The zebrafish *bonnie and clyde* gene encodes a Mix family homeodomain protein that regulates the generation of endodermal precursors

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Vertebrate endoderm development has recently become the focus of intense investigation. In this report, we first show that the zebrafish *bonnie and clyde* (*bon*) gene plays a critical early role in endoderm formation. *bon* mutants exhibit a profound reduction in the number of *sox17*-expressing endodermal precursors formed during gastrulation, and, consequently, a profound reduction in gut tissue at later stages. The endodermal precursors that do form in *bon* mutants, however, appear to differentiate normally indicating that *bon* is not required at later steps of endoderm development. We further demonstrate that *bon* encodes a paired-class homeodomain protein of the Mix family that is expressed transiently before and during early gastrulation in both mesodermal and endodermal progenitors. Overexpression of *bon* can rescue endodermal gene expression and the formation of a gut tube in *bon* mutants. Analysis of a newly identified mutant allele reveals that a single amino acid substitution in the DNA recognition helix of the homeodomain creates a dominant interfering form of Bon when overexpressed. We also show through loss- and gain-of-function analyses that Bon functions exclusively downstream of *cyclops* and *squint* signaling. Together, our data demonstrate that Bon is a critical transcriptional regulator of early endoderm formation.

[*Key Words*: Paired-class homeodomain; *sox17*; *cyclops*; *squint*; gut]

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One of the fundamental problems in developmental biology is to understand how the three germ layers—ectoderm, mesoderm, and endoderm—are established. The molecular mechanisms of ectoderm and mesoderm formation have been analyzed extensively and our understanding of their development is fairly advanced. In contrast, the molecular analysis of endoderm formation has only begun relatively recently, but already several *Xenopus* and zebrafish genes have been implicated in this process.

In *Xenopus*, several related paired-class homeodomain proteins, the Mix and Bix proteins, have been proposed to have important roles in endoderm formation (Rosa et al. 1989; Henry and Melton 1998; Mead et al. 1998; Tada et al. 1998). The genes encoding these proteins are all expressed in the prospective endoderm during gastrulation; most are also expressed in marginal cells that will form mesoderm, with the exception of *Mixer*, which is expressed exclusively in the endoderm (Henry and Melton 1998). When overexpressed in animal caps, these genes exhibit different abilities to activate endodermal gene expression: *Mixer* and *Bix2/Milk* appear to promote endodermal gene expression strongly, and at the expense of mesodermal gene expression (Ecochard et al. 1998; Henry and Melton 1998); *Bix1* overexpression at low and high levels induces mesodermal and endodermal gene expression, respectively (Tada et al. 1998); and *Mix.1* is able to activate endodermal gene expression only when co-expressed with the dorso-vegetal homeobox gene *siamois* (Lemaire et al. 1998). Additionally, *Bix4* is able to restore endodermal gene expression to *VegT* depleted embryos, which lack all endoderm and most mesoderm (Zhang et al. 1998b), indicating that Bix4 acts downstream of *VegT* in endoderm formation (Casey et al. 1999). Therefore, whereas the various *Xenopus Mix* and *Bix* genes can regulate endoderm development, their individual roles and degree of importance in this process remain unresolved.

In contrast to the situation in *Xenopus*, only a single *Mix*-like gene has been identified so far in other vertebrates. The chick *CMIX* gene is expressed in the epiblast of the posterior marginal zone of early chick embryos,

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and starting at the onset of gastrulation, along the entire primitive streak except for its most posterior part (Peale et al. 1998; Stein et al. 1998). In mouse, a *Mix*-like gene, *Mml*, has recently been identified (Pearce and Evans 1999); it is expressed in the visceral endoderm at embryonic day 5.5 (E5.5), and starting at E6.5 throughout the entire primitive streak except for its most anterior part. In zebrafish, a *Mix*-like gene has also been described and provisionally named *mixer*, because of its homology with *Xenopus Mixer* and ability to induce endodermal gene expression in wild-type and mutant zebrafish embryos (Alexander et al. 1999; Alexander and Stainier 1999).

The *Xenopus* genes *Xsox17*α, and *Xsox17*β (here referred to collectively as *Xsox17*), which encode proteins containing a high mobility group DNA-binding domain, are also implicated in endoderm development (Hudson et al. 1997). Expression of *Xsox17* becomes restricted to the endoderm at the onset of gastrulation, and when overexpressed in animal caps, *Xsox17* activates endodermal gene expression. *Mixer* induces *Xsox17* expression but *Xsox17* does not induce *Mixer* expression in animal caps, suggesting that *Mixer* acts upstream of *Xsox17*; however, *Xsox17* expression appears 3 hr before that of *Mixer* during normal development (Henry and Melton 1998). Therefore, it may be that these genes initiate expression independently of each other, with *Mixer* then regulating the maintenance of *Xsox17* expression (Henry and Melton 1998; Yasuo and Lemaire 1999). Additionally, coexpression of *Mixer* and a dominant interfering form of *Xsox17* (*Xsox17–enR*) in animal caps blocks the induction of endodermal gene expression, whereas coexpression of *Xsox17* and *Mixer–enR* does not, suggesting that *Mixer* functions through *Xsox17* in endoderm formation (Henry and Melton 1998). In the absence of genetic analyses of the *Mix*, *Bix*, and *Xsox17* genes, however, it remains difficult to describe precisely the relationships between these various genes.

