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bozozok directly represses *bmp2b* transcription and mediates the earliest dorsoventral asymmetry of *bmp2b* expression in zebrafish

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SUMMARY

Formation of the gastrula organizer requires suppression of ventralizing signals and, in fish and frog, the need to counteract the effect of ubiquitously present maternal factors that activate the expression of Bmps. How the balance between dorsalizing and ventralizing factors is shifted towards organizer establishment at late blastula stages is not well understood. Mutations in zebrafish *bozozok* (*boz*) cause severe defects in axial mesoderm and anterior neurectoderm and affect organizer formation. The *boz* gene encodes the homeodomain protein Bozozok/Dharma and its expression in the region of the organizer is activated through β-catenin signaling. Here, we investigate the molecular mechanism by which *boz* contributes to the establishment of the organizer. We demonstrate that the homeodomain protein Boz acts as a transcriptional repressor in zebrafish: overexpression of an En-Boz fusion protein can rescue the *boz* phenotype, whereas a VP16-Boz fusion protein acts as an antimorph. Expression analysis of *bmp2b* indicates that Boz negatively regulates *bmp2b* in the prospective organizer. We demonstrate that this Boz activity is independent of that of other zygotic genes, because it also occurs when translation of zygotic genes is suppressed by cycloheximide (CHX). We identify two high-affinity binding sites for Boz within the first intron of the *bmp2b* gene. Deletion of these control elements abolishes Boz-dependent repression of *bmp2b* in the early blastula. Thus, Boz directly represses *bmp2b* by binding to control elements in the *bmp2b* locus. We propose that early transcriptional repression of *bmp2b* by Boz is one of the first steps toward formation of a stable organizer, whereas the later-acting Bmp antagonists (e.g. Chordin, Noggin) modulate Bmp activity in the gastrula to induce patterning along the dorsoventral axis. Thus, similar to *Drosophila* Dpp, asymmetry of Bmp expression in zebrafish is initiated at the transcriptional level, and the shape of the gradient and its function as a morphogen are later modulated by post-transcriptional mechanisms.

Key words: Zebrafish, Bmp, *bozozok*, Gastrula organizer, Dorsoventral pattern, Transcription repression

INTRODUCTION

The inhibition of Bmp/Dpp signals by Chordin/Sog and other antagonists is central to dorsoventral patterning mechanisms in animal development (Holley et al., 1995; Sasai et al., 1995; Marqués et al., 1997). In zebrafish, mutations in *bmp2b* (*Swirl*) (Kishimoto et al., 1997; Nguyen et al., 1998), *bmp7* (*Snailhouse*) (Dick et al., 2000; Schmid et al., 2000) and *smad5* (*Somitabun*) (Hild et al., 1999; Kramer et al., 2002) result in strong dorsalization, whereas mutations in *chordin* (*chd*) (Schulte-Merker et al., 1997) produce ventralized phenotypes. Thus, in zebrafish, Bmp2b and Bmp7 cooperate and ventralize the embryo by activating transforming growth factor β (TGFβ) family receptors and signaling via downstream Smads. Whereas *Dpp* expression in *Drosophila* is initiated in a spatially restricted dorsal domain, Bmp2/4 family proteins in vertebrates are initially expressed in a fairly ubiquitous manner throughout the blastoderm, as seen in *Xenopus* (*bmp4*) (Hemmati-Brivanlou and Thomsen, 1995) and zebrafish (*bmp2b* and *bmp7*) (Martinez-Barbera et al., 1997; Nikaido et al., 1997; Dick et al., 2000; Kramer et al., 2002). In *Drosophila*, the dorsally restricted expression of *Dpp* is established by transcriptional repression of *Dpp* by Dorsal on the ventral side of the embryo. In vertebrates, the mechanisms for the initial transcriptional repression of Bmp genes on the dorsal side of the embryo are not well understood. In late blastulae and early gastrulae, Bmp gene expression is limited dorsally by organizer-derived secreted factors such as Chordin and Noggin, which inhibit Bmp signaling and block the feedback loop by which Bmp maintains its own expression

(Onichtchouk et al., 1996; Kishimoto et al., 1997). In *Xenopus*, *chd* expression is directly repressed by the homeodomain-protein Vox, which is produced under the control of Bmp4 (Melby et al., 1999). Because Bmp signaling efficiently represses *chd* expression, it has remained unclear how a dorsal organizer is initiated at blastula stages in the presence of high levels of Bmp activity.

Mutations in *bozozok* (*boz*) affect proper establishment of the zebrafish gastrula organizer. *boz* mutant embryos lack axial mesoderm and have severe patterning defects in the anterior neuroectoderm (Solnica-Krezel et al., 1996; Solnica-Krezel and Driever, 2001). *boz* encodes a homeodomain protein also known as Dharma and Nieuwkoid (Koos and Ho, 1998; Yamanaka et al., 1998; Fekany et al., 1999). It is transcribed in dorsal blastomeres and in the dorsal yolk syncytial layer from the earliest zygotic gene expression until early gastrula (Yamanaka et al., 1998). Hyperdorsalization of zebrafish embryos by incubation in lithium chloride, which activates β-catenin signaling, results in expression of *boz* around the margin (Yamanaka et al., 1998). Active components of β-catenin signaling are required for *boz* expression (Shimizu et al., 2000), which is mediated through TCF/LEF binding sites in the *boz* promoter (Ryu et al., 2001). The function of *boz* is required for expression of *gsc* and many other organizer-specific genes (Fekany et al., 1999). Thus, *boz* might be directly activated by Wnt/β-catenin signaling and might directly mediate some activities of the Nieuwkoop center to establish the organizer. *boz* encodes a homeodomain protein and has been suggested to be involved in regulating expression of zygotic patterning genes acting both in the Nieuwkoop center and in the gastrula organizer. Recent studies indicate that *boz* is required for downregulation of *bmp2b* expression on the dorsal side (Koos and Ho, 1999) and it has also been suggested to interfere with Wnt signaling (Fekany-Lee et al., 2000) and Nodal signaling (Shimizu et al., 2000). However, the mechanism by which *boz* exerts its function at the crossroads of anterioposterior and dorsoventral patterning is not yet understood.

In this study, we investigate the role of *boz* in establishing dorsoventral polarity at the molecular level. We show that Boz acts as a transcriptional repressor, because fusion proteins containing the Boz homeodomain and the *Drosophila* Engrailed repressor domain can rescue the *boz* mutant phenotype. By contrast, a fusion of Boz with the VP16 transcriptional activator domain acts as a Boz antimorph, phenocopying the *boz* mutant phenotype and ventralizing the embryo. Repression of *bmp2b* does not require translation of zygotic gene products and thus must be a direct effect of Boz. We identify two high-affinity binding sites for Boz in the first intron of *bmp2b* and demonstrate by deletion analysis that the Boz-binding sites mediate *bmp2b* repression. We suggest a molecular pathway for initiation of dorsoventral asymmetry of *bmp2b* expression in which β-catenin signaling activates *boz*, which, in turn, represses *bmp2b* transcription in the prospective organizer.

MATERIALS AND METHODS

Genetic strains and phenotypic analysis

The zebrafish *boz*^{m168} allele (Solnica-Krezel et al., 1996) was crossed from its AB/Tü strain background into an India strain wild-type background, in which the *boz* phenotype shows higher penetrance and expressivity. Staging was performed according to Kimmel and colleagues (Kimmel et al., 1995). In situ hybridization was performed as described by Hauptmann (Hauptmann, 1999). Phenotypes of *boz* mutant embryos were classified according to Fekany et al. (Fekany et al., 1999) (Table 1).

