

Direct Binding of Lef1 to Sites in the *boz* Promoter May Mediate Pre–Midblastula-Transition Activation of *boz* Expression

Tinchung Leung, Iris Söll, Sebastian J. Arnold, Rolf Kemler, and Wolfgang Driever*

The Nieuwkoop center provides signals essential for the establishment of the dorsal gastrula organizer in vertebrates. Activation of β -catenin is one of the events in the Nieuwkoop center that lead to activation of dorsal-specific genes during blastula and early gastrula stages. Zebrafish *bozozok* (*boz*) mutant embryos have severe defects in axial mesoderm and anterior neuroectoderm. The *boz* gene is activated in the organizer in response to β -catenin signaling, and Boz protein has been demonstrated to contribute to organizer formation by repression of ventralizing genes, including *bmp2b*, *vega1*, and *vega2*. Here, we investigate the timing and molecular mechanism by which *boz* expression is activated in the organizer. We demonstrate that *boz* is already expressed before midblastula transition (MBT). We further identify high-affinity binding sites for Tcf/Lef1 within the *boz* promoter region. These sites, together with the finding that β -catenin induces *boz* expression, indicate that transcription of *boz* may be activated directly by β -catenin/Lef1. We hypothesize that pre-MBT activation of *boz* may be important to build up a sufficiently strong antagonizing activity against zygotic ventralizing genes activated immediately post-MBT. Thus, the early onset of *boz* expression may be crucial for organizer establishment in the presence of ubiquitous maternal activators of ventralizing genes. *Developmental Dynamics* 228:424–432, 2003. © 2003 Wiley-Liss, Inc.

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INTRODUCTION

The establishment of dorsal identity in amphibians is thought to be accomplished by the Nieuwkoop center, localized in dorsal vegetal blastomeres (Nieuwkoop, 1969a,b). A major role of the Nieuwkoop center is induction of the dorsal gastrula organizer. The Nieuwkoop center is initiated in *Xenopus* and zebrafish during the first cleavages, when dorsal determinants are transported from the vegetal pole toward the pro-

spective dorsal side (Elinson and Rowling, 1988; Jesuthasan and Strähle, 1997; Rowling et al., 1997; Mizuno et al., 1999; Ober and Schulte-Merker, 1999). This process leads to activation of components of the Wnt signaling pathway and accumulation of β -catenin in dorsal blastomeres in *Xenopus* and in nuclei of the dorsal yolk syncytial layer as well as in dorsal blastomeres in zebrafish (Schneider et al., 1996; Larabell et al., 1997; Moon and

Kimelman, 1998). Nuclear localization of β -catenin has been suggested to be the dorsal determinant that can induce secondary axes in *Xenopus* and zebrafish (Funayama et al., 1995; Kelly et al., 1995). In *Xenopus*, β -catenin is thought to activate expression of *siamois* (Lemaire et al., 1995), *twin* (Laurent et al., 1997), and *nodal-related* genes (Yang et al., 2002). The gastrula organizer is located in the embryonic shield of zebrafish and the dorsal

Developmental Biology, Institute Biology 1, University of Freiburg, Freiburg, Germany

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Dr. Leung's present address is Weis Center for Research, Geisinger Health System, 100 North Academy Avenue, Danville, PA 17822.

Dr. Arnold's and Kemler's present address is Max-Planck-Institut für Immunbiologie, Stübweg 51, 79108 Freiburg, Germany.

*Correspondence to: Wolfgang Driever, Developmental Biology, Institute Biology 1, University of Freiburg, Hauptstrasse 1, D-79104 Freiburg, Germany. E-mail: driever@biologie.uni-freiburg.de

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blastopore lip of amphibians (Oppenheimer, 1936; Spemann, 1938; Beddington and Robertson, 1999). The organizer secretes factors that antagonize bone morphogenetic protein (BMP) and Wnt signaling, such as Chordin (Sasai et al., 1995), Cerberus (Piccolo et al., 1999), and Frzb (Leyns et al., 1997; Wang et al., 1997). Wnt and BMP activities, as modulated by their antagonists, establish dorsoventral pattern in the vertebrate gastrula (reviewed in Harland and Gerhart, 1997; Niehrs, 1999).