Several mutations have recently been reported to affect endoderm development in zebrafish (Schier et al. 1997; Feldman et al. 1998; Alexander et al. 1999; Reiter et al. 1999). The *cyclops* (*cyc*) and *squint* (*sqt*) genes encode Nodal-related proteins, and embryos mutant for both of these genes lack all endodermal and most mesodermal derivatives (Feldman et al. 1998). The same phenotype is seen in embryos that lack both maternal and zygotic *one-eyed pinhead* (*oep*), a gene encoding a member of the EGF–CFC protein family essential for Nodal signaling (Zhang et al. 1998a; Gritsman et al. 1999), whereas zygotic *oep* mutants lack endoderm as well as the prechordal plate and ventral neuroectoderm. Nodal-related genes have also been implicated in *Xenopus* endoderm development (Clements et al. 1999; Osada and Wright 1999; Yasuo and Lemaire 1999).

casanova (*cas*) and *faust* (*fau*), two zebrafish mutants that exhibit bilateral hearts (cardia bifida), have also been shown to be essential for endoderm development. In *cas* mutants, the early endodermal expression of genes such as *axial*, *sox17*, and *fkd2* does not initiate, but expression of the *Mix*-like gene is normal, suggesting that *cas* acts downstream of, or in parallel to, this *Mix*-like gene to promote endoderm formation (Alexander et al. 1999). The *cas* gene has not yet been isolated. The *fau* locus is essential for multiple aspects of heart and endoderm development; *fau* mutants exhibit reduced amounts of cardiac and endodermal tissue and abnormal morphogenesis of the heart, pharyngeal endoderm, and gut (Reiter et al. 1999). *fau* encodes Gata5, a zinc finger-containing transcription factor, and is expressed in the endodermal and some mesodermal progenitors before to the onset of gastrulation (Reiter et al. 1999).

The *bonnie and clyde* (*bon*) mutation, like *oep*, *cas*, and *fau*, causes cardia bifida (Stainier et al. 1996). In this report, we show that in *bon* mutants the number of *sox17*-expressing endodermal precursors formed during gastrulation is significantly reduced, and that the gut tube is almost entirely absent at later stages. The endodermal precursors that do form in *bon* mutants, however, appear to differentiate normally—indicating that *bon* is not required at later steps of endoderm development. We also show that *bon* corresponds to the *Mix*-like gene that we isolated previously (Alexander et al. 1999). We further examined the relationship between *bon* and Nodal signaling and found that *bon* can induce a few cells to express *sox17* expression in *cyc;sqt* double mutants, which normally completely lack *sox17* expression. In contrast, *bon* cannot induce ectopic *cyc* and *sqt* expression in wild-type embryos, indicating that *bon* functions exclusively downstream of these *nodal*-related genes. Our results provide the first genetic evidence that *Mix* genes are critical for early endoderm development, show that *bon* is required only at a very early step of endoderm formation (i.e., the generation of *sox17*-expressing endodermal precursors), and suggest that additional factors act downstream of *cyc* and *sqt* to generate the full complement of endodermal precursors.

Results

bon *mutants show severe defects in endoderm development*

The zebrafish *bon* locus is defined by two mutant alleles: bon^{m425} was identified in a large-scale screen for embryonic lethal mutations (Stainier et al. 1996), whereas the second allele, bon^{s9}, was identified in a mosaic F_1 screen for cardiac mutations (Alexander et al. 1998). Both alleles segregate as fully penetrant, completely recessive mutations, and cause essentially identical phenotypes at the morphological level. The transheterozygous phenotype (bon^{m425}/bon^{s9}) is also fully penetrant and indistinguishable from that of either homozygous mutant at the morphological level.

bon^{m425} was originally classified as a cardia bifida mutation as it blocks cardiac fusion and leads to the presence of bilateral myocardial tissue (Stainier et al. 1996). *bon* mutants also exhibit pericardial edema, collapsed brain ventricles, as well as an enlarged yolk and a thickened yolk extension that may be secondary to the pronounced pericardial edema or defects in the yolk syncy-

tial layer (Fig. 1A,B). The hearts, though bifid, contain distinct atrial and ventricular tissues (Fig. 1C,D).

oep, *cas*, and *fau* were also classified as cardia bifida mutations (Chen et al. 1996; Stainier et al. 1996), but recent experiments have shown that all three also cause endoderm defects that may in fact lead to the cardia bifida phenotype (Schier et al. 1997; Peyriéras et al. 1998; Alexander et al. 1999; Reiter et al. 1999). These data prompted us to analyze endoderm development in *bon* mutants. Light microscopic observation at four days post-fertilization (dpf) reveals that most *bon* mutants lack a gut tube, although some display a small amount of peristaltically contracting gut tissue (Fig. 1E,F). We next examined early endodermal gene expression in *bon* mutants and found profound defects. Zebrafish *sox17*, a ho-