Constructs and overexpression of mRNA in fish embryos

boz full-length cDNA was amplified by RT-PCR from 30% epiboly stage embryos, using primers 5′-ATACTCACGCAGCTTTTGGG and 5′-CAAAATGTTGGCATTATTCTGTC (Yamanaka et al., 1998) and subcloned into the pCS2+ expression vector. The *En-boz* construct was generated by recombinant PCR to fuse the Engrailed repressor domain (amino acids 1-296) to the homeodomain of Boz (amino acids 105-192). The *VP16-boz* construct was generated by recombinant PCR to fuse the VP16 activation domain (C-terminal 72 amino acids) to the homeodomain of Boz (amino acids 105-192). Boz(Δ-eh1) was generated by recombinant PCR to delete the eh1 motif (amino acids 9-15). Boz(HD) was generated by subcloning the C-terminal amino acids 105-192 with the homeodomain into pCS2+. Capped sense RNA was synthesized using SP6 RNA polymerase and the mMESSAGE mMACHINE system (Ambion) after *Not*I digestion in pCS2+.

For CHX treatment (modified from Gard et al., 1990), embryos were injected with mRNA at the early one-cell stage, subsequently dechorionated using pronaseE (Sigma, 1 mg ml^{-1}) and incubated in 0.3× Danieau's medium. At 1.5 hours post-fertilization (hpf), the medium was replaced with 0.3× Danieau's medium containing 10 μg ml^{-1} CHX (Calbiochem, diluted from 100 mg ml^{-1} stock in ethanol). Embryos were maintained in CHX until control embryos had reached sphere stage and then fixed for whole-mount in situ hybridization.

bmp2b regulatory region

Genomic PAC clones containing *bmp2b* were isolated by filter hybridization of a zebrafish genomic library (RZPD, Berlin) with a *bmp2b* cDNA probe. The 3.5 kb upstream of the *bmp2b* coding region, including the first intron, were sequenced by primer walking. The

Table 1. Rescue of *boz* mutant phenotype by *En-boz* mRNA

	Amount of mRNA	Number of embryos	Percentage of embryos in phenotypic class							
			Wild type	I	II	III	IV	V	Dorsalized	Other defects*
Control	Non-injected	1072	73.2	13.0	5.4	3.4	5.0	–	–	–
En-boz	0.05 pg	461	79.4	6.3	3.0	3.6	7.4	0.7	–	–
En-boz	0.25 pg	373	76.7	0.8	0.8	1.3	3.2	1.3	14.2	1.6

Varying amounts of *En-boz* mRNA were injected at the one-cell stage into embryos from crosses of *boz* heterozygous parents. Phenotypes were assessed after 1 day of development. Twenty-five percent of the progeny would be expected to develop *boz* mutant phenotypes, and 75% to develop as wild types.

Phenotypic classes: class I (most severe; absence of eyes, most of the forebrain and the notochord); class II (cyclopia with lack of notochord and fused somites); class III (partially fused eyes, truncated notochord in the trunk and fused somites); class IV (head is normal but the notochord has gaps spanning several somites in the trunk); class V (small breaks in notochord).

*Other defects here predominantly include partially duplicated axis.

transcription start site was determined by primer extension analysis (MMLV reverse transcriptase, Ambion. *bmp2b* primer: 5′-TTCC-CGTCGTCTCCTAAGTTC-3′).

Electrophoretic mobility shift assay

The Boz bacterial expression construct was generated by subcloning the *boz* full-length cDNA into the 6×His-tagged bacterial expression vector pET15b (Novagen), followed by transformation into BL21(DE3pLysS) strain bacteria. Bacterial recombinant Boz protein was induced by IPTG at 25°C for 4 hours and then isolated and purified over a Talon (Clontech) affinity column according to the manufacturer's instructions.

For each of the double-stranded oligonucleotides used, one of the two strands was ^{32}P end-labeled by the T4 kinase reaction or by Klenow fill-in reaction. Probe (10,000 cpm per 10 fmoles) was incubated with 0.1 μg of purified Boz protein in the presence of 0.76 μg of poly d(I-C) in binding buffer (20 mM HEPES, 50 mM EDTA, 5 mM $MgCl_2$, 5% glycerol, 1 mM DTT, pH 8) in a total volume of 10 μl as described (Brannon et al., 1997). Samples were incubated on ice for 20 minutes, followed by a further 20-minute incubation with the radiolabeled probe at room temperature. Electrophoresis was performed in 4% polyacrylamide gel with 0.25× TBE buffer at room temperature.

For the analysis of the *bmp2b* promoter, 3.5 kb of upstream region was sequenced and seven ^{32}P end-labeled DNA fragments (F1, –807 to –445; F2, –466 to –124; F3, –151 to +214; F4, +280 to +901; F5, +880 to +1494; F6, +1470 to +1741; F7, +1723 to +2343) were generated by PCR to cover almost the entire region. The fragments F7 and F4 were each digested by two separate sets of enzymes to generate overlapping sets of smaller fragments: (1) *Alu*I (for F7) or *Mbo*I and *Sau*3AI (for F4), followed by radiolabeling with Klenow; (2) *Mse*I digestion, ligation of *Mse*I adapters to the fragments and amplification of the ligated fragments by PCR using end-labeled *Mse*I adapter oligonucleotides. The radiolabeled *Mse*I PCR fragments were confirmed by electrophoretic mobility shift assay (data not shown). Double-stranded oligonucleotides were synthesized to verify binding sites: B1 (+1980 to +2033: 5′-GGTAAGTAAAATAATCTTATT-TCAAACTAAAAGCAAGATTATTTTACTCACCAA), B2 (+1470 to +1494: 5′-AAAGCAAGATTAGTTTACTGGCTTG), B3 (+537 to +565: 5′-GCGTGCCTGCATGTAATGTGTGAGGTCAG), B4 (+430 to +467: 5′-GCATTCAATTACGTGCTTGATATTACGTATTAGC-AAAC), B5 (+343 to +369: 5′-TCGCTTGTGGATTAAAACAC-GAATTCA), B6 (–151 to –124: 5′-GTATTGCGTATACATTA-CATTCTCGTTC) and B7 (–786 to –759: 5′-GATGTAAAGTCA-GAATTATTAGCCCCCT). Bicoid binding site double-stranded oligonucleotide: (5′-GTCACCTCTGCCCATCTAATCCCTTGAC-GC) from the *hunchback* gene (Driever et al., 1989). Underlined nucleotides indicate potential Bicoid-type homeodomain binding sites.

Luciferase assay of promoter activity

The Bmp2b-pLUC promoter fusion (*bmp2b* –806 to +2538) was generated by subcloning the *bmp2b* fragment at the *Bam*HI and *Hin*dIII sites into the pLUC vector (a luciferase derivative of pBL-CAT6 provided by J. Altschmied, Würzburg) (Boshart et al., 1992). Promoter fusions that have the binding sites B1 and /or B4 deleted were constructed by PCR-based subcloning. BmpΔ1pLUC has a deletion (basepairs +1983 to +2030) spanning site B1, BmpΔ4pLUC has a deletion (basepairs +432 to +490) spanning site B4 and BmpΔ1,4pLUC has both of these sites deleted but still contains the sequence +491 to +1982 of the first intron.

Embryos were harvested within 5 minutes of fertilization and the chorions were removed with pronase E (Sigma, 1 mg/ml). The embryos were co-injected with linearized pLUC reporter DNA derivatives and mRNA during early one-cell stage. The embryos were then incubated at 28.5°C for 5 hours. Ten microfuge tubes of ten embryos were collected for each experimental condition, the supernatant was aspirated and the embryos were frozen at –80°C immediately after addition of 80 μl lysis buffer (0.1 M sodium phosphate buffer pH 7.3, 1 mM DTT, 0.2% Triton-X100, 0.2 mM AEBSF [4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride; Sigma]. For the luciferase assays, embryos were thawed and dissolved by repeated pipetting, and the lysate cleared by centrifuging 10 minutes at 16,000 ***g*** (4°C). 60 μl of supernatant were transferred to 96-well microtiter plates on ice. 350 μl of reaction buffer (22.5 mM glycylglycin pH 7.8, 2 mM ATP, 10 mM $MgSO_4$) were mixed (vortex) with 50 μl embryo extract in luminometer tubes and the reaction was started by injection of 0.2 mM D-luciferin in 20 mM glyclglycin pH 7.8. Measurements were recorded using a Lumat LB9501 (Berthold). Each experiment (ten measurements of ten embryos each) was performed at least in triplicate and the standard deviation was calculated.