boz mutant zebrafish embryos lack axial mesoderm and have severe patterning defects in the anterior neuroectoderm (Solnica-Krezel et al., 1996). *boz* encodes a homeodomain protein also known as Dharma or Nieuwkoid (Koos and Ho, 1998; Yamanaka et al., 1998; Fekany et al., 1999). *boz* is expressed in dorsal blastomeres as well as in the dorsal yolk syncytial layer (YSL) during late blastula stages. Hyperdorsalization of zebrafish embryos by incubation in lithium chloride, which activates β -catenin signaling, results in expression of *boz* circumferating the margin (Yamanaka et al., 1998). Active components of β -catenin signaling are required for *boz* expression (Shimizu et al., 2000), and promoter analysis of the *boz* gene indicates that mutations affecting potential Tcf/Lef1 binding sites affect the expression of *boz* (Ryu et al., 2001). The function of *boz* is required for proper expression of *gsc* and many other organizer-specific genes (Fekany et al., 1999). *boz* is also required for down-regulation 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) as well as Nodal signaling (Shimizu et al., 2000; Sirotkin et al., 2000). Analysis of the Boz protein revealed that it has transcriptional repressor activities and that a fusion protein of the Boz homeodomain with the Engrailed repressor domain can rescue *boz* mutant embryos (Leung et al., 2003). Boz protein binds specifically to the *bmp2b* promoter, and binding to these sites is required for the earliest repression of *bmp2b* expression in the organizer (Leung et al., 2003). Therefore, activation of *boz* expres-

sion in the region of the organizer is important for initiating asymmetry in *bmp2b* expression and dorsoventral patterning.

In this study, we further investigate the mechanisms that control expression of *boz*. We show that the *boz* promoter region has several functional high-affinity Tcf/Lef1 binding sites. Thus, the previously suggested activation of *boz* by β -catenin (Ryu et al., 2001) is likely a direct interaction. We further investigate the timing of *boz* activation and find that *boz* transcription starts significantly earlier than midblastula transition (MBT). MBT is a developmental landmark characterized by the onset of zygotic transcription and changes in cell cycle control (Newport and Kirschner, 1982a,b). In zebrafish, the MBT occurs at the tenth cell cycle (Kane and Kimmel, 1993). In *boz* mutant embryos, *boz* expression is reduced much earlier than previously reported, indicating that *boz* activity is already required very early during the activation phase of *boz* expression to achieve a normal expression domain. This early requirement for *boz* activity may reflect a requirement for repression of *vega1*, which in turn is a repressor of *boz* (Kawahara et al., 2000a; Shimizu et al., 2002), already during early post-MBT zygotic transcription of *boz*.

RESULTS

boz Is a Direct Target of the β -Catenin-Tcf/Lef1 Pathway

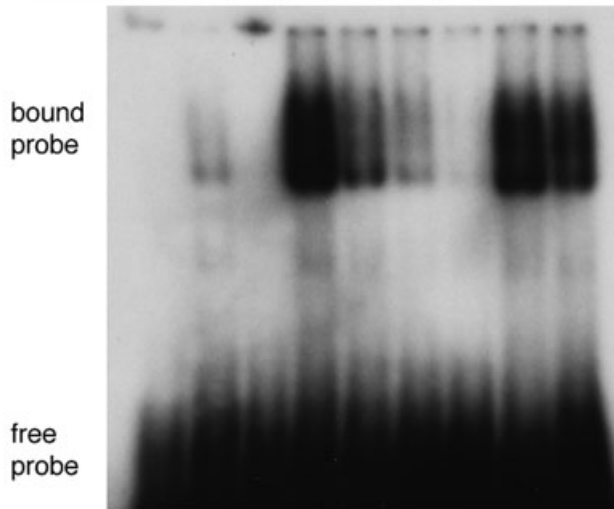
In zebrafish, *boz* is required for the establishment of a functional gastrula organizer (Solnica-Krezel et al., 1996; Fekany et al., 1999). In *boz* mutant embryos, the organizer does not form properly, resulting in absence of axial mesoderm, as well as anterior truncations of the neural plate and cyclopia (Fig. 1A,B). *boz* encodes a paired-class homeodomain protein expressed zygotically in dorsal blastomeres and yolk syncytial layer (Fig. 1C,D; Koos and Ho, 1998; Yamanaka et al., 1998). Because microinjection of *boz* mRNA into the YSL can rescue *boz* mutant embryos and induce secondary axes in wild-type embryos, it has been suggested that *boz* functions

