The homeobox genes vox and vent are redundant repressors of dorsal fates in zebrafish

Yoshiyuki Imai1, Michael A. Gates1,*, Anna E. Melby2, David Kimelman2, Alexander F. Schier3 and William S. Talbot1,‡

1Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, USA
2Department of Biochemistry, University of Washington, Seattle, WA 98195, USA
3Developmental Genetics Program, Skirball Institute of Biomolecular Medicine, Department of Cell Biology, New York University School of Medicine, New York, NY 10016, USA
*Present address: Molecular and Cellular Biology Program, University of Washington, Seattle, WA 98195, USA
‡Author for correspondence (e-mail: talbot@cmgm.stanford.edu)

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SUMMARY

Ventralizing transcriptional repressors in the Vox/Vent family have been proposed to be important regulators of dorsoventral patterning in the early embryo. While the zebrafish genes vox (vega1) and vent (vega2) both have ventralizing activity in overexpression assays, loss-of-function studies are needed to determine whether these genes have distinct or redundant functions in dorsoventral patterning and to provide critical tests of the proposed regulatory interactions among vox, vent and other genes that act to establish the dorsoventral axis. We show that vox and vent are redundant repressors of dorsal fates in zebrafish. Mutants that lack vox function have little or no dorsoventral patterning defect, and inactivation of either vox or vent by injection of antisense morpholino oligonucleotides has little or no effect on the embryo. In contrast, embryos that lack both vox and vent function have a dorsalized phenotype. Expression of dorsal mesodermal genes, including chordin, goosecoid and bozozok, is strongly expanded in embryos that lack vox and vent function, indicating that the redundant action of vox and vent is required to restrict dorsal genes to their appropriate territories. Our genetic analysis indicates that the dorsalizing transcription factor Bozozok promotes dorsal fates indirectly, by antagonizing the expression of vox and vent. In turn, vox and vent repress chordin expression, restricting its function as an antagonist of ventral fates to the dorsal side of the embryo. Our results support a model in which BMP signaling induces the expression of ventral genes, while vox and vent act redundantly to prevent the expression of chordin, goosecoid and other dorsal genes in the lateral and ventral mesendoderm.

Key words: vox, vent, bozozok, chordin, goosecoid, bmp. Gastrulation, Morpholino, Zebrafish

INTRODUCTION

The asymmetric action of β-catenin protein establishes the dorsoventral axis of vertebrate embryos. In zebrafish and Xenopus, maternal β-catenin accumulates in dorsal nuclei, where, in concert with the HMG-box protein Tcf/Lef, it activates the expression of zygotic genes that specify dorsal positional identity (reviewed by Moon and Kimelman, 1998; Kodjabachian et al., 1999). Among the first genes activated by β-catenin in zebrafish is the homeobox gene bozozok (boz), which is expressed in the dorsal blastomeres and dorsal yolk syncytial layer, a structure that can induce and pattern the mesoderm and endoderm in teleost embryos (Yamanaka et al., 1998; Koos and Ho, 1998). Mutational analysis indicates that boz is required for the development of dorsal structures, including the axial mesoderm and anterior central nervous system (Fekany et al., 1999).

Ventral fates are induced by members of the bone morphogenetic protein (BMP) family, a subclass of the transforming growth factor β superfamily, including BMP2, BMP4 and BMP7 (for reviews, see Harland and Gerhart, 1997; Dale and Wardle, 1999; Kodjabachian et al., 1999). In Xenopus, explant and overexpression assays show that BMPs have ventralizing activity, promoting the formation of epidermis and ventral mesoderm at the expense of neuroectoderm and dorsal mesoderm. The zebrafish mutations swirl (swr) and snailhouse (snh) inactivate the bmp2b and bmp7 genes, respectively, and produce strongly dorsalized embryos in which paraxial mesoderm and the neural plate are expanded at the expense of ventral structures (Mullins et al., 1996; Kishimoto et al., 1997; Nguyen et al., 1998; Dick et al., 2000; Schmid et al., 2000). A number of dorsally expressed proteins antagonize the ventralizing action of BMPs, thereby promoting the formation of dorsal fates. One such BMP antagonist is Chordin (Sasai et al., 1994), a secreted factor that binds and inactivates BMPs (Piccolo et al., 1996). Zebrafish chordin (chd) mutants are ventralized, such that ventral cell types, including blood and epidermis, are expanded, while
more dorsal structures such as neural plate and paraxial mesoderm are reduced (Hammerschmidt et al., 1996a; Schulte-Mmerker et al., 1997).

The vox/vent-class homeobox genes have also been implicated in patterning the dorsoventral axis. In Xenopus, Vox/Xvent-2/Xom (referred to here as Vox) is expressed ubiquitously in the late blastula, and soon thereafter transcripts are excluded from dorsal territories including the dorsal mesoderm and presumptive neural plate (Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996). Transcripts of Xvent-1 (and the closely related gene PV1) are also excluded from dorsal territories, but, in contrast to Vox, expression is largely confined to the margin (Gawantka et al., 1995; Ault et al., 1996). Overexpression of either Vox or Xvent-1 can ventralize the embryo, mimicking the effects of BMP overexpression, but differences in the extent of ventralization suggest that there may be quantitative or qualitative differences in the activities of the two genes (Gawantka et al., 1995; Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996). Analysis of fusion proteins with transcriptional activator and repressor domains shows that Vox and Xvent-1 can act as transcriptional repressors (Onichtchouk et al., 1998; Melby et al., 1999; Trindade et al., 1999), although one study has suggested that Vox directly activates transcription of the Xvent-1 and BMP4 genes (Schuler-Metz et al., 2000). Vox and Xvent-1 were proposed to be key transcriptional mediators of ventralizing BMP signals, because BMP4 induces expression of both genes, and overexpression of either gene can counteract the dorsalizing effects of a dominant negative BMP receptor (Gawantka et al., 1995; Ault et al., 1996; Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996; Onichtchouk et al., 1998). There is also evidence, however, that Vox and Xvent-1 act upstream of BMP4, supporting the possibility that BMP4 and Vox/Vent transcription factors may promote each other’s expression in a positive feedback loop (Onichtchouk et al., 1996; Schmidt et al., 1996; Onichtchouk et al., 1998). Despite the evidence that Vox and Xvent-1 have similar activities as transcriptional repressors in overexpression assays, previous work has not established whether these genes have distinct or redundant functions.

Overexpression studies have also implicated two zebrafish vox/vent-class homeobox genes, known as vox (or vega1) and vent (or vega2), in dorsoventral patterning (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). The homeodomains of the Vox and Vent proteins are about 70% identical to each other and to Xenopus Vox and Xvent-1. Based on overall sequence identity, it is difficult to determine which zebrafish and frog genes are orthologous, but similarities in expression pattern suggest that zebrafish vox corresponds to Vox and that zebrafish vent corresponds to Xvent-1 (Melby et al., 2000). The vox gene is widely expressed soon after the midblastula transition, and transcripts are not detectable in dorsal cells by the late blastula stage (Kawahara et al., 2000a; Melby et al., 2000). Expression of vent is first detected at the late blastula stage in the ventrolateral marginal region (Kawahara et al., 2000b; Melby et al., 2000). BMP signals are required for the maintenance of vox and vent expression in the late gastrula, and the Boz homeodomain protein acts in the late blastula to establish the asymmetric distribution of vox and vent by inhibiting their expression in dorsal marginal cells. As with the Xenopus genes, overexpression of vox or vent can ventralize the embryo, and analysis of activator and repressor domain fusion proteins suggests that Vox and Vent can act as transcriptional repressors (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). Possible downstream genes include dorsal genes such as dkk1, squint, cyclops, floating head (flh), chd and goosecoid (gsc) (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000), but it is not clear which of these are crucial targets for Vox or Vent. Although vox and vent may control similar or identical target genes, differences in the activities of vox and vent in overexpression assays suggest that these proteins may have different regulatory functions (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). Loss-of-function studies are required to address what aspects of dorsoventral patterning require the functions of vox and vent, to determine the extent to which these functions overlap, and to provide crucial tests of the proposed regulatory interactions among the vox, vent, boz, chd and bmp genes.