RESULTS

Boz acts as a transcriptional repressor during zebrafish organizer formation

The severe dorsoanterior patterning defects in *boz* mutant embryos (Solnica-Krezel et al., 1996; Fekany et al., 1999) could be caused by lack of activation of dorsoanterior genes, by lack of repression of ventralizing genes or by a combination of both. We assayed Boz for its potential effects on transcription in two heterologous systems, yeast and *Drosophila* (see supplemental data S1 and S2 at http://dev.biologists.org/supplemental/). In both assay systems, Boz or Boz fusion proteins did not appear to be activators of target genes but rather affected target gene expression as repressors or as competitors of endogenous activators.

To determine whether Boz functions as a transcriptional repressor in zebrafish, we analysed the effects of repressor and activator fusion constructs on embryonic axis formation. We created a fusion of the Engrailed (En) repressor domain (amino acids 1-296) (Han and Manley, 1993) and the homeodomain of Boz (amino acids 105-192) (Fig. 1). Overexpression by microinjection of 2 pg *En-boz* synthetic mRNA at the one-cell stage induced dorsalization (Fig. 1A-D), which, at 24 hpf, is similar to that resulting from overexpression of wild-type *boz* (Yamanaka et al., 1998). Injection of 0.05-0.25 pg of *En-boz* mRNA into progeny of *boz* heterozygous parents provides some phenotypic rescue, shifting the distribution of *boz* phenotypic classes from severe class I and II mutant phenotypes to the less severe classes IV and V (Table 1). We observed a reduction in the penetrance of the mutant phenotype, indicating complete rescue in some cases. In addition, at the higher concentration, a large proportion of embryos become dorsalized, resembling *snailhouse* or *piggytail* phenotypes (Mullins et al., 1996). This dorsalizing activity occurs during early patterning. Overexpression of either *En-boz* (Fig. 2E,F,M,N) or *boz* mRNA (Fig. 2C,D,K,L) induced ectopic *chd* and *gsc* expression in 50% epiboly stage wild-type embryos. Thus, the En-Boz fusion protein has activities similar to wild-type Boz: overexpression of either *En-boz* or *boz* mRNA can rescue *boz* mutant phenotypes and hyperdorsalize wild-type embryos.

The N-terminus of Boz contains an eh1 motif (FSIDYIL; see supplemental data S1 at http://dev.biologists.org/supplemental/) that is conserved among genes homologous to *Engrailed* (Koos and Ho, 1999). The eh1 motif is also present

in other classes of homeodomain proteins that have been shown to act as repressors, such as Gsc (Ferreiro et al., 1998), Hex (Ho et al., 1999), Xvent1 (Friedle et al., 1998; Rastegar et al., 1999), Vox (Melby et al., 1999), Xanf1 (Ermakova et al., 1999) and Xvent2 (Trindade et al., 1999). To investigate the function of this motif we created a Boz variant with the eh1 motif deleted, Boz(Δ-eh1). When injected into one-cell stage embryos, *boz(Δ-eh1)* mRNA lacks dorsalizing activity, even when as much as 25 pg mRNA were injected (data not shown). Thus, the eh1 motif is essential for the dorsalizing activity of Boz in zebrafish embryos. A Boz derivative lacking the homeodomain, Boz(Δ-HD), has been reported to show no biological activity in zebrafish embryos (Koos and Ho, 1999). We overexpressed the Boz homeodomain region alone and could not induce abnormal development (amino acids 105-192; up to 25 pg mRNA injected; data not shown). Thus, the endogenous activity of Boz might be exclusively mediated through the eh-1 domain and the DNA binding homeobox domain.

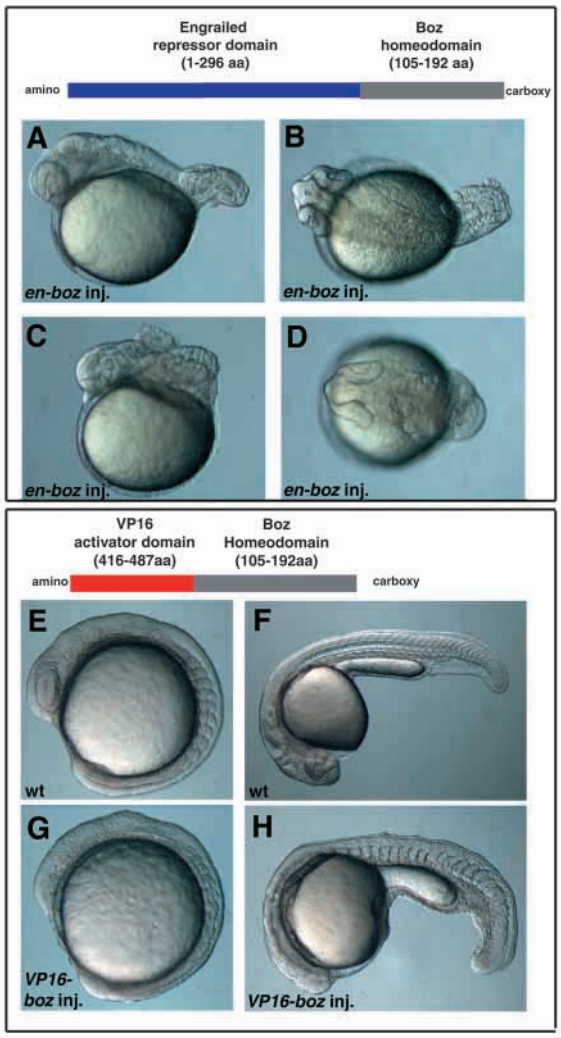


Fig. 1. A fusion of the Boz-homeodomain with the En repressor domain causes dorsalization, whereas Boz fused to the VP16 activator ventralizes developing embryos. (A-D) An En-Boz repressor domain fusion protein dorsalizes the zebrafish embryo. Top: structure of the En-Boz fusion protein. (A-D) Embryos injected at the one-cell stage with 2 pg *En-boz* mRNA. Lateral views (A,C) and dorsal views (B,D) of live embryos at 24 hpf. Injected embryos develop a severely dorsalized phenotype. For comparison, see wild-type embryo of same age in (F). (E-H) A VP16-Boz activator-domain fusion protein ventralizes the zebrafish embryo. Top: structure of the VP16-Boz fusion protein. Wild-type embryos at 12-somite stage (E) and 24 hpf (F). (G,H) Embryos were injected at the one-cell stage with 5 pg *VP16-boz* mRNA. Lateral view at the 12-somite stage (G) and at 24 hpf (H). Injected embryos lack axial mesoderm and develop strong anterior patterning defects including absence of the eye cup (12 som) or eye (24 hpf) and reduced size of the diencephalon.

VP16-Boz fusion protein functions as a Boz antimorph

We created the transcriptional activator fusion construct *VP16-boz* by fusing the VP16 activator domain (C-terminal 72 amino acids; Sadowski et al., 1988) to the Boz homeodomain (amino acids 105-192; Fig. 1). Injection of 5 pg *VP16-boz* mRNA into one-cell-stage embryos produced the full range of *boz* mutant phenotypes (Fig. 1G,H). Increasing amounts of injected *VP16-boz* mRNA resulted in a gradual increase in the incidence of severe *boz* mutant phenotypes (Table 2). In addition, some embryos were ventralized, showing an expansion of ventral fates such as the blood-forming region and enlarged ventral tail fins. Consistent with the morphological defects, expression of the organizer specific genes *gsc* and *chd* was reduced in *VP16-boz* injected wild-type embryos at 50% epiboly (Fig. 2G,H,O,P). Taken together, these data suggest that the VP16-Boz fusion protein acts like a *boz* antimorph, induces ventralization and phenocopies the *boz* mutant phenotype. As VP16 fusion proteins cause increased levels of transcription of target genes, our finding of diminished expression of *chd* and *gsc* indicates that VP16-Boz only indirectly affects these genes. We postulate that VP16-Boz activates expression of ventral factors, which in turn repress *chd* and *gsc* transcription.