in the Nieuwkoop center (Yamanaka et al., 1998; Fekany et al., 1999). Microinjection of synthetic RNA encoding zebrafish β -catenin or an activated form of β -catenin into one-cell stage zebrafish embryos causes an expanded *boz* expression domain (Shimizu et al., 2000; Ryu et al., 2001). Here, we show that, by injection of approximately 50 pg of capped β -catenin mRNA, it is even possible to induce an ectopic *boz* expression domain on the ventral side of sphere stage embryos (Fig. 1E,F). Thus, β -catenin is not only able to expand the *boz* expression domain but may induce ectopic ventral expression. These findings indicate that *boz* acts downstream of β -catenin.

To determine whether *boz* is a direct target of the β -catenin/Tcf transcriptional complex, we analyzed the *boz* promoter. PAC clones containing the *boz* gene were isolated from a genomic library by filter hybridization with a *boz* cDNA. Primer extension analysis revealed three putative transcription start sites at +1, -159, and -444 (78, 237, and 522 base pairs upstream of the ATG start codon, respectively). The site +1 appears to be used predominantly; data not shown). A stretch of 2.5 kb upstream of the coding region was sequenced and analyzed for the presence of potential Tcf/Lef-1 binding sites containing the consensus sequence CTTG(A/T)(A/T) (Travis et al., 1991; Brannon et al., 1997). A total of seven potential Tcf/Lef-1 sites were identified, all within the proximal 1.2 kb (highlighted blue in Fig. 2). The figure also shows sites predicted by Ryu et al. (2001), termed T1 through T9; we did not study potential sites T3 and T7, because they deviate from the consensus sequence. We investigated whether recombinant murine Lef-1 protein can bind to double-stranded (ds) oligonucleotides containing these sites by electrophoretic mobility shift assay (Travis et al., 1991). We found that Lef-1 strongly binds to three of the ds oligonucleotides: S2, S3 (with two potential Tcf/Lef-1 sites), and S6; but only weakly to the other three sites: S1, S4, and S5 (Fig. 2). The specificity of binding is supported by our observation that excess Tcf/Lef-1 consensus site ds oligonucleotides could efficiently compete for binding of

A

probe site	-	-	S1	S1	S1	S1	S1	S1	S1
probe mutant site	m1	m1	-	-	-	-	-	-	-
competitor Tcf site	-	-	-	-	50	150	500	-	-
competitor mut. Tcf	-	-	-	-	-	-	-	150	500
Lef1 protein	-	+	-	+	+	+	+	+	+



B

probe site	-	-	S2	S2	S2	S2	S2	S2	S2	S2
probe mutant site	m2	m2	-	-	-	-	-	-	-	-
competitor Tcf site	-	-	-	-	100	300	1000	-	-	-
competitor mut. Tcf	-	-	-	-	-	-	-	100	300	1000
Lef1 protein	-	+	-	+	+	+	+	+	+	+