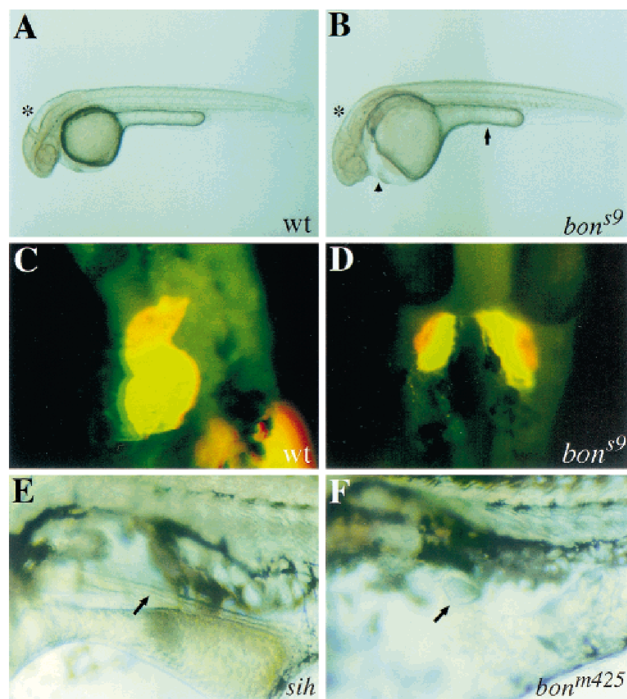

Figure 1. The *bon* mutations affect gut tube formation and heart morphogenesis. Light microscopic and immunofluorescent images of wild-type and *bon* mutant embryos at 30 hpf (*A–D*) and four days-postfertilization (*E*,*F*). (*A*,*B*) Compared with wild-type siblings (*A*), *bon* mutants (*B*) show pericardial edema (arrowhead), collapsed brain ventricles (asterisk), an enlarged yolk and a thickened yolk extension (arrow). (*C*,*D*) Ventral views of wild-type (*C*) and *bon* mutant (*D*) embryos stained with MF20 (TRITC) and S46 (FITC) antibodies. Red fluorescence indicates MF20 staining of ventricular tissue, whereas yellow fluorescence indicates the overlap of MF20 and S46 staining in atrial tissue. Wild-type embryos have a single heart tube with the ventricle (red) anterior to the atrium (yellow). *bon* mutants display cardia bifida, with the two separate hearts exhibiting distinct atrial and ventricular tissues. (*E*,*F*) *bon* mutants generally lack a gut tube, although sometimes a small amount of peristaltically contracting gut tissue is present (arrow in *F*). For comparison, we show a *silent heart* (*sih*) mutant where a similar extent of edema allows easy visualization of the gut tube (arrow in *E*).

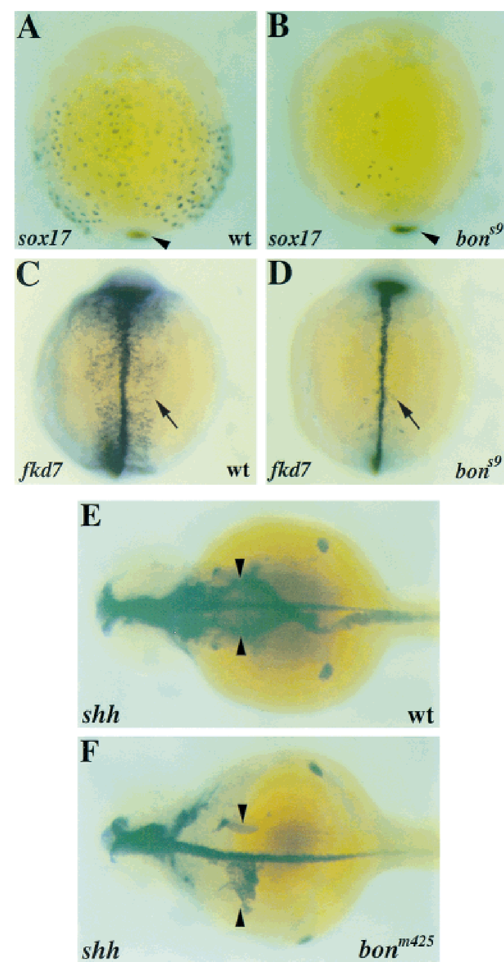

Figure 2. Endoderm development is defective in *bon* mutants. Wild-type (*A*,*C*,*E*) and *bon* mutant (*B*,*D*,*F*) embryos were examined for expression of *sox17* at 90% epiboly (*A*,*B*), *fkd7* at the 10-somite stage (*C*,*D*), and *shh* at 36 hpf (*E*,*F*). (*A*,*B*) During gastrulation *sox17* is expressed in the endodermal precursors throughout the hypoblast as well as in the forerunner cells (arrowhead in *A*). Endodermal *sox17* expression is greatly reduced in *bon* mutants (*B*), whereas *sox17* expression in the forerunner cells is normal (arrowhead in *B*). (*C*,*D*) During mid-somitogenesis *fkd7* is expressed in the endoderm (arrow in *C*) as well as in the floor plate and hypochord. In *bon* mutants (*D*), there are only a few *fkd7*-expressing endodermal cells (arrow in *D*), whereas expression in the floor plate and hypochord is normal. (*E*,*F*) Similarly, in 36 hpf wild-type embryos, *shh* is expressed throughout the endoderm (arrowheads in *E*); *bon* mutants exhibit small patches of *shh*-expressing endoderm (arrowheads in *F*). *shh* is also expressed in the floor plate, forebrain, and fin buds. All panels show dorsal views, with anterior to the *top*, except *E* and *F*, where anterior is to the *left*.

molog of *Xenopus Xsox17*, is expressed in endodermal precursors as well as in the forerunner cells (Alexander and Stainier 1999). In *bon* mutants, the number of *sox17*-expressing endodermal precursors is dramatically reduced compared with wild-type, whereas the number of forerunner cells appears to be unaffected (Fig. 2A,B; Table 1). The number of *axial*-expressing endodermal