Regulation of *bmp2b* expression by *boz*

boz RNA can dorsalize zebrafish embryos, and so we determined the effects of *boz*, *En-boz* and *VP16-boz* on *bmp2b* expression. Wild-type gastrula stage embryos express a gradient of *bmp2b* mRNA, with the highest concentration at the ventral side (Fig. 3A,B). Injections of 2 pg of synthetic *boz* or *En-boz* mRNA into one-cell-stage wild-type embryos almost completely abolished *bmp2b* expression at 50% epiboly (Fig. 3C-F). To determine whether *boz* influences initiation of *bmp2b* expression, we analysed embryos at sphere stage, when zygotic *bmp2b* expression begins. Overexpression of *boz* caused a dramatic reduction of *bmp2b* expression at sphere stage (Fig. 3J,K) but did not change the expression level of *chd* (Fig. 3I). These findings indicate that Boz might directly repress *bmp2b* but does not directly affect the expression of *chd*. Following overexpression of *boz*, we could not detect any changes in the expression of *bmp7* at sphere stage (data not shown). If *boz* directly represses *bmp2b* then *VP16-boz* might activate it. Indeed, we found that overexpression of *VP16-boz* clearly enhances *bmp2b* expression by sphere stage (Fig. 3G,H).

To further analyse the function of *boz* in *bmp2b* regulation during early development, we compared *bmp2b* expression at sphere stage in wild-type and *boz* mutant embryos. In wild-

Table 2. *VP16-boz* mRNA overexpression ventralizes wild-type embryos

	Amount of mRNA (pg)	Number of embryos	Percentage of embryos in phenotypic class							
			Wild type	I	II	III	IV	V	Ventralized	Other defects*
VP16-boz	10	526	62.4	–	–	0.6	4.4	3	27.4	2.3
VP16-boz	25	605	54	0.7	0.7	1.2	8	2	32.6	0.8
VP16-boz	50	479	37.6	3.1	2.1	3.5	10.9	2.1	37.6	11.7

Varying amounts of *VP16-boz* mRNA were injected at the one-cell stage into wild-type embryos. Phenotypes were assessed after 1 day of development. For *boz* phenotypic classes, see Table 1.

*Other defects here include predominantly single-eyed embryos.

type embryos, *bmp2b* expression is ubiquitously initiated in the blastoderm, except for a small domain at the dorsal blastoderm margin (Fig. 3L,N). The early dorsal repression does not depend on *chd* function (Hild et al., 1999). In *boz* mutants, the repression of *bmp2b* expression in this dorsal domain is abolished (Fig. 3M,O) (see also Koos and Ho, 1999). This early *bmp2b* expression phenotype is found in all *boz* mutant embryos, although they later exhibit highly variable morphological phenotypes (classes I-V; Table 1) (Fekany et al., 1999). In crosses between heterozygous *boz* parents, one-quarter of the progeny lack repression of *bmp2b* at the dorsal margin between sphere stage and 30% epiboly. Taken together, these findings suggest that *boz* functions to repress *bmp2b* during the initial phase of its expression from sphere stage to 30% epiboly.

The complexity of dorsoventral patterning interactions could still make the interaction between *boz* and *bmp2b* an indirect one, by Boz repressing an activator of zygotic *bmp2b* transcription. To investigate whether other early zygotic genes contribute to the effect of Boz on *bmp2b* transcription, we inhibited translation of zygotically expressed mRNAs with CHX by incubating embryos from 1.5 hpf onwards in 10 μg ml^{-1} CHX. In this experiment, maternal and injected mRNAs are translated during the first 90 minutes before CHX is added but zygotic transcripts generated after the mid-blastula transition are not translated in the presence of CHX. At this CHX concentration, we find that *gsc* expression is not detectable and thus depends on the presence of other zygotic gene products (Fig. 4B,H). *bmp2b* is not affected by CHX and is thus activated by maternal factors (Fig. 4A,G). Repression of *bmp2b* transcription by overexpression of *boz* is not affected by CHX (Fig. 4C,I). Similarly, activation of *bmp2b* expression by overexpression of *boz-VP16* also occurs in the presence of CHX (Fig. 4E,K). This demonstrates that zygotic gene products are not required to mediate the repressive effect of Boz on *bmp2b* transcription. Further, our data on *gsc*

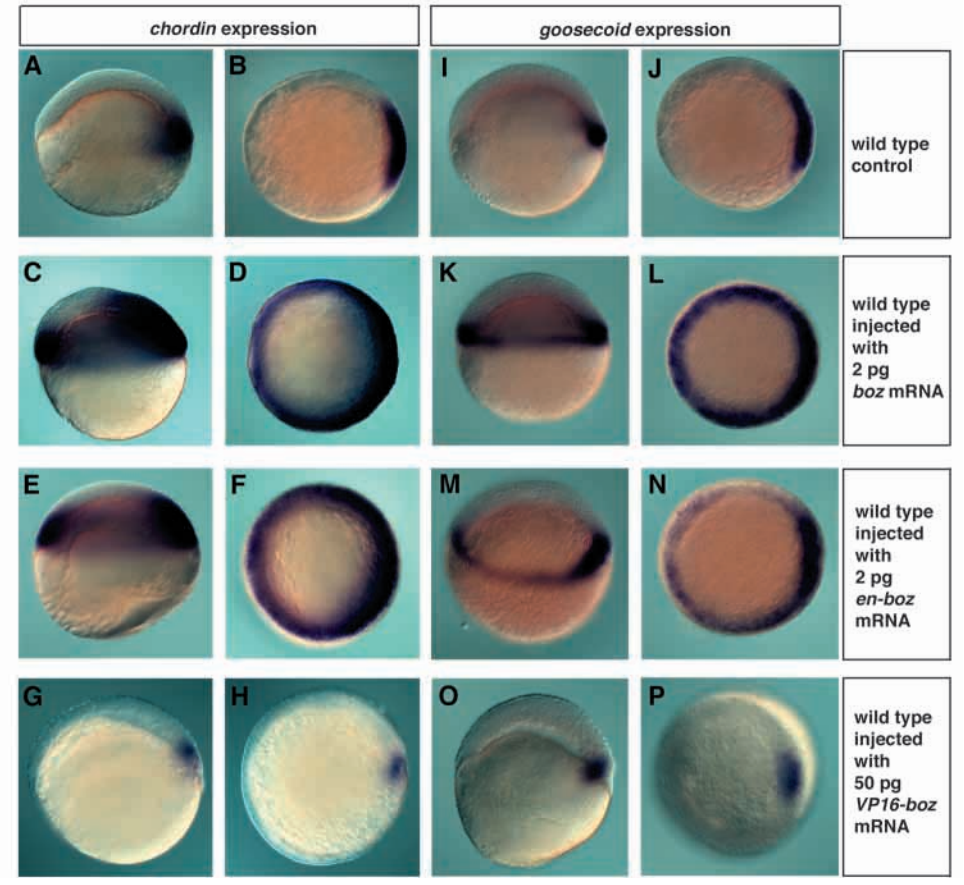


Fig. 2. Effect of En-Boz repressor and VP16-Boz activator fusion proteins on *gsc* and *chd* expression. Wild-type embryos were injected with in vitro transcribed mRNA at the one-cell stage and allowed to develop to 50% epiboly. Expression of *chd* (A-H) or *gsc* (I-P) was visualized by in situ hybridization. (A,B,I,J) Uninjected wild-type embryos. *chd* and *gsc* are restricted to the dorsal margin of the embryo where the shield will form. (C,D,K,L) Embryos injected with 2 pg *boz* mRNA. *chd* and *gsc* expression expands to the ventral side of the embryo. (E,F,M,N) Embryos injected with 2 pg *En-boz* mRNA. Similarly, *chd* and *gsc* expression expand to the ventral side of the embryo. (G,H,O,P) Embryos injected with 50 pg *VP16-boz* mRNA. Expression of both *chd* and *gsc* at the dorsal margin is strongly reduced. (A,C,E,G,I,K,M,O, lateral views, dorsal at right, animal pole at top; B,D,F,H,J,L,N,P, animal pole views, dorsal at right).