We report that vox and vent are redundant repressors of dorsal fates in zebrafish. In a genetic screen for mutants with altered dorsoventral patterning, we recovered two deletions of LG13, which our mapping experiments show is the location of both vox and vent. In some genetic backgrounds, homozygous deletion mutants are strongly dorsalized, and the expression of dorsal marker genes expands to include lateral and ventral blastomeres. In a second screen, we recovered a point mutation, voxst9, that inactivates vox. Most homozygous voxst9 individuals have a wild-type phenotype, but a few are dorsoverted to a variable degree. Transheterozygotes for the point mutation and deletion are also variably dorsalized, but the fraction of mutants displaying a dorsIALIZED phenotype is higher than for homozygous voxst9 mutants. Whereas most vox mutants are wild type in appearance, injection of an antisense morpholino oligonucleotide (MO) that reduces vent function, strongly dorsalizes vox mutants. Similarly, wild-type embryos injected with MO for both vox and vent are strongly dorsalized, while injection of either MO alone has little or no effect. We show that the redundant action of vox and vent is required to restrict dorsal mesodermal genes to their appropriate territories. Using both mutants and MO injection, we have analyzed the interactions between vox, vent, boz and chd. Our results demonstrate that a key function of boz is to repress the expression of vox and vent, suggesting that boz promotes dorsal fates indirectly, by inhibiting the ventralizing action of vox and vent. The redundant action of vox and vent antagonizes chordin expression, and the expanded action of chd is a key reason that ventral gene expression is reduced in embryos that lack vox and vent function. Our results provide genetic evidence that vox and vent function in parallel with bmp2b signaling to establish ventral fates in the late blastula. BMP signaling may act to induce the expression of ventral gene expression, while vox and vent act redundantly to prevent the expression of chordin, goosecoid and other dorsal genes in the lateral and ventral mesendoderm.

MATERIALS AND METHODS

Mutagenesis and screening
To isolate new mutations that disrupt dorsoventral patterning in
zebrafish, we sought mutants among the F2 haploid offspring of F1 fish generated by postmeiotic mutagenesis with the chemical mutagen ENU as described (Imai et al., 2000). To detect mutants with dorsoventral patterning defects, we analyzed haploid embryos by in situ hybridization with a probe for gsc (51 clutches), by morphological inspection (23 clutches), or both (187 clutches). Four mutations recovered from this screen have been described previously (Dick et al., 2000; Imai et al., 2000). We now report two new mutations, T(LG13)st7 and T(LG13)st8, that strongly expand gsc expression and produce a dorsalized phenotype. In the original F2 haploid clutches screened, these mutations caused expanded gsc expression in a small fraction of the embryos (5/62 for st7, 5/73 for st8). In subsequent generations, both mutations produced dorsalized phenotypes in non-Mendelian ratios in haploid embryos that are characteristic of translocations (st7: 24% dorsalized, n=715; st8: 15%, n=399). Mapping studies (see below and data not shown) indicate that both mutations are translocations involving LG13, consistent with previous evidence that postmeiotic mutagenesis with ENU can produce chromosomal rearrangements in addition to point mutations (Riley and Grunwald 1995; Imai et al., 2000). For both mutations we obtained unbalanced translocation heterozygotes that transmit deletions as Mendelian recessive lethal mutants (st7: 50% dorsalized in haploid embryos, n=324; st8: 50%, n=70; see Table 1 for segregation data in diploids). We refer to these deletions as Df(LG13)st7 and Df(LG13)st8, or, for simplicity, Df(st7) and Df(st8).

We conducted a second genetic screen to identify point mutations in genes located within the region deleted by Df(st7). In this screen, heterozygous Df(st7)/+ male tester fish were crossed to female F1 fish generated by premeiotic ENU treatment, which induces almost exclusively point mutations. To mutagenize spermatogonial cells, we were able to obtain unbalanced translocation heterozygotes that transmit deletions as Mendelian recessive lethal mutations (st7: 50% dorsalized in haploid embryos, n=324; st8: 50%, n=70; see Table 1 for segregation data in diploids). We refer to these deletions as Df(LG13)st7 and Df(LG13)st8, or, for simplicity, Df(st7) and Df(st8).

Genetic mapping and analysis of mutant alleles

Genetic mapping and analysis of translocations and deletions were done as described (Talbot and Schier, 1999; Imai et al., 2000; Kelly et al., 2000). Primers for simple sequence length polymorphism (SSLP) markers were obtained from Research Genetics (Huntsville, AL). Polymorphisms in vox and vent were scored in the HS meiotic mapping panel as described (Kelly et al., 2000; http://zebrafish.stanford.edu); the primers used for vox were 5'-CAATCGACG- CCTAAAACTCG-3' and 5'-GGAGTCTTGATCAGCTTG-3'; and for vent the primers were 5'-GAACTAGCCGGAAGAC-GGTG-3' and 5'-GATTTCTGGGATAGGATATTTCA-3'. The vox expression was identified by direct sequencing of RT-PCR products as described (Talbot and Schier, 1999). To confirm the mutation, a vox fragment was amplified from genomic DNA of wild-type and dorsalized mutant (Df(st7) vox st9) siblings, and the PCR fragments were sequenced directly.

To genotype vox st7, a vox DNA fragment was amplified with PCR primers 5'-CTATAATACGCGCCCTTTGGGACCC-3' and 5'-TTTCCGCTTGGACGACTCGACAG-3' and digested with NcoIII. Df(st7) was scored by the presence or absence of vent (using the same primers as for meiotic mapping) or SSLP marker Z10362. Methods for genotyping boc st9, cdp st8, and svr st7, previously identified mutations used in this study, have been described (Fekany et al., 1999; Hild et al., 1999; Dick et al., 2000).

In situ hybridization

Probe synthesis and in situ hybridization were carried out as described (Sirokin et al., 2000). For genotyping after photography, genomic DNA was extracted as described (Sirokin et al., 2000).

Plasmid constructs

To construct the wild-type vox and vox st7 expression constructs, pCS2-vox and pCS2-vox-GTG, the coding region of the vox cDNA (Melby et al., 2000) was amplified by PCR and cloned into pCS2+ using oligonucleotides, 5'-CTGGGATCCATGTTGAGAAACTTTCTCGTG-3' (vox) and 5'-CTGGGATCCATGTTGAGAAACTTTCTCCTCGTG-3' (vent), for sense primers, and 5'-AGGCTGAGTCA-GTGATTAATGATGCTGAGC-3' for antisense primer. The italicized nucleotides are restriction sites used for cloning the PCR products and the underlined nucleotides are the initiation codon (ATG) and its mutant version (GTG). The insert sequences of these plasmids were confirmed by DNA sequencing.

