, since vox transcripts are still present in the bmp mutants at the margin (Fig. 3), Boz does not simply regulate vox by inhibiting bmp expression. We therefore suggest that Boz regulates the expression of vox through multiple mechanisms.

Conversely, Vox and Vent are proposed to be repressors of boz expression since ectopic vox and vent eliminated the appearance of boz, although we do not know if they directly or indirectly repress boz expression. These results suggest that mutually repressive interactions between the dorsally activated boz and the vox and vent genes are used to precisely regulate the domain of boz expression. We suggest that vox may be the more important factor in these interactions, since the limit of its expression domain is more dorsal than that of vent, although both genes are effective at inhibiting boz expression. While previous studies in Xenopus assigned a role for Xvent-1 and Vox in repressing the expression domains of genes that are part of the organizer such as goosecoid, chordin, and XFD-1 (Friedle et al., 1998; Melby et al., 1999; Onichtchouk et al., 1998; Trindade et al., 1999), our studies indicate that in zebrafish, vox and vent have an important early role in regulating the initial establishment of the organizer through their interaction with boz.

Interestingly, while boz is initially expressed in boz mutants, the expression of boz subsequently declines, suggesting the presence of an autoregulatory loop (Fekany et al., 1999; Koos and Ho, 1999). Our results provide a molecular explanation for this effect. We suggest that boz is initially activated by the Wnt pathway on the dorsal side of the embryo. In that region, Boz represses the expression of vox, vent, and bmp2b, which function together elsewhere in the embryo to maintain ventral and lateral fates. In a boz mutant, boz transcription is still activated dorsally by the Wnt pathway, but as no functional Boz protein is synthesized, vox, vent, and bmp2b are now expressed dorsally where Vox and Vent act to suppress boz transcription.

It will be of great interest to identify mutants in vox and vent to test these ideas. We have found that none of the published dorsalized mutants have mutations in vox or vent (M.M. and D.K., unpublished). While it may not be easy to identify a single mutant in vox or vent due to some potential redundant functions, a mutant that eliminates both vox and vent would be predicted to show expanded boz expression and therefore a dorsalized phenotype. It will be important to map the location of these two genes. If, as in Xenopus, they are closely linked (Rastegar et al., 1999), it may be possible to identify a small deletion that removes both of them.

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