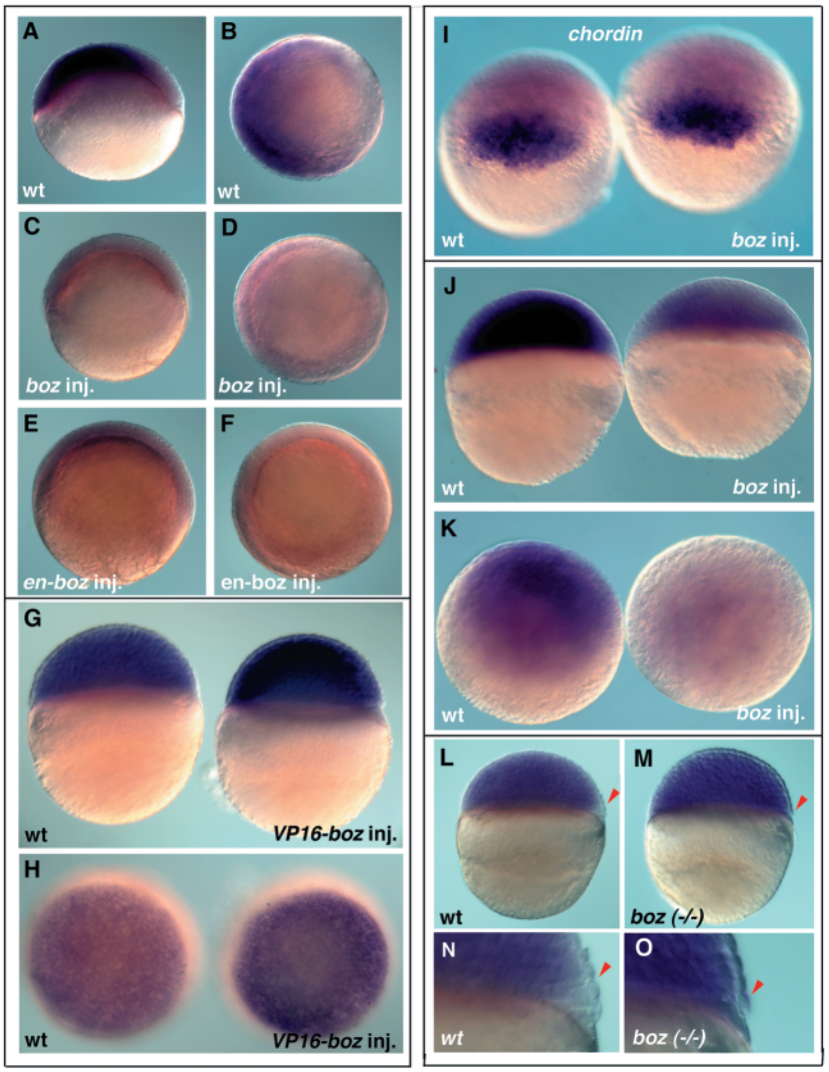


Fig. 3. Control of *bmp2b* expression by Boz, En-Boz and VP16-Boz. (A-F) Boz and En-Boz can repress *bmp2b* expression early during gastrulation. Expression of *bmp2b* at 50% epiboly in wild-type embryos (A,B), embryos injected with 2 pg *boz* mRNA (C,D) and embryos injected with 2 pg *En-boz* mRNA (E,F). (G,H) Embryos injected with 50 pg *VP16-boz* mRNA (right) express increased levels of *bmp2b* at sphere stage compared with uninjected controls (left). (I) Expression of *chd* at sphere stage is not affected in embryos injected with 2 pg *boz* mRNA (right) compared with the uninjected control (left). (J,K) Expression of *bmp2b* at the sphere stage is severely reduced in embryos injected with 2 pg *boz* mRNA (right) compared with the uninjected controls (left). (L-O) Comparison of *bmp2b* expression in wild-type (L,N) and *boz* mutant embryos (M,O) at sphere stage (red arrowheads mark dorsal margin in L and N, margin in M and O). A,C,E,G,J,L,M: lateral views; B,D,F,H,K: animal pole views; A-H,J-M, dorsal side to the right when the dorsal side could be visually identified; I is an oblique view onto the animal pole from the dorsal side onto the prospective shield region. N and O are enlargements of the dorsal or marginal regions of the embryos pictured in L and M, respectively.

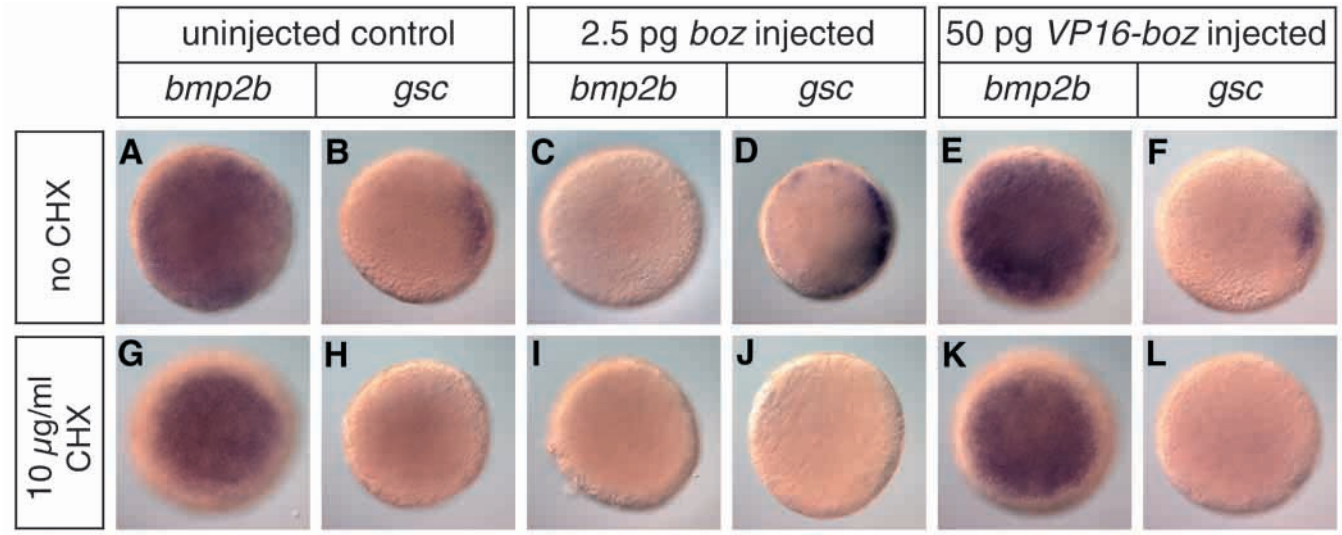


Fig. 4. The effect of Boz on *bmp2b* transcription is independent of other zygotic gene expression. Expression of *bmp2b* and *gsc* at sphere stage in wild-type control embryos (A-F) and in embryos treated with cycloheximide (CHX) between 1.5 hpf and fixation at sphere stage (G-L). Expression of *bmp2b* (A,G) and *gsc* (B,H) in uninjected embryos. Expression of *bmp2b* (C,I) and *gsc* (D,J) in embryos injected with 2.5 pg *boz* mRNA. Expression of *bmp2b* (E,K) and *gsc* (F,L) in embryos injected with 50 pg *VP16-boz* mRNA. *gsc* expression is influenced by *boz* already at sphere stage (D) and is also used here as a control to determine the effectiveness of cycloheximide treatment (H,J,L).