RNA and morpholino oligonucleotide microinjections

To make vox, vox st7 and vent mRNA, pCS2-vox, pCS2-vox-GTG and ZV86 (see above and Melby et al., 2000) were linearized and transcribed with SP6 polymerase using the mMessage mMachine kit (Ambion). Morpholino oligonucleotides were obtained from Gene Tools LLC. Antisense vent morpholino (5'-CCACTGAGAATCTGGTGATCATC-3'), antisense vox morpholino (5'-AGTCCAGGGAAAGTCTCTTACCAT-3') and standard control morpholino (5'-CCTCCTACCTACGTCAATATATTACA-3') were used. RNA and morpholino oligonucleotides were diluted in 5 mg/ml Phenol Red, 0.2 M KCl prior to microinjection. 100 pl of RNA or morpholino oligonucleotide was microinjected into 1-8-cell stage embryos that had been dechorionated by Pronase treatment.

RESULTS

Isolation of new dorsalizing mutations

In a genetic screen for zebrafish mutants with early patterning defects, we isolated two new mutations that disrupt the development of the dorsoventral axis (see Materials and Methods). These mutations, T(LG13)st7 and T(LG13)st8, strongly expand expression of the dorsal mesoderm marker gsc and produce a dorsalized phenotype. Although these mutations are translocations (see Materials and Methods), in both cases we were able to obtain unbalanced translocation heterozygotes that transmit simple Mendelian deletions (see below), which are called Df(LG13)st7 and Df(LG13)st8. Because it is more rewarding to analyze these Mendelian mutations than balanced translocations, Df(LG13)st7 and Df(LG13)st8 were used for most of the experiments described below. For simplicity, we refer to these mutations as Df(st7) and Df(st8) hereafter.

Homozygotes for the Df(st7) mutation have a marked truncation of the body axis at about 24 hours after fertilization (hours) (Fig. 1B; Table 1). In some crosses, Df(st7) mutants have an elongated shape at the end of gastrulation and lyse during somitogenesis (data not shown). In addition to these zygotic effects, the Df(st7) mutation has a weak dominant maternal effect (Fig. 1D; Table 1), at least in some genetic backgrounds. Following the classification of Mullins et al. (Mullins et al., 1996), in which dorsalized phenotypes range from class 1 (C1, weak) to class 5 (C5, strong), Df(st7) homozygotes display C4/C5 dorsalized phenotypes. Df(st8) mutants and Df(st7)/T(st8) transheterozygotes also have a C4/C5 dorsalized phenotype (Fig. 1E; Table 1). Because these results suggest that Df(st7) and Df(st8) delete at least some of the same essential genes, we have used Df(st7) for most of our phenotypic studies. In stocks derived from crosses to the TL strain (Haffter et al., 1996), the Df(st7) dorsalized phenotype is fully penetrant (Table 1). In other genetic backgrounds, the Df(st7) mutation displays lower
penetrance and much more variable expressivity, such that some mutants do not have a dorsalized morphology (e.g. TL/WIK; Table 1). There is also an effect of maternal age on the maternal and zygotic effects of Dfst7; progeny of older females tend to have more severe phenotypes than progeny of younger females (data not shown). In backgrounds with a low frequency of dorsalized mutants, Dfst7 homozygotes display extensive cell death at about 1 day after fertilization (Fig. 1C; Table 1). Regardless of the background and the extent of dorsalization, most Dfst7 mutants display extensive cell death and degeneration by 30 hours (not shown). These results indicate that the region deleted in Dfst7 contains loci essential for embryogenesis, including a gene or genes required for development of the dorsoventral axis.

Dfst7 and Dfst8 mutations delete a region of LG13 that includes vox and vent

As a first step to identify the critical gene(s) inactivated by Dfst7 and Dfst8, we mapped the mutations. Analysis of simple-sequence length polymorphism (SSLP) markers (Shimoda et al., 1999) indicated that segments of LG13 are deleted in Dfst7 and Dfst8 mutants (Fig. 2A and data not shown). As shown in Fig. 2B, the Dfst7 mutation stretches over at least 29 cM from SSLP marker Z17223 to Z4252, whereas the Dfst8 mutation is somewhat longer, spanning a 35 cM interval defined by markers Z9423 and Z4252. The finding that Dfst7 and Dfst8 remove overlapping segments of LG13 supports the conclusion that these mutations inactivate the same gene(s) with crucial function in dorsoventral patterning. Furthermore, the mapping studies indicate that the Dfst7 and Dfst8 mutations do not inactivate bmp2b/swr, bmp7/snhs or smad5/sbn, because these genes reside at different locations (LG20, LG11 and LG14, respectively).

Analysis of another LG13 rearrangement, Df(LG13)st4 (Dfst4 hereafter), was useful in defining the region of LG13-containing genes with important functions in dorsoventral patterning. In previous work (Imai et al., 2000), we showed that Dfst4 deletes approximately 20-27 cM of LG 13, covering an interval that overlaps extensively but not completely with the region deleted in Dfst7 and Dfst8 (Imai et al., 2000; Fig. 2B). These three mutations were recovered in the same screen (Imai et al., 2000; this work), and they have been maintained on similar genetic backgrounds, suggesting that the genes affected by the rearrangements rather than genetic background are responsible for phenotypic differences among these mutants. Dfst4, Dfst4/Dfst7 and Dfst4/Dfst8 mutants are not

### Table 1. Phenotypes produced by mutant crosses

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
<th>Genetic background</th>
<th>Temperature (ºC)</th>
<th>Age (months)</th>
<th>Wild type (%)</th>
<th>C1 (%)</th>
<th>C2 (%)</th>
<th>C3 (%)</th>
<th>C4/C5 (%)</th>
<th>Degeneration by 24 hours (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(LG13)st7/+</td>
<td>Df(LG13)st7/+</td>
<td>TL</td>
<td>28</td>
<td>67.8</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>26</td>
<td>0</td>
<td>0</td>
<td>345</td>
</tr>
<tr>
<td>Df(LG13)st7/+</td>
<td>Df(LG13)st7/+</td>
<td>TL/WIK</td>
<td>28</td>
<td>71.9</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>8.1</td>
<td>19</td>
<td>395</td>
<td></td>
</tr>
<tr>
<td>Df(LG13)st7/+</td>
<td>Df(LG13)st7/+</td>
<td>TL</td>
<td>28</td>
<td>92.1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>31</td>
<td>0</td>
<td>835</td>
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<td>0</td>
<td>0</td>
<td>32</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

*The same stocks of fish were used for these crosses at different times, minimizing differences due to genetic background.*
and mutant alleles (vox st9). A TG occurs at codon 170, near the end of the homeodomain.