Table 1. bon^{s9} *causes a stronger phenotype than* bon^{m425}

	No. of *sox17*-expressing endodermal precursors		
	shield (6 hpf)	75% epiboly (8 hpf)	90% epiboly (9 hpf)
Wild type	150	300	500
bon^{m425}	15.2 ± 1.1 (n = 12)	39.0 ± 3.9 (n = 9)	52.4 ± 3.0 (n = 8)
bon^{s9}	4.6 ± 0.9 (n = 8)	18.5 ± 2.1 (n = 11)	24.4 ± 4.7 (n = 5)

The total number of *sox17* expressing endodermal precursors was counted in *bon* mutants and estimated in wild-type embryos. *P* values (*t*-test) of the differences between *bon*^{m425} and *bon*^{s9} are 0.0007 (shield), 0.0035 (75% epiboly), and 0.0075 (90% epiboly).

precursors is similarly reduced in *bon* mutants (data not included). Quantitation of *sox17*-expressing endodermal precursors in both *bon* alleles demonstrates that *bon* is required for the generation of normal numbers of *sox17*-expressing endodermal precursors and not for their subsequent proliferation, and that the *bon*^{s9} endodermal defect is stronger than the *bon*^{m425} defect (Table 1).

At later stages, the *forkhead*-related gene *fkd7* is expressed in the forming gut tube, as well as in the hypochord and floor plate (Odenthal and Nüsslein-Volhard 1998). In *bon* mutants, endodermal *fkd7* expression is almost entirely absent, whereas hypochord and floor plate expression appears to be unaffected (Fig. 2C,D). We also examined the expression of *sonic hedgehog* (*shh*), which is expressed throughout the gut tube at larval stages (Krauss et al. 1993). Although *shh* expression in the floor plate, forebrain, and fin buds appears normal in *bon* mutants, endodermal *shh* expression is largely absent, although in some mutants, small clusters of *shh*-expressing endodermal cells can be seen (Fig. 2F). Together, these data indicate that *bon* has a critical role in the early events of endoderm development in zebrafish embryos.

The bon *locus encodes a Mix-like paired-class homeodomain protein*

Using half-tetrad analysis (Johnson et al. 1995), we mapped *bon*^{m425} to LG20. Further studies revealed that *bon* is located ~2.2 cM and 3.9 cM proximal to the simple sequence repeat marker Z13626 (Shimoda et al. 1999) and *bmp2b*, respectively, and ~12 cM distal to Z20046 (Fig. 3A). Radiation hybrid mapping using the Goodfellow panel placed the zebrafish *Mix*-like gene (Alexander et al. 1999) 34.6 cR proximal to *bmp2b*, that is, in the same region as *bon*. Linkage analysis showed no recombination between *bon* and the *Mix*-like gene in 51 meiotic events (data not shown), and prompted us to sequence the *Mix*-like gene in both *bon*^{m425} and *bon*^{s9} mutants. Sequencing the *bon*^{m425} allele revealed a T to A transversion (TAT → TAA) that introduces a premature translational stop at codon 86 and leads to a truncation in the homeodomain (Fig. 3B). In *bon*^{s9}, we found a T to C substitution (GTC → GCC) that introduces an alanine in place of a highly conserved valine at codon 104 (Fig. 3B) within the predicted third (DNA recognition) helix of the homeodomain (Kissinger et al. 1990; Wintjens and Rooman 1996).

Using restriction fragment length polymorphisms to analyze linkage, we found that the mutant alleles *bon*^{m425} and *bon*^{s9} segregated with the *bon* phenotype in 500 and 200 meiotic events, respectively. Together, the genetic data and identification of severe molecular lesions in this gene indicate that *bon* corresponds to the *Mix*-like gene we had provisionally named *mixer* (Alexander et al. 1999). According to the zebrafish nomenclature guidelines (Westerfield 1995), this gene will now be renamed *bon*.

As previously reported (Alexander et al. 1999), *bon* expression is first detected at sphere stage in a small group of dorsal cells, and by dome stage has spread throughout the marginal zone where it is expressed in both mesodermal and endodermal progenitors (Fig. 3D,E). By 60% epiboly *bon* expression is undetectable. *bon* expression is indistinguishable between wild-type, *bon*^{m425} and *bon*^{s9} mutant embryos (data not shown), indicating that initiation and maintenance of *bon* expression does not require Bon function.

Overexpression of bon *mRNA rescues* sox17 *expression and gut tube formation in* bon *mutants*

To test further whether the *bon* phenotype is caused by the identified mutations, we injected synthetic wild-type *bon* mRNA (100 pg) into embryos derived from both *bon*$^{m425/+}$ and *bon*$^{s9/+}$ heterozygote intercrosses. The injected embryos were processed for in situ hybridization at 75%–80% epiboly and the number of *sox17*-expressing endodermal precursors on the dorsal side was counted. As a control, we counted the number of *sox17*-expressing endodermal precursors in β-*galactosidase* (*lacZ*) mRNA-injected *bon* mutants. We found that the number of *sox17*-expressing endodermal precursors in *bon* mutants was significantly increased in both alleles following *bon* mRNA injection (Fig. 4A–D; Table 2). These data indicate that *bon* mRNA can rescue the early endodermal defect of *bon* mutants. To analyze whether this rescue was dose-dependent, we also injected 200 pg of *bon* mRNA and found that injection of a higher dose did not increase the average number of *sox17*-expressing endodermal precursors (data not shown).