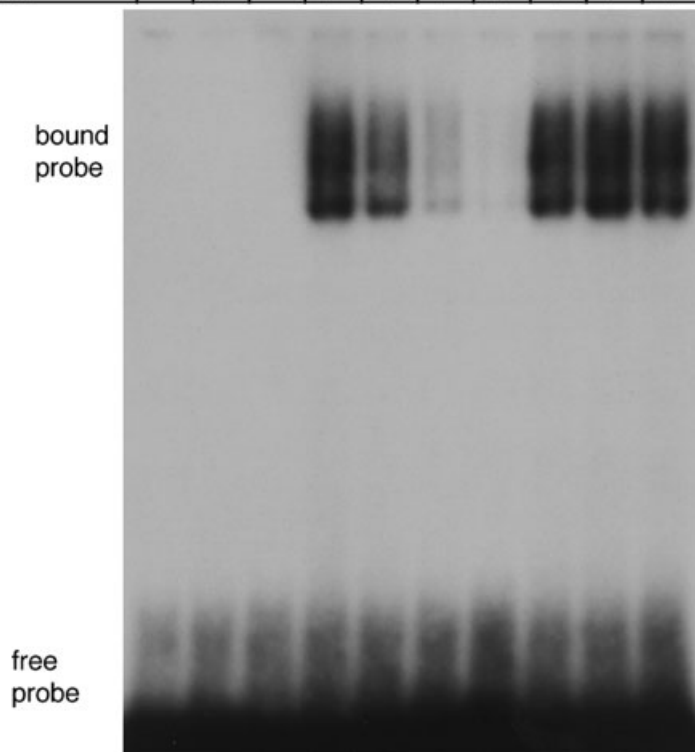


Fig. 3. Specificity of Lef1 binding to sites in the *boz* promoter. **A,B:** The specificity of Lef-1 binding to S1 (A) and S2 (B) sites was tested by including competitor double-stranded oligonucleotides in the binding reactions. The binding of Lef-1 to *boz* sites in which the consensus Lef-1 site had been mutated (m1 for S1, m2 for S2) was first tested (leftmost two lanes). A consensus Tcf/Lef-1 binding site was used to compete for S1 (50- to 500-fold) or S2 (100- to 1,000-fold molar excess) binding. At highest competitor concentration, Lef-1 binding to S1 and S2 sites was completely abolished. Tcf/Lef-1 binding sites for which the core of the consensus sequence had been mutated were not able to compete for S1 or S2 binding at 500- and 1,000-fold molar excess, respectively. For details, see Experimental Procedures section.

tion of *boz* transcription. While reverse transcriptase-polymerase chain reaction (RT-PCR) may be the most sensitive technique to detect

mRNA, the PCR result does not provide for means of exact control of embryonic stages used to extract mRNA. Therefore, we decided to use

whole-mount in situ hybridization to detect *boz* mRNA during each stage of zebrafish blastula development (Fig. 4; for staging, see Kimmel

et al., 1995). We were not able to detect any *boz* mRNA before the 256-cell stage. At the 256-cell stage, *boz* mRNA could not be detected significantly above background levels (data not shown). However, at the 512-cell stage, we detected a strong signal using an antisense probe against *boz* mRNA (Fig. 4A–C). The 512-cell stage can be easily distinguished from the 1,000-cell stage (Fig. 5), because at the 512-cell stage, the enveloping layer has not yet formed: compare the round surface of animal blastomeres in Figure 5C with the flat shape of the enveloping layer cells seen at the 1,000-cell stage (Fig. 5D). Furthermore, the YSL forms only after MBT (Fig. 5B,F). At the 512-cell stage, *boz* is predominantly expressed in blastomeres in dorsolateral positions as well as cells located to more dorso-animal positions (Fig. 4A,B) but is not detectable in the cytoplasmic layer. *boz* expression remains confined predominantly to dorsal blastomeres until high stage (Fig. 4D–I), and from the oblong stage onward, more and more transcript is detectable in the YSL at the blastoderm margin (Fig. 4J–L). At the sphere stage, levels of *boz* transcript appear to be similar in dorsal blastomeres and the yolk syncytial layer (Fig. 4M,N). The spatial pattern of activation of *boz* transcription may reveal that the pattern of β -catenin translocation to the nucleus may extend further to the animal pole than previously reported (Schneider et al., 1996). In *Xenopus* blastula embryos, activated β -catenin can also be observed in dorsal blastomeres in a broader zone extending several cell layers away from the marginal zone toward the animal and vegetal pole (Schneider et al., 1996).

It has been reported previously that *boz* expression is strongly reduced or not detectable at sphere stage in *boz* mutant embryos. We investigated whether a sequential requirement for β -catenin and Bozok can be detected by comparing *boz* expression in precisely staged wild-type and *boz* mutant embryos (Fig. 6). We used crosses between genotypically homozygous *boz*^{m168} fish to generate groups of embryos that were all homozygous mutant.

Therefore, we were certain about the genotype of the embryos even before mutant phenotypes become visible. In *boz* mutant embryos at the 512-cell and 1,000-cell stage, *boz* is expressed temporally and spatially similar to wild-type; however, transcript levels appear to be somewhat lower in most embryos (Fig. 6A–L). From the high stage onward and continuing through oblong stage, *boz* expression is significantly reduced in *boz* mutant embryos (Fig. 6M–X). We were not able to detect any *boz* expression at sphere stage in *boz* mutant embryos. Thus, *boz* expression is reduced in *boz* mutant embryos slightly earlier than previously reported (Fekany et al., 1999; Koos and Ho, 1999). Our data reveal that there may be two phases of regulation of *boz* expression: an early pre-MBT activation that depends on β -catenin activity, and immediately after MBT a requirement for Boz activity to maintain *boz* expression. However, we are aware that the reduced levels of *boz* mRNA from high stage on may also be caused by reduced stability of the mutant *boz* mRNA.