A TG of the open reading frame to GTG. The second in-frame phenotype: only the wild-type allele (+/+ or vox). All dorsalized embryos (n linked to vox = 291) had only the mutant (Df st7). Phenotype. Analysis of SSLP markers confirmed that the penetrant, because some of these embryos have a wild-type phenotype resides in the region deleted in the distal region of LG13 (data not shown). (B) Linkage map of LG13 showing the positions of vox and vent and the regions deleted in Df st7, Df st8 and Df st4 (vertical lines indicate deleted regions, broken vertical lines denote uncertainty in the locations of the breakpoints). Marker distances are based on Shimoda et al. (Shimoda et al., 1999). We localized vox and vent by scoring polymorphisms in both genes in the HS meiotic mapping panel (Kelly et al., 2000). (C) The vox st9 mutation is tightly linked to vox. All dorsalized embryos (n = 291) had only the mutant vox allele, indicating that their genotype was Df st7/vox st9. Three different genotypes were observed in embryos with a wild-type phenotype: only the wild-type allele (+/+) or Df st7/+), both wild-type and mutant alleles (vox st9/+; arrowhead), and only the mutant allele (Df st7/vox st9; asterisk). The Df st7/vox st9 genotype is only partially penetrant, because some of these embryos have a wild-type phenotype. Analysis of SSLP markers confirmed that the vox st9 mutation resides in the distal region of LG13 (data not shown). (D) Schematic representation of Vox protein and the lesion in the vox st9 mutation. The vox st9 mutation changes the initiation codon (ATG) of the vox open reading frame to GTG. The second in-frame ATG occurs at codon 170, near the end of the homeodomain.

dorsalized (Imai et al., 2000; data not shown), suggesting that the gene(s) whose deletion is responsible for the dorsalized phenotype resides in the region deleted in Df st7 but not in Df st4.

By scoring polymorphisms in our meiotic mapping panel (Kelly et al., 2000), we localized two candidate genes, the ventrally expressed homeobox genes vox and vent (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000), to the distal region of LG13, within the interval deleted in Df st7 but not Df st4 (Fig. 2B). These results show that vox and vent reside in the region of LG13 that contains a gene or genes with important functions in dorsoventral patterning.

**Isolation of a point mutation in vox**

To identify point mutations in genes responsible for the dorsalized phenotype of the deletions, we conducted a screen to find new mutations that fail to complement Df st7 (see Materials and Methods). In crosses of 448 F1 females to Df st7/ males, we identified one mutation, vox st9, that failed to complement Df st7. A variable fraction of progeny (2-14%) from crosses of vox st9/+/ females to Df st7/ males had C1 to C4 dorsalized phenotypes (Fig. 1G-I), but many Df st7/vox st9 individuals are wild-type in appearance (Table 1). The penetrance and expressivity of the Df st7/vox st9 genotype are stronger at 31°C than 28°C (Table 1), as was shown by scoring the phenotypes of embryos from the same clutches incubated at the two temperatures. Most vox st9 homozygotes display no mutant phenotype and can grow to fertile adults (Fig. 1F; Table 1; see also Fig. 5B,J), but a variable dorsalized phenotype was apparent in some vox st9 homozygotes that were progeny of older females (Table 1). We noted a similar effect of maternal age on the penetrance and expressivity of the Df st7/vox st9 genotype. An effect of maternal age has previously been noted in the analysis of boz mutants, which have more severe phenotypes when they are produced by young females and relatively less severe phenotypes when produced by older females (Fekany et al., 1999).
Mapping, molecular analysis, and functional studies demonstrated that the vox\textsuperscript{gtg} mutation reduces or eliminates vox function. Genetic linkage analysis mapped vox\textsuperscript{gtg} to the distal region of LG13, near vox and vent (Fig. 2B,C; data not shown). To search for a possible lesion, we sequenced the coding sequences of vox and vent from vox\textsuperscript{gtg} mutants. Although no lesions were detected in the vent open reading frame, the vox gene contained an A→G change that disrupted the translational initiation codon (ATG→GTG). This ATG→GTG change was not present in genomic DNA (prepared from tail 2C). The second in-frame A TG in the vent initiation codon (A TG GTG; Fig. 2D). This A TG mutation reduces or eliminates vox function, owing to morpholino oligonucleotide injection.

(A) Uninjected wild-type control embryo. Wild-type (B-D) or vox\textsuperscript{gtg} (E,F) embryos were injected at the 1-4-cell stage with 840 pg vox antisense MO (B,F), 840 pg vent MO (C,E), or co-injected with 420 pg vox and 420 pg vent MO (D). Photographs of living embryos were taken at approximately 30 hours. Lateral views, anterior towards the left.

Fig. 4. Dorsalized phenotype of embryos lacking vox and vent function. (A) Uninjected wild-type control embryo. Wild-type (B-D) or vox\textsuperscript{gtg} (E,F) embryos were injected at the 1-4-cell stage with 840 pg vox antisense MO (B,F), 840 pg vent MO (C,E), or co-injected with 420 pg vox and 420 pg vent MO (D). Photographs of living embryos were taken at approximately 30 hours. Lateral views, anterior towards the left.

(D) F1 st7 enzyme test for the A fin of the mutagenized male. Using a restriction change, and that, instead, this mutation was induced by ENU in the germline of the mutagenized male (G0) that gave rise to the change was not present in genomic DNA (prepared from tail 2C). The second in-frame A TG in the vox initiation codon (A TG GTG; Fig. 2D). This A TG mutation, or in genomic DNA from 8 F1 siblings of the original F1 vox\textsuperscript{gtg}/+ heterozygote (not shown). These results indicate that the parental chromosomes did not contain the A TG mutation, and the vox construct engineered to contain the A TG mRNA from a wild-type construct or mRNA transcribed from a vox construct engineered to contain the ATG→GTG change.

 Vox\textsuperscript{gtg} suggested that loss of vox function contributes to the dorsalized phenotype of Df\textsuperscript{st7} mutants. To determine whether expression of vox could rescue the dorsalized phenotype of the deletion mutants, we injected synthetic vox mRNA into embryos from Df\textsuperscript{st7}/+ intercrosses and assayed gsc (Fig. 3) and chd (data not shown) expression at the late blastula stage (40% epiboly, 5 hours). In wild-type embryos at this stage, gsc is expressed specifically in dorsal mesendodermal precursors, and chd is expressed more broadly, in a dorsal domain that includes cells in presumptive neural plate and paraxial mesoderm in addition to axial mesendoderm (Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997). In Df\textsuperscript{st7} mutants injected with a control RNA (Fig. 3E; data not shown), gsc and chd expression was strongly expanded, such that transcripts were detected in the ventral marginal region. Overexpression of vox reduced the expansion of gsc and chd in Df\textsuperscript{st7} mutants (Fig. 3F; data not shown), and, consistent with previous studies (Kawahara et al., 2000a; Melby et al., 2000), also reduced the domains of expression in wild-type embryos (Fig. 3B; data not shown). Overexpression of vent also reduced the expanded domains of gsc and chd in Df\textsuperscript{st7} mutants (Fig. 3H; data not shown) and reduced the dorsal domains of gsc and chd expression in wild-type embryos (Fig. 3D; data not shown). Synthentic vox RNA with the vox\textsuperscript{gtg} ATG→GTG mutation had no detectable activity in this assay (Fig. 3C,G). These results demonstrate that vox and vent have similar ability to counteract the expansion of dorsal gene expression in Df\textsuperscript{st7} mutants.