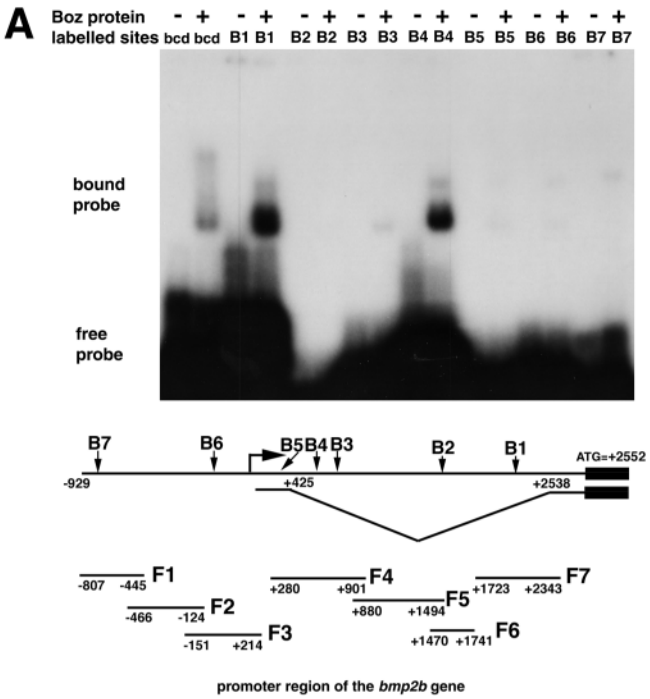
expression (Fig. 4D,J,F,L) demonstrate that the effect of Boz on *gsc* requires the expression of zygotic proteins.

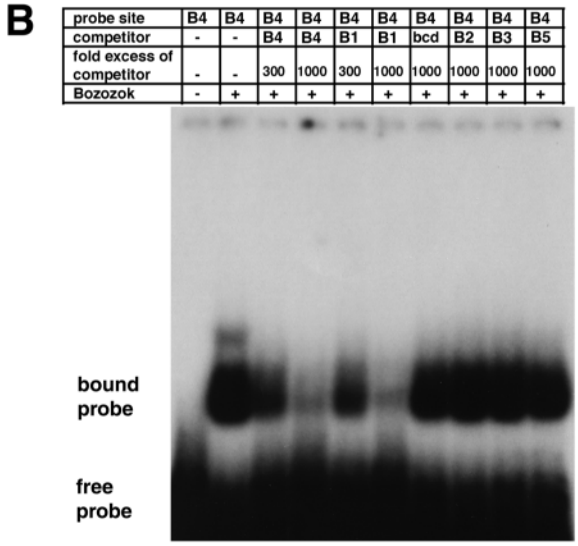
Boz binds to regulatory elements in *bmp2b*

To determine whether *boz* affects *bmp2b* expression by directly binding to the *bmp2b* regulatory region, we characterized the genomic organization of *bmp2b* (Fig. 5A). Primer extension analysis revealed the putative transcriptional start site 2551 bp upstream of the ATG (data not shown) and we identified a 2.1 kb intron 15 bp upstream of the start codon. Seven PCR fragments were generated to cover the 3.5 kb region upstream of the ATG (Fig. 5A) and these were subjected to DNA electrophoretic mobility shift assays (EMSA). Two DNA fragments (F4, +280 to +901; and F7, +1723 to +2343) showed changes in electrophoretic mobility in the presence of Boz, indicating specific binding (data not shown). These large fragments were further digested into sets of smaller fragments to identify Boz binding sites by EMSA (data not shown) and two smaller fragments (B1, +1980 to +2033; B4, +430 to +467) were identified that were strongly bound by Boz. Five other sites containing a potential Bicoid-class homeodomain binding motif (C)TAAT(C) were identified in the promoter and analysed by EMSA (double-stranded oligonucleotides B2, B3, B5, B6, B7; Fig. 5A) but they were bound only very weakly or not at all by Boz. Only the B1 and B4 double-stranded oligonucleotides showed specific binding to recombinant Boz protein (Fig. 5B,C). The binding of Boz to the radiolabeled double-stranded oligonucleotides B1 and B4 could be specifically competed by either B4 or B1 competitor oligonucleotides, but not efficiently by the Bicoid-site, B2, B3 or B5. The specific binding of Boz to B1 and B4 sites in *bmp2b* supports the hypothesis that *bmp2b* is a direct target of the transcriptional repressor Boz.

Boz binding sites in *bmp2b* confer Boz-dependent repression of *bmp2b*

To investigate whether the B1 and B4 sites mediate Boz-dependent *bmp2b* repression in vivo, we performed luciferase reporter assays in blastula stage embryos. A fusion of the luciferase reporter (BmppLUC) with 3.5 kb of sequences 5′ of the Bmp2b ATG, including the first intron and 929 bp upstream of the transcription start site, was injected into one-cell-stage embryos and found to drive expression of luciferase after mid-blastula transition. Co-injection of BmppLUC with *boz* mRNA or *En-boz* mRNA resulted each in about 6.5-times repression, whereas co-injection with *VP16boz* mRNA resulted in 8.8-





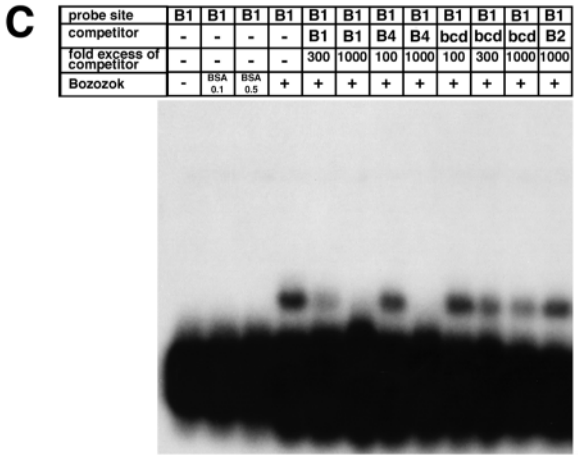


Fig. 5. Boz binds to control elements in the zebrafish *bmp2b* gene. (A) Several different end-labeled PCR fragments (F1-F7) covering 3.5 kb of *bmp2b* genomic DNA, including the first intron, were generated and analysed for the binding of Boz. Further digests of the fragments identified potential binding sites B1-B7, for which electrophoretic mobility shift assays are shown. Only B1 and B4 show efficient Boz binding. The leftmost lanes reveal shift of double-stranded oligonucleotides containing the Bicoid (Bcd) consensus binding site in the presence of Boz. (B,C) The specificity of binding was analysed using various competitor double-stranded oligonucleotides. Putative Boz binding sites B1 and B4 successfully compete both their own and each other's binding to Boz. By contrast, neither oligonucleotide with a Bcd consensus site nor extra *bmp2b* sites containing TAAT motifs (B2, B3 or B5) can efficiently compete for Boz binding to sites B1 or B4.