DISCUSSION

Pattern formation along the dorsoventral axis is characterized by antagonisms of ventralizing and dorsalizing activities at various control levels. The default fate of a blastomere in fish and frogs may be considered as ventral, because maternal factors, like *smad5/sbn* in zebrafish (Kramer et al., 2002), lead to ubiquitous zygotic *bmp2b* expression in the absence of dorsalizing activities. Thus, BMPs are initially expressed in a fairly ubiquitous manner throughout the blastoderm in vertebrates, 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). Counteracting this ventral default state are activities of the Nieuwkoop center and the gastrula organizer. Dorsal development in amphibians and fish is initiated by dorsal activation and enrichment of β -catenin in the nuclei (Gerhart et al., 1989; Schneider et al., 1996; Harland and

Gerhart, 1997; Heasman, 1997). β -Catenin acts as an activator mediating Wnt-dependent transcription when bound to members of the Tcf/Lef1 family of DNA binding proteins (Molenaar et al., 1996). In *Xenopus*, β -catenin is thought to activate expression of *siamois* (Lemaire et al., 1995), *twin* (Laurent et al., 1997), and nodal-related genes *Xnr5* and *Xnr6* (Yang et al., 2002).

In zebrafish, *boz* has been suggested to be a target of β -catenin mediated activation, based on LiCl experiments (Yamanaka et al., 1998; Fekany et al., 1999), overexpression of β -catenin or activated β -catenin (Shimizu et al., 2000; Ryu et al., 2001), as well as promoter studies, which suggested a role of putative Tcf/Lef1 binding sites in the *boz* promoter toward the control of *boz* expression (Ryu et al., 2001). Here, we show specific binding of Lef1 protein to several sites in the *boz* promoter and, thus, corroborate evidence that the predicted sites are indeed functional. Our DNA binding data, together with the previously published promoter analysis (Ryu et al., 2001), demonstrate that *boz* may indeed be a direct target of β -catenin. The finding that *boz* is a downstream target of β -catenin is consistent with reports that overexpression of β -catenin mRNA cannot rescue the *boz* mutant phenotype (Fekany et al., 1999).

Boz acts as a transcriptional repressor directly on the *bmp2b* promoter (Leung et al., 2003) and is required to clear the nascent organizer from Bmp2b activity. *boz* mediates the earliest dorsoventral asymmetry of *bmp2b* expression and, thus, contributes to the initiation of dorsoventral pattern in a crucial manner. Furthermore, it has been shown previously 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 like *gsc* and *chd*. Such multiple inhibitory interactions may be essential to control the extent of dorsal and ventral territories, which otherwise would