Fig. 5. Expression of goosecoid and chordin is ventrally expanded in embryos lacking vox and vent function. Expression of gsc (A-H) and chd (I-P) at 40% epiboly (5 hours) was examined by whole-mount in situ hybridization in wild type (A,I), vox\textsuperscript{gtg} mutants (B,J), Df\textsuperscript{st7}/vox\textsuperscript{gtg} mutants (C,K), Df\textsuperscript{st7} mutants (D,L), control MO-injected embryos (E,M), vox MO-injected embryos (F,N), vent MO-injected embryos (G,O), and in embryos co-injected with vox and vent MO (H,P). In B,C,J,K, genotypes were determined by PCR assay after the photographs were taken. Animal pole views, dorsal towards the right.
The overexpression experiments, together with the analysis of the $vox^{st9}$ mutation support the idea that the loss of $vox$ function contributes to the dorsalized phenotype of $Df^{st7}$ homozygotes. $vox$, however, cannot be the only important gene removed by the deletion, because $Df^{st7}/vox^{st9}$ mutants have a weaker and more variable phenotype than $Df^{st7}$ homozygotes, and most $vox^{st9}$ homozygotes are not detectably dorsalized (Fig. 1; Table 1). Because $vox$ and $vent$ have similar ventralizing activities in overexpression assays (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000; Fig. 3) and because both $vox$ and $vent$ are deleted in $Df^{st7}$ mutants, we hypothesized that $vox$ and $vent$ have overlapping activities, such that both genes must be inactivated to produce a strong dorsalized phenotype.

**Table 2. Dorsalization of embryos by injection of $vox$ and $vent$ morpholino oligonucleotides**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Morpholino</th>
<th>Dose</th>
<th>Wild type (%)</th>
<th>C1 (%)</th>
<th>C2 (%)</th>
<th>C3 (%)</th>
<th>C4-C5 (%)</th>
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<tr>
<td></td>
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<td>5</td>
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<td>62</td>
</tr>
<tr>
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<tr>
<td></td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
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<tr>
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<tr>
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*Five of these strongly dorsalized embryos were genotyped by PCR assay: all were $vox^{st9}$ homozygotes.
As another approach to examine the phenotypic effects of reducing vox and vent function, we employed antisense morpholino oligonucleotides (MO). In Xenopus and zebrafish, microinjection of MO complementary to the region of the translational initiation codon or 5’ untranslated region can reduce protein levels and produce specific loss-of-function phenotypes (Heasman et al., 2000; Nasevicius and Ekker, 2000). Injection of 1-8-cell wild-type (TL strain) embryos with 840 pg (0.1 pmol) of antisense MO for either vox or vent individually produced only a low frequency of weakly dorsalized embryos (Fig. 4B,C; Table 2). In contrast, coinjection of wild-type (TL strain) embryos with 420 pg of vox MO and 420 pg of vent MO produced a strong dorsalized phenotype at very high frequency (Fig. 4D; Table 2). Coinjection of embryos from a different wild-type strain (AB) with vox and vent MO produced weaker and more variable phenotypes (Table 2), reminiscent of the effect of genetic background on the phenotype of Df(st7) mutants (Table 1). As a control for the specificity of the MO, we injected embryos from intercrosses of vox<sup>st9</sup> homozygotes with the vox and vent MO, individually or in combination. Although injection of 840 pg of vent MO had a very weak effect on wild-type embryos (Fig. 4C; Table 2), this treatment strongly dorsalized vox<sup>st9</sup> mutants (Fig. 4E; Table 2). Injection of 840 pg of vox MO had little effect on vox<sup>st9</sup> mutants (Fig. 4F; Table 2), and coinjection of vox and vent MO dorsalized vox<sup>st9</sup> mutants, just as with wild-type embryos (Table 2). These experiments demonstrate vox and vent have redundant functions and that the loss of both vox and vent produces a strong but strain-dependent dorsalized phenotype.

**Loss of vox and vent function expands dorsoanterior cell types**

To determine what cell types are affected by the loss of vox and vent function, we examined marker gene expression in Df(st7) homozygotes and wild-type embryos co-injected with vox and vent MO (which we refer to as ‘MO vox + vent embryos’). For these experiments, a TL-derived genetic background was used, in which the Df(st7) mutation produces a fully penetrant C4-C5 dorsalized phenotype (Table 1). Similarly, the MO injections were conducted with a wild-type control for the specificity of the MO, we injected embryos from a different wild-type strain (AB) with vox and vent MO, individually or in combination. Although injection of 840 pg of vent MO had a very weak effect on wild-type embryos (Fig. 4C; Table 2), this treatment strongly dorsalized vox<sup>st9</sup> mutants (Fig. 4E; Table 2). Injection of 840 pg of vox MO had little effect on vox<sup>st9</sup> mutants (Fig. 4F; Table 2), and coinjection of vox and vent MO dorsalized vox<sup>st9</sup> mutants, just as with wild-type embryos (Table 2). These experiments demonstrate vox and vent have redundant functions and that the loss of both vox and vent produces a strong but strain-dependent dorsalized phenotype.

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Assays of marker gene expression revealed that vox and vent are required for proper patterning of the dorsoventral axis in the late blastula. In both Dfst7 mutants and MO vox + vent embryos at the late blastula stage (40% epiboly, 5 hours), expression of gsc (Fig. 5D,H) and chd (Fig. 5L,P) was strongly expanded. The expression domains of flh and axial, two other dorsally restricted genes, were also expanded in Dfst7 mutants during gastrulation (data not shown). Consistent with the morphological phenotypes at later stages, gsc and chd are expressed normally in wild-type embryos injected with either vox MO (Fig. 5F,N) or vent MO (Fig. 5G,O) and in vox+ embryos (Fig. 5B,J); partially expanded domains were observed in some Dfst7/vox+ mutants (Fig. 5C,K). At an earlier stage (sphere stage, 4 hours), Dfst7 mutants appear to have normal expression of the dorsally restricted genes gsc, boz, and chd (Fig. 6B,F,J). Slightly later (dome stage, 4.3 hours), however, Dfst7 mutants have a pronounced expansion of gsc and chd expression (Fig. 6D,H), such that transcripts were detected in some ventral marginal cells. At dome stage, boz was expressed in its normal dorsal domain in Dfst7 mutants (data not shown), but at 30% epiboly (4.7 hours) boz expression was expanded (Fig. 5L). These results indicate that the redundant action of vox and vent is required for maintenance, but not initiation, of spatially restricted dorsal gene expression in the late blastula.

The function of vox and vent is also required for proper expression of ventrally restricted genes during gastrulation. As in wild type, bmp2b and bmp4 are strongly expressed in the ventral but not dorsal regions of Dfst7 mutants (Fig. 6N) and MO vox + vent embryos (data not shown) at the late blastula stage (40% epiboly, 5 hours). At the mid-gastrula stage (75% epiboly, 8 hours), however, ventral expression of bmp2b and bmp4 was strongly reduced in Dfst7 mutants and MO vox + vent embryos (Fig. 6P and data not shown). Similarly, the ventral marker gata2 was reduced at the mid-gastrula stage (75% epiboly, 8 hours), but it was expressed normally at the start of gastrulation in Dfst7 mutants and in MO vox + vent embryos (see Figs 8K,L, 9B,F; data not shown). Thus, the redundant action of vox and vent is required for maintenance of ventral gene expression at the mid-gastrula stage, but vox and vent are not required for proper expression of ventral markers at earlier stages.