To determine whether gut tube formation could also be rescued by *bon* mRNA injections, we injected 100 pg of *bon* or *lacZ* mRNA into *bon*$^{s9/+}$ heterozygote intercrosses, and examined *fkd7* expression at 24 hpf. Out of a total of 105 injected embryos, we identified 23 *bon* mutants by genotyping, 18 of which showed a completely continuous gut tube as assessed by *fkd7* expression (Fig. 4F). These successful rescue experiments provide further evidence that the *bon* phenotype is caused by the identified mutations.

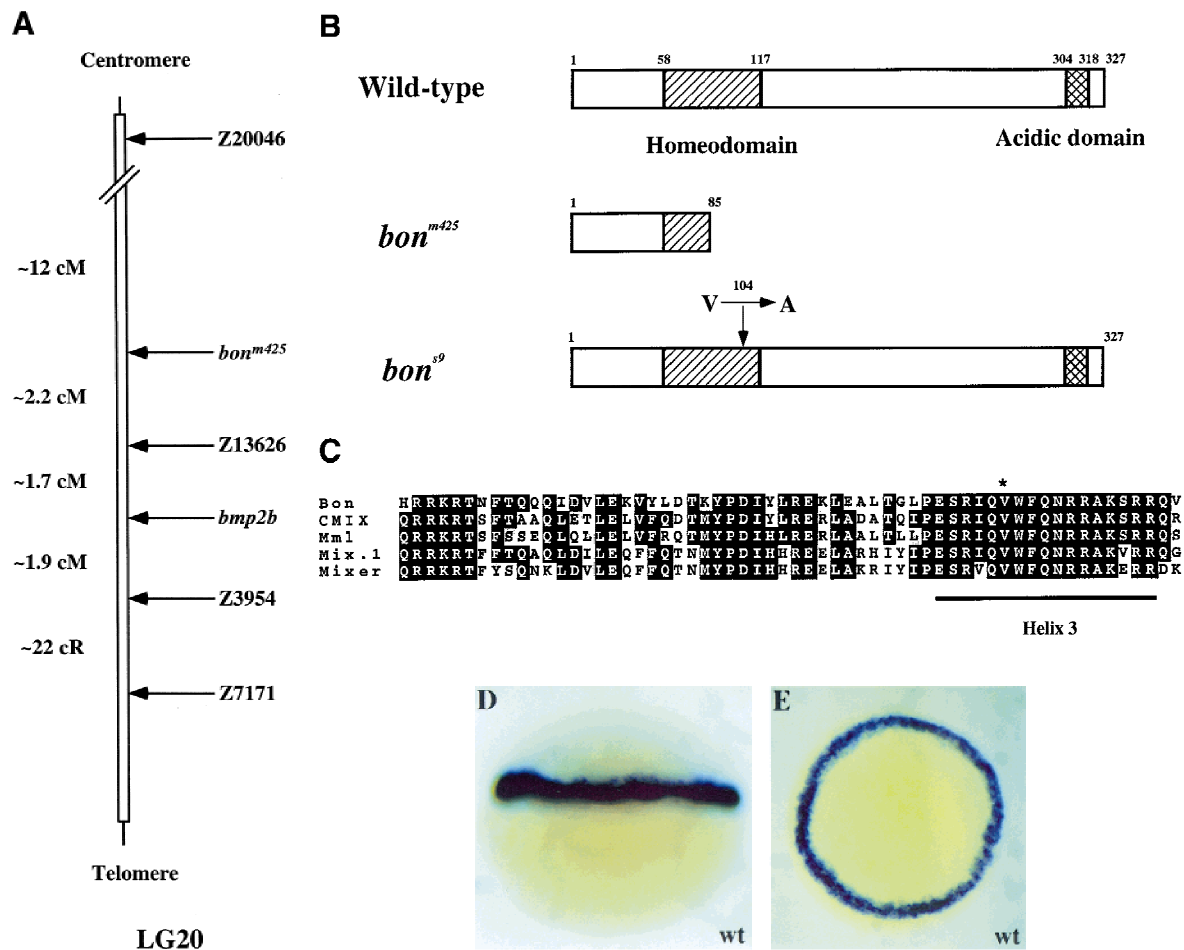

Figure 3. *bon* maps to LG20. (*A*) Schematic representation of part of LG20. *bon* maps ~2.2 cM proximal to the SSR marker Z13626 (134 meioses), 3.9cM proximal to *bmp2b* (153 meioses), 5.8 cM proximal to Z3954 (134 meioses) and ~12 cM distal to Z20046 (25 meioses). Radiation hybrid mapping using the Goodfellow panel placed Z7171 about 22 cR distal to Z3954. (*B*) Schematic representation of wild-type and mutant Bon proteins. The bon^{m425} mutation leads to a truncation in the homeodomain, whereas the bon^{s9} mutation introduces a single amino acid substitution (valine-to-alanine) in the third (DNA recognition) helix of the homeodomain. (*C*) Comparison of the Bon, CMIX, Mml, Mix.1, and Mixer homeodomains. The Bon homeodomain shares the highest level of identity (70%) with the CMIX and Mml homeodomains. The asterisk indicates the highly conserved valine residue mutated in bon^{s9}. (*D*,*E*) Lateral view (*D*) and animal pole view (*E*) of wild-type embryos at 50% epiboly. *bon* is expressed in all cells of the blastoderm margin.