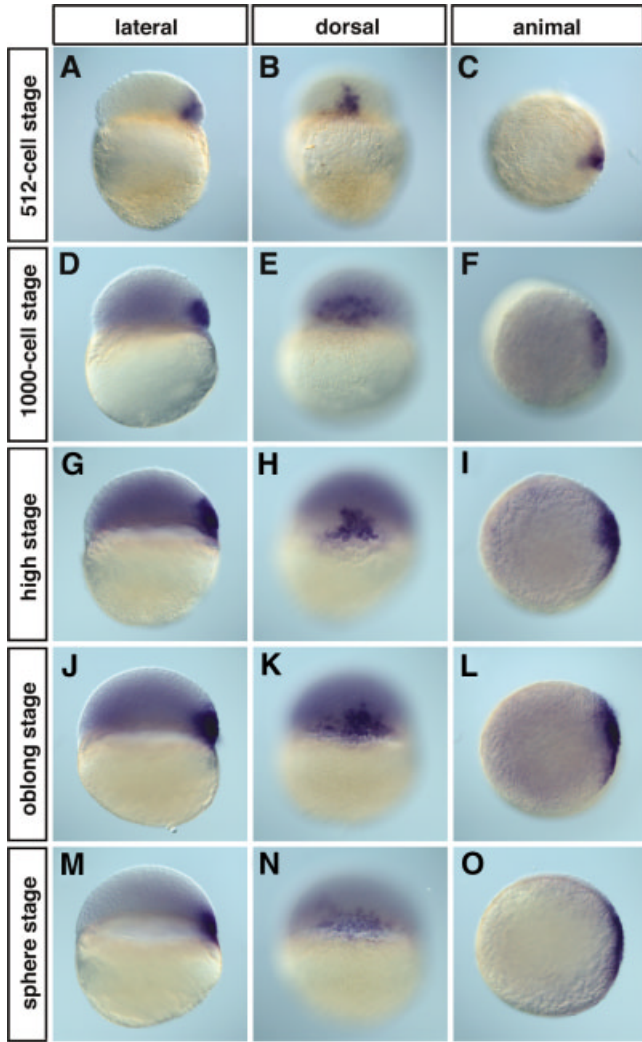


Fig. 4. Pre-midblastula transition activation of *boz* expression. A–O: Detection of *boz* expression by whole-mount in situ hybridization. Boxes at left indicate stages of embryos. Boxes at top indicate orientation of embryo: lateral, lateral view with animal pole at top and dorsal right; dorsal, dorsal view with animal pole at top; animal, animal pole view with dorsal at right.

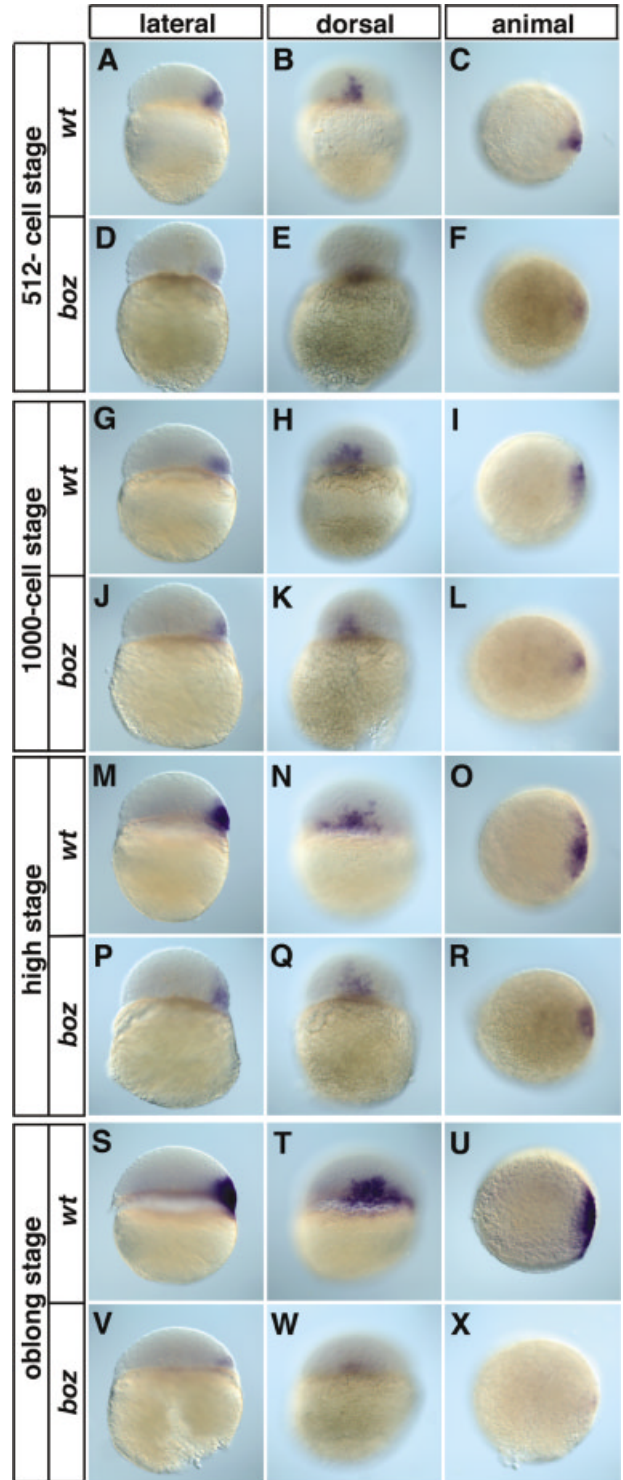


Fig. 6.