To investigate whether vox and vent function in the development of ventrolateral mesodermal derivatives, we examined markers of pronephros, blood, and heart in Dfst7 mutants and MO vox + vent embryos (Fig. 7). Analysis of pax2.1 expression revealed that the developing pronephros, a derivative of intermediate mesoderm, was absent in Dfst7 mutants and MO vox + vent embryos (Fig. 7B,D). Similarly, expression of the erythroid lineage marker gata1 was not detected (Fig. 7F,H), indicating that vox and vent are essential for the development of blood. To investigate whether vox and vent function in development of the heart, we examined nksx2.5, a gene expressed in cardiac precursors. Expression was not detected in Dfst7 mutants (Fig. 7I), but nksx2.5 transcripts were evident in MO vox + vent embryos (Fig. 7L,M). Similarly, Dfst7 mutants did not form a beating heart by approximately 30 hours, in contrast to MO vox + vent embryos, which did have a beating heart at this stage (data not shown). This difference between the cardiac phenotypes of Dfst7 mutants and MO vox + vent embryos suggests that another gene within the Dfst7 deletion may be required for heart development, or perhaps that vox + vent MO co-injection does not eliminate the functions of these genes in cardiac precursors.

Examination of neural markers shows that dorsoanterior territories are expanded in embryos that lack vox and vent function. Expression of pax2.1 and krox20, which mark the midbrain-hindbrain boundary and hindbrain rhombomeres 3 and 5, respectively, was expanded to ventral regions in Dfst7 mutants and MO vox + vent embryos (Fig. 7B,D,O,Q). The expression domains of these genes were also shifted posteriorly (Fig. 7B,D,O,Q), suggesting that more anterior territories are expanded in embryos lacking vox and vent function.

To investigate the possibility that BMP signals and Vox/Vent have different functions in neural patterning, we examined neural markers in swr/bmp2b and Dfst7 mutants (Fig. 8). In wild-type embryos at the mid-gastrula stage, otx2 is expressed in a dorsal animal domain that includes presumptive forebrain and midbrain territories. In swr/bmp2b mutants (Fig. 8F), otx2 was expanded ventrally but not vegetally, such that the expression domain encompassed both dorsal and ventral regions, but the vegetal border was at the wild-type position (arrowhead in Fig. 8F). In Dfst7 mutants and MO vox + vent embryos, otx2 expression was expanded vegetally (arrowheads in Fig. 8G,H); expression was also expanded ventrally, but not to the same extent as in bmp2b/swr mutants. In Dfst7 mutants and MO vox + vent embryos, the ventral (non-neural) ectoderm marker gata2 was reduced from wild-type levels (Fig. 8K,L), and the expression domain was roughly complementary to that of otx2 (compare Fig. 8G,H with 8K,L). As in previous studies (Nguyen et al., 1998), we did not detect gata2 expression in swr/bmp2b mutants (Fig. 8J). Analysis of markers at later stages confirmed that anterior neural territories are expanded in Dfst7 mutants and MO vox + vent embryos. The telencephalon marker emx1 is strongly expanded in Dfst7 mutants and MO vox + vent embryos (Fig. 8O,P), but not in bmp2b/swr mutants (Fig. 8N). Similarly, six3.2, a marker of forebrain, optic primordia and axial mesendoderm, was expressed much more strongly in Dfst7 mutants and MO vox + vent embryos (Fig. 8S,T) than in wild-type (Fig. 8Q) and swr/bmp2b mutants (Fig. 8R).

The finding that anterior neural territories are enlarged in Dfst7 mutants and in MO vox + vent embryos (Figs 7, 8) suggested that vox and vent may repress the expression of signals that induce anterior neural identity. Supporting this possibility, we found that Dfst7 mutants and MO vox + vent embryos had greatly increased expression of dkk1 (Fig. 8C,D), which encodes a secreted Wnt-antagonist with anteriorizing activity (Glinka et al., 1998; Hashimoto et al., 2000; Shinya et al., 2000). In bmp2b/swr mutants at the early gastrula stage (shield stage, 6 hours), dkk1 was not expanded (Fig. 8B). These results indicate that the redundant action of vox and vent is required for the repression of anterior neural territories, and that vox/vent and bmp2b/swr have distinct functions in neural patterning.

**Interactions between vox, vent and chordin:**

**Increased activity of chordin represses ventral gene expression in embryos that lack vox and vent**

Our results show that vox and vent act redundantly to
antagonize chordin expression (Fig. 5). Hence, aspects of the dorsIALIZED phenotype caused by the lack of vox and vent might result from ectopic expression of the dorsaling factor chordin. To investigate this possibility, we examined the ventral marker gata2 in Df\textsuperscript{st7};chd double mutants and in chd single mutants co-injected with vox and vent MO. chd mutants are ventralized, and gata2 is expressed in all but the most dorsal cells in chd mutant embryos (Hammerschmidt et al., 1996a; Hammerschmidt et al., 1996b; Fig. 9C). In Df\textsuperscript{st7};chd double mutants (Fig. 9D) and in chd single mutants co-injected with vox and vent MO (Fig. 9H), gata2 is strongly expanded, indicating that chd is epistatic to vox and vent when assayed with this marker. These results support the model that vox and vent act to promote ventral gene expression by antagonizing the action of chd (Fig. 10).

To determine whether the increased expression of dorsal mesodermal genes in embryos lacking vox and vent function is caused by expanded expression of chd, we examined gsc expression in Df\textsuperscript{st7};chd double mutants. Consistent with previous studies (Hammerschmidt et al., 1996a), gsc expression was normal in chd single mutants (Fig. 9K) at the late blastula stage (40% epiboly, 5 hours). In Df\textsuperscript{st7};chd double mutants (Fig. 9L), gsc transcripts were detected in lateral and ventral marginal cells, such that gsc expression was very similar in Df\textsuperscript{st7};chd and Df\textsuperscript{st7} mutant embryos (Fig. 9J). Thus mutation of chd did not affect gsc expression in embryos lacking vox and vent function, suggesting that vox and vent do not regulate gsc transcription via antagonism of chd action. Instead it is likely that the Vox and Vent proteins directly repress the transcription of gsc and chd (Melby et al., 1999; Trindade et al., 1999; Kawahara et al., 2000b).

**Interactions between vox, vent and boz: increased activity of vox and vent represses dorsal mesoderm in boz mutants**

We have shown that boz expression is expanded in embryos that lack vox and vent function (Fig. 6), and previous work shows that expression of vox and vent is expanded in boz mutants (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). To investigate the order in which these genes act, we examined gsc expression in embryos lacking the function of vox, vent and boz. Expression of gsc was reduced in boz mutants at the late blastula stage (Fig. 9O) – the opposite phenotype of Df\textsuperscript{st7} mutants (Fig. 9N). In Df\textsuperscript{st7};boz double mutants (Fig. 9P) and in boz single mutants that were co-injected with vox and vent MO (data not shown), gsc expression was expanded, indicating that vox and vent are epistatic to boz. Thus, boz function is not required to activate gsc expression at normal levels if vox and vent are inactivated by mutation or morpholino oligonucleotide injection. These results support a model in which boz promotes dorsal mesodermal gene expression by antagonizing the action of vox and vent (Fig. 10).