The Bon^{s9} protein acts in a dominant interfering fashion when overexpressed

The early endoderm defects in bon^{s9} mutants are more severe than those in bon^{m425} mutants, yet the bon^{s9} mutation causes a single amino acid substitution in the third helix of the homeodomain, whereas the bon^{m425} mutation causes a premature stop codon at the beginning of the homeodomain. As we would expect, the bon^{m425} mutation to be a null or near null mutation, we hypothesized that the Bon^{s9} mutant protein functions as a dominant interfering form or antimorph. To test this hypothesis, we introduced an s9-type point mutation in wild-type *bon* by site-directed mutagenesis and analyzed the consequences of overexpressing bon^{s9} mRNA in both wild-type and bon^{m425} mutant embryos. Overexpression of bon^{s9} mRNA significantly reduced the average number of *sox17*-expressing endodermal precursors in both wild-type and bon^{m425} mutant embryos (Fig. 5A–D; Table 3). These data indicate that the Bon^{s9} protein acts in a dominant interfering fashion when overexpressed.

bon *can induce* sox17 *expression in* cyc;sqt *double mutants*

cyc;sqt double mutants lack all endodermal and most mesodermal derivatives and accordingly, lack endodermal *sox17* and *axial* expression (Feldman et al. 1998; Alexander and Stainier 1999; Fig. 6B). In this report, we have shown through loss-of-function analyses that *bon* is necessary for the generation of *sox17*-expressing endodermal precursors. To analyze whether *bon* is sufficient to induce the formation of *sox17*-expressing endodermal precursors in the absence of Nodal signaling, we injected

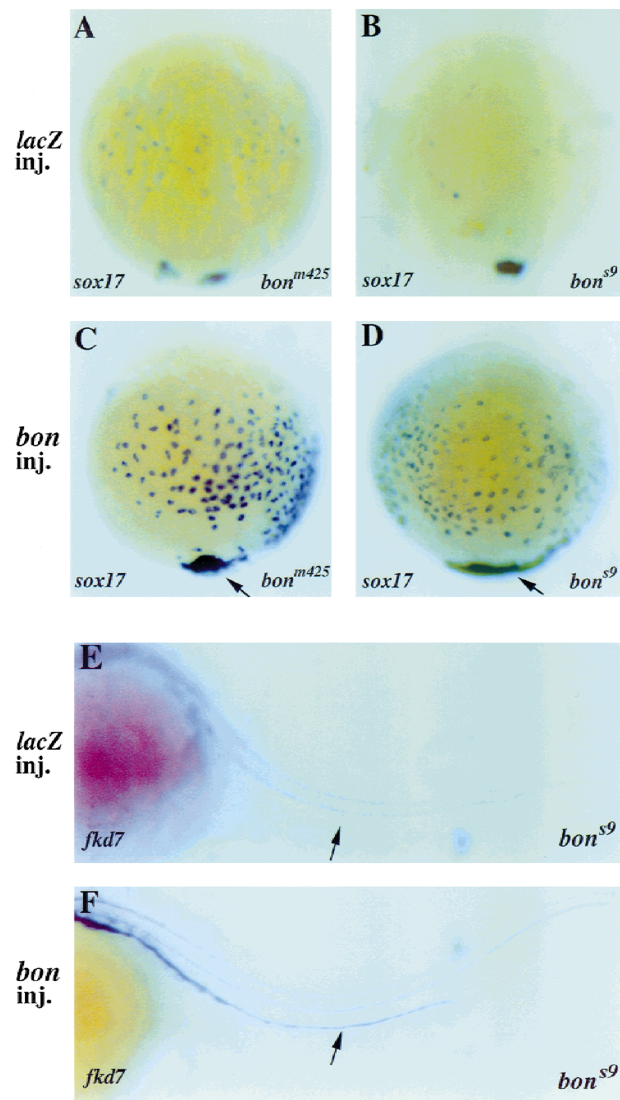

Figure 4. Overexpression of *bon* mRNA rescues endoderm formation in *bon* mutants. Embryos were injected with 100 pg of β-*galactosidase* (*lacZ*) or *bon* mRNA at the 1–4 cell stage (*A–F*). Gut tube formation was assessed by analyzing *fkd7* expression in 24 hpf embryos (*E*,*F*). (*A–D*) Dorsal views. *lacZ* overexpression does not affect *sox17* expression in bon^{m425} (*A*) or bon^{s9} (*B*) mutants. The number of *sox17*-expressing endodermal precursors is significantly increased in bon^{m425} (*C*) and bon^{s9} mutants (*D*) following *bon* overexpression. An enlargement of the most vegetal *sox17*-expressing population of cells is also seen in both alleles following *bon* overexpression (arrows in *C* and *D*). (*E*,*F*) Lateral views, anterior to the *left* and dorsal to the *top*. *fkd7* expression in the gut tube, which is absent in *lacZ*-injected bon^{s9} mutants (arrow in *E*), is restored following *bon* overexpression (arrow in *F*).