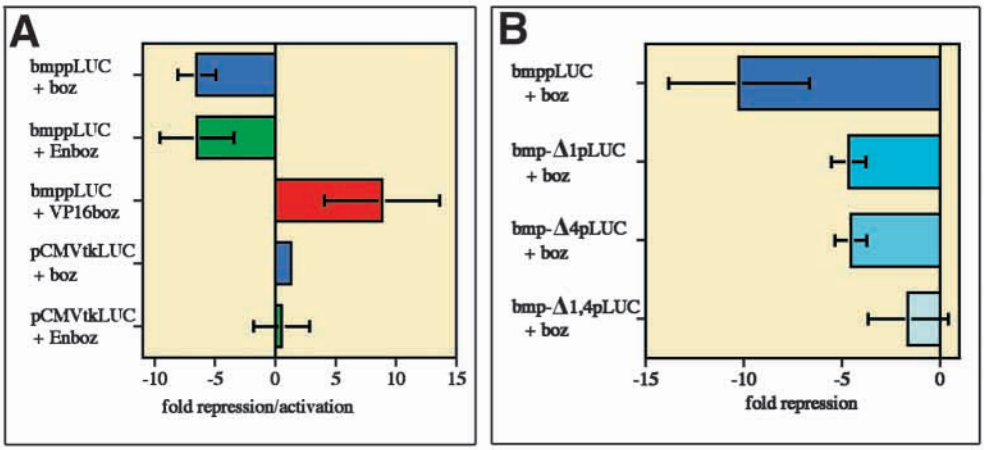


Fig. 6. Binding to sites B1 and B4 mediates Boz-dependent repression of *bmp2b*. Luciferase reporter fusion constructs containing *bmp2b* sequences from –806 to +2538 fused to the luciferase reporter (BmppLUC) or a control (pCMVtkLUC) were injected into wild-type embryos at the one-cell stage and the amount of luciferase activity was determined at 5 hpf (30% epiboly). The amount of luciferase activity recorded for BmppLUC itself was set as 1. (A) Coinjection of BmppLUC (20 ng μl^{-1}) with *boz* mRNA or *Enboz* mRNA (7 ng μl^{-1}) resulted in about 6.5-times repression, whereas co-injection of BmppLUC (5 ng μl^{-1}) with *VP16boz* mRNA (5 ng μl^{-1}) showed roughly 8.8-times activation. Coinjection of pCMVtkLUC (20 ng μl^{-1}) with *boz* mRNA or *En-boz* mRNA (7 ng μl^{-1}) did not have a significant effect on the expression of LUC from the pCMVtkLUC control plasmid. (B) In a separate set of experiments, the contribution of the Boz binding sites B1 and B4 to Boz-dependent repression of BmppLUC was investigated. When injected at a concentration of 20 ng μl^{-1} together with *boz* mRNA (7 ng μl^{-1}), BmppLUC showed 10.2-times repression, B1 deletion (BmpΔ1pLUC) showed 4.6-times repression, B4 deletion (BmpΔ4pLUC) showed 4.5-times repression and deletions of both B1 and B4 (BmpΔ1,4pLUC) showed only 1.6-times repression. Thus, most of the Boz-dependent repression of *bmp2b* appears to be mediated by the sites B1 and B4.

times increase in luciferase activity (Fig. 6A). Neither *boz* mRNA nor *En-boz* mRNA has a significant effect on the expression of LUC from the pCMVtkLUC control plasmid (Fig. 6A). In a separate set of experiments, deletions of Boz binding sites B1 (BmpΔ1pLUC; 4.6-times repression) or B4 (BmpΔ4pLUC; 4.5-times repression) each reduced the repression observed with BmppLUC (10.2-times repression) by more than half (Fig. 6B). Deletion of both B1 and B4 (BmpΔ1,4pLUC; 1.6-times repression) reduced the repression six times to levels within the error margin of control injections. Thus, we show that Boz can repress *bmp2b* transcription directly by binding to sites B1 and B4 within the first intron of *bmp2b*.

DISCUSSION

Boz acts as a transcriptional repressor in organizer development

The N-terminus of Boz contains an eh1 motif, which is conserved among *Engrailed* genes and contributes substantially to the ability of Engrailed to repress transcription in *Drosophila* (Smith and Jaynes, 1996; Koos and Ho, 1998). We tested the functional significance of the eh1 motif in Boz and found that its deletion completely abolished the dorsalizing activity of Boz. We compared the activity of Boz with the activity of fusion proteins in which the Boz homeodomain is fused to the Engrailed repressor domain or the VP16 activator domain. The En-Boz repressor fusion mimicked the endogenous activity of Boz. En-Boz caused dorsalization with activation of the organizer genes *gsc* and *chd*, and, significantly, could rescue the *boz* mutant phenotype. By contrast, overexpression of the VP16-Boz activator fusion phenocopied *boz* mutant defects including downregulation of the dorsal organizer genes and resulted in ventralization of the embryo (Shimizu et al., 2002). These data support the hypothesis that Boz acts as a transcriptional repressor required for proper organizer formation. How does the repressor activity of Boz promote organizer formation and expression of organizer-specific genes? Because the removal of Bmp activity from the dorsal side of the embryo is crucial for dorsal development, we hypothesize that *bmp2b* is a direct target of Boz repression.

Transcriptional repression of *bmp2b* in the nascent organizer by Boz

Bmp2 and Bmp4 function as instructive signals that determine positional identities along the dorsoventral axis (Dosch et al., 1997). Zebrafish *swirl*/*bmp2b* mutants (Kishimoto et al., 1997; Nguyen et al., 1998) share common phenotypic features with *bmp4* mutant mice (Winnier et al., 1995) and the *Swirl*/*bmp2b* expression pattern is similar to that of *Xenopus bmp4* (Nikaido et al., 1997). Thus, zebrafish *bmp2b* seems to be similar in function to mouse or *Xenopus bmp4*, playing essential roles in the establishment of the dorsoventral axis in these organisms. In zebrafish, *bmp2b* is initially ubiquitously expressed at sphere stage. Only a small domain at the dorsal margin is devoid of *bmp2b* expression. *bmp2b* expression becomes progressively restricted to the ventral region during late blastula and early gastrula stages to form the Bmp gradient (Nikaido et al., 1997). Expression in the yolk syncytial layer (YSL) is maintained during gastrulation. Although the initiation of *bmp2b* expression does not depend on its function, the maintenance of *bmp2b* expression from shield stage on requires functional Bmp2b protein and its downstream effector *Somitabun/Smad5* (Kishimoto et al., 1997; Hild et al., 1999; Kramer et al., 2002). Although the Bmp antagonist Chordin is required to form and maintain the dorsoventral gradient of *bmp2b* expression during gastrulation, *bmp2b* expression at sphere stage is normal in *chd* mutant embryos (Miller-Bertoglio et al., 1997). Likewise, *chd* expression is normal through early shield stage in the dorsalizing mutants *swr*, *sbn* and *snh*. In these mutants, *chd* expression expands in lateral and ventral directions only as epiboly proceeds (Miller-Bertoglio et al., 1997). The initiation of the dorsoventral asymmetry of *bmp2b* expression at sphere stage is thought to

depend on dorsally localized factor(s), but the exact mechanism by which *bmp2b* expression is initially blocked on the dorsal side was previously unclear.

Our data provide five lines of evidence that the earliest repression of *bmp2b* in the presumptive organizer is a direct activity of Boz at sphere stage.

(1) Although overexpression of *boz* or *En-boz* represses *bmp2b*, it does not affect *chd* expression at sphere stage. Thus, the repressor activity of Boz on *bmp2b* during sphere stage does not depend on Chordin.

(2) Overexpression of *VP16-boz* causes increased levels of *bmp2b* transcription from mid-blastula transition onward. Together with the reciprocal effect (repression) of *boz* or *En-boz* overexpression on *bmp2b*, this suggests a direct interaction.

(3) Boz exerts its repression of *bmp2b* even when translation of zygotic gene products is inhibited by CHX. Thus, the effect of Boz is not mediated through a relay mechanism, in which Boz would repress a zygotic activator of *bmp2b*.

(4) We have identified binding sites for Boz in the *bmp2b* control region. These Boz binding sites are located close to splice donor and splice acceptor sites of the first intron, a location in which repressor sites for eukaryotic gene regulation are frequently found.

(5) Deletion analysis and reporter assays in zebrafish embryos reveal that the two Boz binding sites in the *bmp2b* control region are required for normal levels of *bmp2b* repression.