**DISCUSSION**

**Redundant ventralizing functions of vox and vent**

Our results demonstrate that the vox and vent homeobox genes have redundant functions in dorsoventral patterning in the zebrafish embryo. Evidence for this conclusion derives from our analysis of a point mutation that disrupts vox, chromosomal rearrangements that delete vox, vent and numerous other genes on LG13, and antisense morpholino oligonucleotides that inactivate vox and vent. Inactivation of either vox or vent by MO injection has little phenotypic effect on the embryo. Similarly, most homozygotes for the loss-of-function allele vox\textsuperscript{st9} are wild type in appearance and viable to adulthood. In contrast, embryos were strongly dorsIALIZED when we inactivated both vox and vent by any of three approaches: coinjection of vox and vent MO into wild type, injection of vent MO into vox\textsuperscript{st9} homozygotes, or deletion of the vox\textsuperscript{vent} region of LG13 in Df\textsuperscript{st7} and Df\textsuperscript{st8} mutants. The combined results show that inactivation of either vox or vent has little effect, whereas the disruption of both genes produces a severely dorsIALIZED phenotype. The phenotype of embryos that lack vox and vent function is strongly affected by genetic background. Our results suggest that the AB background has one or more dominant genes that suppress the dorsIALIZED phenotype caused by loss of vox and vent function. Preliminary analysis suggests that the background effect may be due to one major gene in some crosses, but the penetrance in other crosses suggests that multiple genes may be responsible (Y. Imai, unpublished). Furthermore, the penetrance and expressivity are also strongly affected by maternal age, making it difficult to determine the number of the genes involved in the background effect. Further analysis is required to determine the nature of the genetic differences between strains that affect the phenotype caused by the loss of vox and vent function.
Consistent with our genetic evidence for the redundant functions of vox and vent, previous work shows that vox/vent family genes have similar activities in overexpression assays (Gawantka et al., 1995; Ault et al., 1996; Ladher et al., 1996; Onichtchouk et al., 1996; Onichtchouk et al., 1998; Schmidt et al., 1996; Melby et al., 1999; Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). Vox, Vxent-1, vox and vent can each ventralize the embryo and repress the expression of dorsal genes such as chd and gsc in overexpression assays. In addition, we found that injection of synthetic mRNA encoding either Vox or Vent could counteract the expansion of dorsal marker gene expression in Dfso7 mutants, providing further evidence that function of either gene is sufficient to repress dorsal gene expression. Our results suggest that the differences between the activities of Vox and Vent are largely quantitative, and that both proteins are capable of repressing the same set of crucial target genes. Our results also suggest that the combined level of vox and vent activity is crucial for establishing normal dorsoventral pattern: voxso9 homozygotes have little or no dorsoventral patterning defect, Dfso7 deletion homozygotes have a strongly dorsalized phenotype and Dfso7/voxso9 transheterozygotes, which lack vox function and have reduced vent function, have a range of phenotypes between these extremes.

**vox and vent act in the marginal region to repress dorsal gene expression**

Our results suggest that the requirement for vox and vent function is largely limited to the marginal cells. While vox expression encompasses all but the dorsal marginal cells of the late blastula, expression of vent is predominantly restricted to the ventrolateral marginal region (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). Despite the widespread expression of vox, our results indicate that the vent gene can compensate for the loss of vox in voxso9 homozygotes. Because the expression domains of these redundant genes overlap predominantly in marginal territories, the action of vox and vent is apparently not essential for the repression of dorsal genes in animal territories. Thus, other ventrally expressed transcription factors may limit neural genes to their proper dorsal ectodermal territories. One candidate for such a factor is gata2, expression of which is roughly complementary to otx2 in wild type and in embryos that lack vox and vent function. Another candidate is a zebrafish counterpart of Xenopus Msx-1, which is a transcriptional repressor induced by ventralizing BMP signals (Yamamoto et al., 2000).

**vox and vent redundantly repress chordin and other dorsal genes**

Our loss-of-function analysis and previous overexpression assays (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000) show that many dorsally expressed genes, including dkk1, squint, cyclops, flh, chd and gsc, are repressed by Vox and Vent. Our genetic analysis indicates that chd is a key target of vox and vent. Expression of the ventral marker gata2 is reduced in mid-gastrula stage embryos lacking vox and vent function and strongly expanded in chd mutant embryos. Expression of gata2 is also strongly expanded in Dfso7;chd double mutants, supporting the model that vox and vent activate gata2 expression indirectly, via the inhibition of chd (Fig. 10). In Xenopus, Vox directly represses transcription of the chd gene (Melby et al., 1999), suggesting that Vox may act directly to inhibit chd expression in zebrafish. Interestingly, expression of the dorsal mesoderm marker gsc is expanded in Dfso7;chd double mutants at the late blastula stage, indicating that the expansion of gsc expression in embryos lacking vox and vent function is not a consequence of expanded chd activity. Evidence in frog and zebrafish supports the possibility that Vox and Vent directly repress the transcription of gsc (Melby et al., 1999; Trindade et al., 1999; Kawahara et al., 2000a). Anterior neural territories are expanded in embryos that lack vox and vent function, possibly due to increased expression of dkk1 or another dorsally expressed factor with anteriorizing activity. These results demonstrate that the redundant action of vox and vent is required to restrict expression of chd and other key dorsal genes to the appropriate territories.

**Different roles of zygotic BMP signals and Vox/Vent in promoting development of ventral fates**

Our results suggest that Vox/Vent homeodomain proteins and zygotic BMP signals play different roles in the establishment of ventral positional identity in the late blastula. We suggest that vox and vent function in the ventral marginal region to restrict the expression of the dorsal mesendodermal genes to the proper dorsal marginal territories, while the main role of zygotic BMP signals may be to activate ventral gene expression in ventrolateral territories (Fig. 10). This model is based on the following observations.

First, dorsal mesodermal fates are markedly expanded at early stages in embryos lacking vox and vent function, but not in bmp2b/swr mutants. In Dfso7 mutants and wild-type embryos co-injected with vox and vent MO, genes characteristic of prospective dorsal mesoderm, including gsc, chd and boz, are expressed widely along the margin. In some cases, even the most ventral cells contain transcripts for these genes. In contrast, expression of gsc is normal in swr mutants at the early gastrula stage (Mullins et al., 1996). Thus, vox and vent act redundantly in the late blastula to restrict the expression of dorsal mesendodermal genes to the appropriate dorsal marginal territories. bmp2b/swr is not required for this restriction, indicating that the Vox and Vent repressors do not act simply as effectors of Bmp2b. Instead, we propose that Vox/Vent and zygotic BMP signals have distinct, partially parallel functions required for the development of ventrolateral mesendoderm. In the ventral marginal region, we suggest that bmp2b activates genes required for differentiation of ventrolateral mesodermal cell types, while the redundant action of vox and vent restricts dorsal mesendodermal genes to the proper dorsal marginal territories (Fig. 10).