Table 2. *Overexpression of* bon *mRNA rescues* sox17 *expression in* bon *mutants*

Injected mRNA	Genotype	No. of *sox17*-expressing endodermal precursors on the dorsal side of the embryo
lacZ	wild type	164.1 ± 5.3 (n = 10)
lacZ	bon^{m425}	35.8 ± 3.0 (n = 20)
bon	bon^{m425}	103.7 ± 4.8 (n = 14)
lacZ	bon^{s9}	7.5 ± 0.6 (n = 15)
bon	bon^{s9}	80.1 ± 5.4 (n = 16)

The number of *sox17*-expressing endodermal precursors was counted at 75%–80% epiboly on the dorsal side of the embryo, which we determined to be representative of the whole embryos. *P* values (*t*-test) of the differences between *bon* and *lacZ* injections are 0.0001 for both sets of embryos (bon^{m425} and bon^{s9}).

bon mRNA into *cyc;sqt* double mutants, in which *bon* expression is barely detectable (Alexander and Stainier 1999). We injected *bon* mRNA into 393 embryos from *cyc*/+, *sqt*/+ heterozygote intercrosses (the expected number of double mutants being 24 or 25) and genotyped 93 embryos that showed reduced or absent *sox17* expression. We found that *bon* mRNA induced *sox17* expression in 14 out of 24 *cyc;sqt* double mutants. The average number of *sox17*-expressing cells in these 14 embryos was very low (7.0 ± 1.6) (Fig. 6C,D), indicating that although *bon* can induce the formation of *sox17*-expressing endodermal precursors, its activity in the absence of Nodal signaling is quite weak.

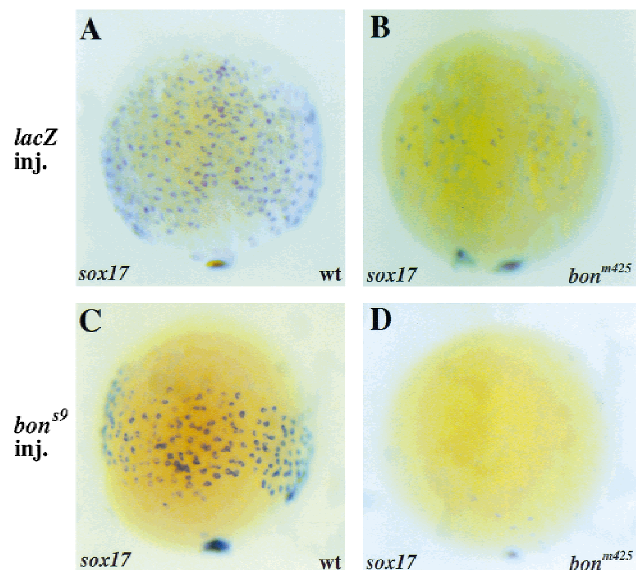

Figure 5. Overexpression of bon^{s9} reduces the number of *sox17*-expressing endodermal precursors in wild-type and mutant embryos. Embryos were injected with 100 pg of *lacZ* or bon^{s9} mRNA at the 1–4 cell stage. (*A–D*) Dorsal views. *lacZ* overexpression does not affect the number of *sox17*-expressing endodermal precursors in wild-type (*A*) or bon^{m425} mutant (*B*) embryos. The number of *sox17*-expressing endodermal precursors in wild-type (*C*) and bon^{m425} mutant (*D*) embryos is reduced following bon^{s9} overexpression.

Table 3. Bon^{s9} *acts in a dominant interfering fashion when overexpressed*

Injected mRNA	Genotype	No. of *sox17*-expressing endodermal precursors on the dorsal side of the embryo
lacZ	wild type	164.1 ± 5.3 (n = 10)
bon^{s9}	wild type	119.4 ± 11.1 (n = 10)
lacZ	*bon*^{m425}	35.8 ± 3.0 (n = 20)
bon^{s9}	*bon*^{m425}	8.5 ± 1.3 (n = 24)

The number of *sox17*-expressing endodermal precursors was counted at 75%–80% epiboly on the dorsal side of the embryo, which we determined to be representative of the whole embryo. *P* values (*t*-test) of the differences between *bon*^{s9} and *lacZ* injection are 0.0009 (wild type) and 0.0001 (*bon*^{m425}).

Nodal-related signals have been shown to induce *bon* expression in zebrafish embryos (Alexander and Stainier 1999). To test whether *bon* may act reciprocally to promote or maintain *cyc* and *sqt* expression, we examined *cyc* and *sqt* expression in *bon* mRNA injected wild-type embryos. Overexpression of *bon* mRNA did not induce ectopic expression of *cyc* or *sqt* in wild-type embryos (Fig. 7C,D). Also, *cyc* and *sqt* expression is normal in *bon* mutants (data not included). These data, together with the fact that *bon* expression is barely detectable in *cyc;sqt* double mutants (Alexander and Stainier 1999), indicate that *bon* functions exclusively downstream of *cyc* and *sqt* and does not have a role in regulating their expression.