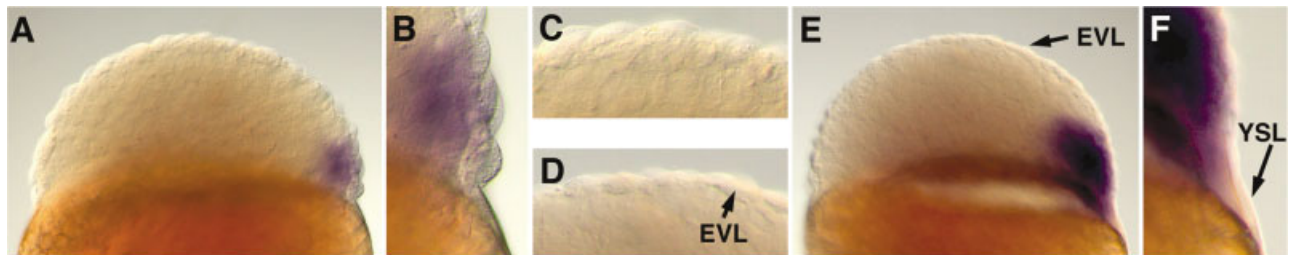


Fig. 5.

predominantly depend on extracellular diffusion of the Bmp2b morphogen—a mechanism that may not be sufficient to generate stable expression borders of organizer genes.

When we investigated the precise temporal progress of *boz* expression in wild-type embryos, we discovered that *boz* transcription is activated earlier than previously assumed, already at the 512-cell stage, and thus before MBT. Although some low-level pre-MBT transcription has been reported for *Xenopus* and *Drosophila* (reviewed in Yasuda and Schuberger, 1992), a specific role and pathway for pre-MBT transcription have so far only been demonstrated for β -catenin-mediated activation of *Xnr5* and *Xnr6* transcription in *Xenopus* (Yang et al., 2002). Experiments in *Xenopus* also indicate that β -catenin activity is required pre-MBT: pre-MBT activation of Tcf-dependent transcription can rescue dorsal development in ventralized embryos, whereas post-MBT activation of Tcf-dependent transcription cannot (Yang et al., 2002). We hypothesize that pre-MBT activation of *boz* may be important for efficient suppression of zygotic ventralizing genes activated post-MBT and, thus, crucial to organizer establishment in the presence of ubiquitous maternal activators of ventralizing genes, such as *smad5/sbn* (Kramer et al., 2002).

In *boz* mutant embryos, *boz* expression is reduced earlier than previously reported. This finding may be due to nonsense-mediated mRNA decay (Peltz et al., 1993; Whitfield et al., 1994) caused by the nonsense mutation in the *boz^{m168}* allele. However, the rapid disappearance of *boz* mRNA may also indicate that *boz* activity is already required very

early during the activation phase of *boz* expression to achieve a normal expression domain. As *boz* is a transcriptional repressor (Leung et al., 2003), it is unlikely that this is autoregulation. Rather, the early requirement for *boz* activity may reflect a requirement for repression of *vega1*, which is activated just after MBT, and later also of *vega2* and *ved*, which all three in turn are repressors of *boz* (Kawahara et al., 2000a, b; Shimizu et al., 2002). In the absence of functional Boz protein, *Vega1*, *Vega2*, and *Ved* may accumulate to levels that may efficiently suppress *boz* transcription, overriding the activating signal from β -catenin/Tcf. Thus, vertebrates may have two mechanisms to initialize dorsal pattern, both of which are mediated by β -catenin-dependent pre-MBT activation of dorsal genes: activation in the Nieuwkoop center and organizer of high levels of signals that promote dorsal development, like *Xnr5* and *Xnr6* in *Xenopus* (Yang et al., 2002), as well as activation of repressors of ventralizing genes, like *boz* in zebrafish.

EXPERIMENTAL PROCEDURES

The zebrafish mutant *boz^{m168}* allele was originally identified in an ENU screen (Solnica-Krezel et al., 1996). In situ hybridization was performed as described by (Hauptmann, 1999). Homozygous mutant *boz^{m168}* adult zebrafish were generated by injection of in vitro transcribed *boz* mRNA (Fekany et al., 1999) into one-cell stage embryos from crosses between heterozygous parents. Progeny was genotyped by using DNA prepared from tail fin biopsies by PCR-based genotyping using an

HaeIII-restriction fragment length polymorphism caused by the mutation in the *boz^{m168}* allele (Fekany et al., 1999). Homozygous *boz^{m168}* males and females are viable and fertile. For the β -catenin overexpression experiment, capped sense RNA was synthesized using SP6 RNA polymerase and the mMMESSAGE mMACHINE system (Ambion) after *NotI* digestion in pCS2+. In vitro synthesized mRNA was microinjected into the one-cell stage embryos.