Second, phenotypic differences in the neural development of embryos that lack vox and vent function and of bmp2b/swr mutants indicate that Vox/Vent and Bmp2b have different roles in patterning the ectoderm. During gastrulation, the dorsalization of the ectoderm is more complete in bmp2b/swr mutants than in embryos that lack vox and vent function. At mid-gastrulation in bmp2b/swr mutants, the ventral marker gata2 is not expressed, and the dorsal marker otx2 is strongly expressed even in ventral ectoderm. In contrast, gata2 is expressed ventrally in embryos that lack vox and vent function, although at less than wild-type levels, and otx2 expression is not expanded to the same extent as bmp2b/swr mutants. Thus, Bmp2b appears to be more important than Vox and Vent to
promote ventral gene expression and to restrict the expression of neural genes within the ectoderm, an activity that may be mediated by BMP-dependent ventral expression of transcription factors such as Gata2.

**A positive feedback loop between Vox/Vent and BMP signals in the gastrula**

vox/vent and bmp2/boz are initially expressed independently of each other’s action. In the late blastula, bmp2/boz is expressed normally in embryos that lack vox and vent function, and bmp2/boz mutants have normal expression of vox and vent (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). As embryogenesis proceeds, however, expression of vox/vent and bmp2/boz genes becomes interdependent, apparently through a positive feedback loop established during gastrulation (Fig. 10). At mid-gastrulation, expression of bmp2/boz is reduced in embryos that lack vox and vent function, and vox and vent are likewise reduced in bmp2/boz mutants (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). These results show that zygotic BMP signals are required for normal levels of vox and vent expression during gastrulation. We suggest that vox and vent act to promote bmp2/boz and bmp4 expression by inhibiting the expression of chd, which is known from previous work to block a positive autoregulatory activity of BMP signals (Hammerschmidt et al., 1996b; Schulte-Merker et al., 1997).

Accordingly, vox and vent do not activate bmp and other ventrally expressed genes directly, but instead repress chordin, an inhibitor of bmp expression (Fig. 10). In embryos that lack vox and vent, we propose that interruption of this vox/vent-bmp2/boz positive-feedback loop is responsible for the diminution of ventral gene expression at mid-gastrulation. The timing of changes in gene expression in embryos lacking vox and vent function is consistent with this model, because expression of chd is expanded significantly before the expression of bmp and other ventral genes is reduced. Interruption of the vox/vent-bmp2/boz positive-feedback loop may also lead to the expansion of some dorsal genes in bmp2/boz mutants. Accordingly, chd expression is normal in bmp2/boz mutants at the onset of gastrulation, but expanded by mid-gastrulation (Miller-Bertoglio et al., 1997), so that the expansion of chd coincides with the reduction of vox and vent expression in bmp2/boz mutant embryos.

Our results suggest that the vox/vent-bmp2/boz positive feedback loop maintains ventral positional identity during gastrulation. The participation of the extracellular factors Chordin and BMP incorporates flexibility and sensitivity to cellular environment into the mechanism that maintains dorsoventral identity. For example, a cell moving from ventral to dorsal territories during gastrulation would reduce its expression of vox and vent in response to increased levels of Chd and reduced levels of BMP activity. The reduction of Vox and Vent levels would in turn permit the expression of dorsal genes appropriate for the cell’s new environment.

**bozo** is required to antagonize the action of vox and vent

The homeobox gene boz is required for the normal expression of gsc, a gene that is repressed by vox and vent. Our genetic evidence indicates that boz promotes dorsal mesodermal gene expression indirectly, by repressing vox and vent expression in the late blastula (Fig. 10). The gsc gene is expressed, and indeed greatly expanded, in boz embryos that also lack vox and vent function, indicating that the function of boz is not needed to promote gsc expression if vox and vent are inactive. Consistent with our evidence that a crucial function of boz is to repress the action of vox and vent, previous work indicates that the expression of vox and vent is expanded to include dorsal territories in boz mutants (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). In boz mutants, expression of bmp2/boz is also expanded dorsally (Koos and Ho, 1999), and the evidence that bmp2/boz and vox/vent are not required for each other’s expression in the late blastula suggests that boz represses these genes in parallel (Fig. 10). The Boz protein has a motif that is conserved among transcriptional repressors (Koos and Ho, 1998), supporting the possibility that Boz may directly repress genes with ventralizing activity such as vox, vent, and bmp2b. Conversely, our results show that vox and vent repress boz expression in a negative regulatory loop established in the late blastula (Fig. 10). Thus genetic analysis shows that the antagonistic transcription factors Vox/Vent and Boz are essential components of the mechanism that establishes and maintains dorsoventral asymmetry in the zebrafish.

**Role of vox and vent in maintaining dorsoventral asymmetry**

Characterization of embryos that lack vox and vent function demonstrates that the redundant action of these genes antagonizes expression of a number of dorsally expressed genes including gsc, chd, and boz. Interestingly, we found that chd, gsc, and boz are expressed only on the dorsal side of Df(st7) mutants at sphere stage (4 hours). Slightly later (dome stage, 4.3 hours), expression of gsc and chd is strongly expanded, and boz expression is expanded at 30% epiboly (4.7 hours). Thus it seems that the action of vox and vent is not required to repress dorsal genes at the time of their initial activation. Soon after initiation of dorsal gene expression, however, the redundant action of vox and vent is required to prevent expansion of dorsal genes into ventrolateral territories.

Why are vox and vent needed to repress dorsal gene expression in the late blastula but not in the initial phase of dorsal gene expression? We propose that an inducer of dorsal genes is present in the ventral marginal region of the late blastula, when and where vox and vent are required, but not at the midblastula period, when chd and gsc are dorsally restricted even in the absence of vox and vent function. β-catenin is a candidate for such an inducer. After its early role in development of dorsal axial structures (Heasman et al., 1994), β-catenin is required for development of ventral structures during gastrulation (Pelegri and Maischein, 1998; Heasman et al., 2000). In zebrafish, β-catenin begins to accumulate in ventrolateral marginal nuclei in the late blastula stage (Warga, 1996), approximately coinciding with the onset of the requirement for vox and vent. Thus vox and vent may prevent ectopic activation of dorsal gene expression by β-catenin acting in the ventral region of the late blastula. It is also likely that Vox and Vent counteract other activators of dorsal gene expression, including the Nodal-related signals Squint and Cyclops (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998). Like β-catenin, Nodal-related signals are potent inducers of chd and gsc expression in
overexpression assays. squint and cyclops are expressed around the circumference of the margin, where they induce mesendodermal formation, in the late blastula but not at earlier stages. Thus, Vox and Vent may be required to prevent Nodal-related signals from ectopically activating dorsal genes during mesendodermal induction. This model predicts that the Vox/Vent repressors counteract the effects of transcriptional mediators of Nodal signals and/or β-catenin, thereby restricting expression of genes such as gsc to the dorsal marginal region.

Conclusions

In summary, our results support the following model (Fig. 10) of the interactions among genes that establish dorsoventral asymmetry in zebrafish. The maternal dorsal determinant β-catenin activates expression of boz, which in turn represses vox, vent, and bmp2b in dorsal cells. vox and vent have redundant activities that repress genes characteristic of dorsal mesoderm, including chd and gsc. Zygotic BMP signals are not required for the repression of the dorsalmost mesendodermal fates, but these signals are essential activators of ventral genes. Chordin antagonizes BMP signals, thereby inhibiting a positive feedback loop between vox/vent and bmp2b during gastrulation. Biochemical characterization of the target genes of Vox, Vent and Boz will elucidate the mechanisms by which the antagonistic activities of these genes establish the dorsoventral axis in the zebrafish embryo.

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