Model for the role of *boz* during initiation of the organizer

Our data show that dorsoventral asymmetry of *bmp2b* expression is initiated at the level of transcription (Fig. 7). In the absence of *boz*, maternal factors like *Smad5/Sbn* (Kramer et al., 2002) lead to ubiquitous zygotic expression of *bmp2b*. The presence of *bmp2b* in the organizer region of *boz* mutants interferes with the establishment of the organizer because Bmp2b mediates repression of *chd* and other organizer genes. In the presence of Boz, a *bmp2b*-free dorsal marginal zone is generated, which promotes proper organizer formation through other targets of β-catenin/Tcf/Lef signaling. The variable expressivity of the *boz* mutant phenotype can be understood as the result of the competition between Nieuwkoop-center signaling activity to establish the organizer and Bmp2b-mediated repression of organizer genes. This competition might have a variable outcome, as reflected by the variable penetrance and expressivity of the *boz* mutant phenotype (Fekany et al., 1999): occasionally, β-catenin signaling is strong enough to induce a near-normal organizer even in the absence of functional Boz. More often, *bmp2b* repression by Boz is required to permit the formation of a potent organizer. Thus, the Nieuwkoop center itself is involved in two activities: (1) induction of organizer genes (e.g. *chd*, *noggin* and *gsc*); and (2) direct repression of ventralizing signals at the dorsal margin.

Boz thus is a component of a complex network of negative regulatory interactions between dorsalizing and ventralizing activities. It was previously shown that mutual repression exists between Boz and the ventralizing transcription factors Vega1 (Vox) and Vega2 (Vent) (Kawahara et al., 2000a,b; Melby et al., 2000; Imai et al., 2001) as well as *ved* (Shimizu et al., 2002). Vega1 (Vox), Vega2 (Vent) and Ved also repress other dorsal genes, such as *gsc* and *chd*. Such multiple inhibitory interactions might be essential to control the extent of dorsal and ventral territories, which otherwise would predominantly depend on extracellular diffusion of the Bmp2b morphogen – a mechanism that might not be sufficient to generate stable borders of organizer gene expression.

Several questions remain. How can transcriptional repression of *bmp2b* by overexpression of *boz* explain the concurrent expansion of *gsc* and *chd* expression? Interestingly, following *boz* overexpression, *gsc* and *chd* expression expand to marginal and dorso-animal positions, but not to ventro-animal positions. We suggest that this ventrolateral expansion of *gsc* and *chd* is not a consequence of repressed *bmp2b* expression, because *gsc* and *chd* expression are normal in *bmp2b/swr* mutants during late blastula and early gastrula stages (Miller-Bertoglio et al., 1997; Mullins et al., 1996). Instead, we postulate that the expansion of *gsc* and *chd* results from the repression of additional *boz* target genes like *vega1* (*vox*) and *vega2* (*vent*), which are ventrolaterally expressed and can repress *gsc* and *chd* (Imai et al., 2001). In the zebrafish Df^{st7} mutant, which lacks both *vox* and *vent* loci, *chd* and *gsc* expression are ventrolaterally expanded (Imai et al., 2001). Following *boz*-mediated repression of *vox* and *vent*, and the elimination of their repressive effect on *chd* and *gsc*, β-catenin or its effectors might induce *chd* and *gsc* expression around the margin. The accumulation of β-catenin even at ventrolateral positions has been reported for late blastula and early gastrula stage zebrafish (R. Warga, Origin and specification of the endoderm in the zebrafish, *Danio rerio*. PhD thesis, Eberhardt-Karls-Universität Tübingen, Germany, 1996). Similarly, enhanced levels of nuclear translocation of β-catenin have

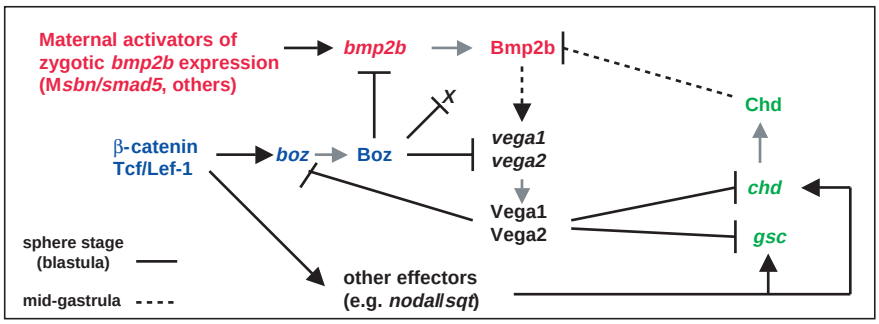


Fig. 7. Role of *boz* during early dorsoventral patterning. Simplified pathways of regulatory interactions. Solid lines represent interactions during blastula stages, dotted lines represent interactions the significance of which begins during mid-gastrulation. The interactions with *vega1* and *vega2* are modified from Imai et al. (Imai et al., 2001). Work by Kramer et al. (Kramer et al., 2002) indicates that maternal Smad5 is an ubiquitous activator of *bmp2b* expression. 'X' represents targets of Boz other than *bmp2b*, which might mediate the function of Boz in anterioposterior patterning. Other effectors of β-catenin signaling include the probable activators of *chd* expression not yet identified in zebrafish (i.e. functional homologs of *Xenopus Siamois* and *Twin*) as well as Nodal signals, which co-activate *gsc* through FAST/FoxH1 (Pogoda et al., 2000). Gene names are italicized, protein names begin with capital letters; translation is indicated by gray arrows. Black arrows indicate activation, whereas 'T' bars indicate repression.

been observed in *Xenopus* early gastrulae in a region extending to more lateral positions than those of *gsc* or *chd* expression (Schneider et al., 1996). Homologs of *Siamois* (Lemaire et al., 1995) and *Twin* (Laurent et al., 1997), which mediate the effect of β-catenin signaling to cause activation of organizer genes like *gsc* (Cho et al., 1991; Laurent et al., 1997) in *Xenopus*, have not yet been described for zebrafish.

Our findings correlate with data obtained from experiments in *Xenopus*, which indicate that inhibition of Bmp and Nodal, Bmp and Wnt signaling, or of Bmp, Nodal and Wnt pathways is sufficient for head induction (Glinka et al., 1997; Piccolo et al., 1999). Induction of neurectoderm in *Xenopus* can be promoted by Wnt/β-catenin signaling (Baker et al., 1999). This suggests that, in zebrafish, *boz* might serve as a downstream effector of Wnt signaling in this process. Early expression of *β-catenin* induces the expression of neural-specific markers and inhibits the expression of *bmp4* in *Xenopus* ectoderm. Further, Wnt, but not the Bmp antagonist Noggin, can inhibit *bmp4* expression at early gastrula stages. Boz could be the functional homolog of an as-yet-unidentified gene in *Xenopus* that mediates Wnt-signaling-dependent repression of *bmp4* expression.

The alteration in *bmp2b* expression in *boz* mutants from that of the wild type is not sufficient to explain the observed degree of anterioposterior and dorsoventral patterning defects. The *boz* mutants have less severe expansion of *bmp2b* expression than do *chd* mutants, but more severe dorsoventral patterning defects. Further, overexpression of zebrafish *dkk1*, a Wnt antagonist, can rescue anterior neural plate and axial mesoderm defects in *boz* mutant embryos (Hashimoto et al., 2000). Our finding that Boz acts as a repressor suggests that Boz might also directly interact with some component of the zygotic Wnt signaling pathway, that functions in the dorsal blastula and early gastrula.

From an evolutionary viewpoint, our findings point at potential similarities in the initiation of dorsoventral Bmp/Dpp asymmetry in both vertebrates and invertebrates. In *Drosophila*, transcriptional repression by Dorsal initiates dorsally restricted expression of the potent Dpp morphogen and our data show that an analogous strategy is applied to restrict the early source of Bmp2b locally in zebrafish. The later Dpp/Bmp2b – Sog/Chd antagonism then fine tunes and maintains the activity of the Dpp/Bmp2b morphogen on the appropriate side of the embryo. The requirement for a combination of initial restriction by transcriptional control and later inhibition of protein function by antagonists to mediate the initiation, establishment and maintenance of the Dpp/Bmp2b morphogen gradient is thus a feature found in both arthropods and vertebrates.

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