Primer Extension Analysis

Total RNA was isolated from sphere stage embryos (4 hpf). One microgram of total RNA was used for reverse transcription with Moloney murine leukemia virus reverse transcriptase (Ambion) at 42°C for 1 hr in the presence of radiolabeled gene-specific primers to *boz* (primer: 5'TGT-TCAAGTGTACGGGTGC 3'). The synthesized labeled cDNA was analyzed on a 6% denaturing polyacrylamide gel, and the size was compared with a sequencing ladder.

Isolation of *boz* Genomic Clones

Genomic PAC clones containing *boz* were isolated by filter hybridization of a zebrafish genomic library (RZPD, Berlin) with a *boz* cDNA probe. The 2.5 kb upstream of the *boz* coding region were accessed by primer walking and sequenced by using a Licor 2000L automatic sequencer.

Recombinant Protein Purification

Recombinant GST-tagged full-length mouse Lef-1 (1 to 387 -amino acid) fusion protein was prepared as described (Huber et al., 1996).

Electrophoretic Mobility Shift Assay

For each of the ds oligonucleotides used, one of the two strands was ³²P end-labeled by the T4 kinase reaction or by Klenow fill-in reaction. Probe (10,000 cpm /10 fmol) was incubated with 0.1 μ g of purified recombinant Lef-1 protein in the presence of 0.76 μ g of poly d(I-C) in binding buffer (20 mM HEPES, 50 mM

Fig. 5. *boz* expression at 512- and 1,000-cell stage. A-F: Detection of *boz* expression by whole-mount in situ hybridization. A-C: A 512-cell-stage embryo. D-F: A 1,000-cell-stage embryo. B,F: Magnifications of dorsal zone: the 1,000-cell stage in F has already formed the yolk syncytial layer (YSL), which is absent at the 512-cell stage in B. C,D: The 1000-cell stage in D has already formed the enveloping layer (EVL), while round blastomeres are still at the surface of the blastula in the 512-cell-stage embryo in C. Lateral, lateral view with animal pole at top and dorsal right.

Fig. 6. *boz* expression depends on *boz* activity from high stage onward. A-X: Detection of *boz* expression by whole-mount in situ hybridization. Boxes at left indicate stages of embryos as well as genotypes: wt, wild-type (A-C,G-I,M-O,S-U); *boz*, *boz^{m168}* homozygous mutant embryos (D-F,J-L,P-R,V-X). Boxes at top indicate orientation of embryo: lateral, lateral view with animal pole at top and dorsal right; dorsal, dorsal view with animal pole at top; animal, animal pole view with dorsal at right.

ethylenediaminetetraacetic acid, 5 mM MgCl₂, 5% glycerol, 1 mM dithiothreitol, at pH 8) in a total volume of 10 µl as described (Brannon et al., 1997). Samples were incubated on ice for 20 min, followed by a further 20-min 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 *boz* genomic region, six putative oligonucleotides were synthesized (Lef-1 sites are underlined; positions are highlighted in Fig. 2): S1, 5'TGACITCCACITCTACAAAGAGGTGTAATGGGCC; S2, 5'GGGTTTCAGGGTCTTCAAAGTGGATCTT; S3, 5'ACGCTTTCATTTCAAAGCGCGCAATTCTTTGAACAAGTTTCGT; S4, 5'TCGTCTATTTAAATTCAAAGAACGGACAA; S5, 5'TTAGCTTTATCAAAGTTTGATAAGAAACCA; and S6, 5'CTGTTATAATCATAATCAAAGCAGTTATTGGGTGCTG.

Competition analysis was performed by incubation in the presence of double-stranded competitor oligonucleotides: wild-type competitor Tcf/Lef-1 binding oligonucleotides (consensus underlined) 5'TCGACCTGCAAGTAGGGCACCCCTTGAAGCTCTCCC and mutated (bold) competitor Tcf/Lef-1 binding oligonucleotide 5'TCGACCTGCAGGTAGGGCAC: **AATTTC**AAGCTCTCCC as described (Travis et al., 1991).

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