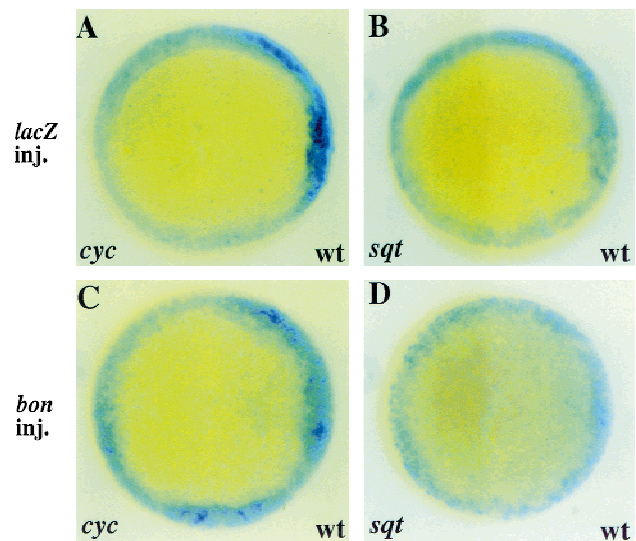

Figure 7. *bon* overexpression does not induce ectopic *cyc* or *sqt* expression in wild-type embryos. Wild-type embryos were injected with 100 pg of *lacZ* or *bon* mRNA and examined for *cyc* or *sqt* expression at 50% epiboly. (*A–D*) Animal pole views, dorsal to the *right*. *lacZ* overexpression does not affect *cyc* or *sqt* expression in wild-type embryos (*A*,*B*). Ectopic *cyc* or *sqt* expression was not observed in *bon*-injected wild-type embryos (*C*,*D*).

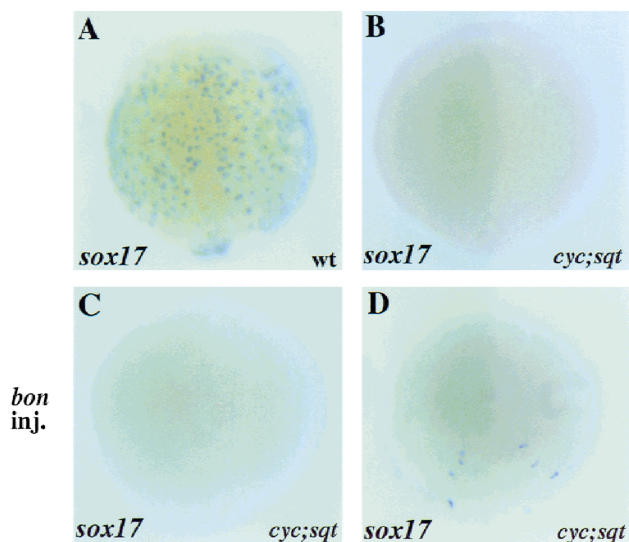

Figure 6. *bon* overexpression can induce *sox17* expression in *cyc;sqt* double mutants. (*A*,*B*) Wild-type (*A*) and *cyc;sqt* double mutant (*B*) embryos were examined for *sox17* expression at 90% epiboly. (*A–D*) Dorsal views. (*A*,*B*) *sox17* expression is completely absent in *cyc;sqt* double mutants (*B*) as determined by genotyping (n = 10). (*C*,*D*) Embryos from *cyc*/+, *sqt*/+ heterozygote intercrosses were injected with 100 pg of *bon* mRNA at the 1–4 cell stage, examined for *sox17* expression at 75%–80% epiboly and subsequently genotyped. Of 24 *cyc;sqt* double mutants, no *sox17* expression was found in 10 (*C*), whereas some *sox17* expression was observed in 14 (*D*).

Discussion

bon *is a critical regulator of early endoderm formation*

In *bon* mutants, the number of *sox17*-expressing endodermal precursors is dramatically reduced during gastrulation, and later the gut tube is almost entirely missing. The number of *sox17*-expressing endodermal precursors does increase as development proceeds, however, suggesting that endodermal proliferation is not affected. Furthermore, the *sox17*-expressing endodermal precursors in *bon* mutants appear to differentiate normally, as they go on to express *fkd7* and form peristaltically contracting gut tissue. Therefore, *bon* appears to regulate the initial formation of endodermal precursors but not their subsequent proliferation or differentiation. In *Xenopus* animal caps, overexpression of *Mix* and *Bix* family genes can induce the expression of endodermal genes and overexpression of *Mixer–enR* mRNA can block endodermal gene expression (Henry and Melton 1998; Mead et al. 1998; Tada et al. 1998; Casey et al. 1999), indicating that these genes are important determinants of endoderm development. The severe endoderm defects seen in *bon* mutants provide the first ge-

netic evidence that a *Mix*-like gene, *bon*, is a critical determinant of early endoderm formation in vertebrates, and a detailed analysis of the *bon* phenotype brings us closer to understanding the role *Mix* genes have in endoderm development.

Whereas a small number of endodermal precursors develop in *bon* mutants, endodermal expression of *sox17*, *axial*, and *fkd2* is absent completely in *cas* mutants (Alexander et al. 1999; Alexander and Stainier 1999). There are at least two possible explanations for the fact that some endodermal precursors remain in *bon* mutants. First, *bon*^{m425} and *bon*^{s9} may not be null mutations. Alternatively, other factors may regulate the generation of *sox17*-expressing endodermal precursors. For example, the number of *sox17*- and *axial*-expressing endodermal precursors is also reduced in *faust/gata5* mutants, and overexpression of *gata5* mRNA can increase the number of endodermal precursors in wild-type and *bon* mutant embryos (Reiter et al., in prep.), indicating that at least one other transcription factor is important for early endoderm development in zebrafish embryos. *gata5* expression is normal in *bon* mutants (Reiter et al., in prep.), and its activity may account for the residual endodermal development in these embryos. The *cas* gene product itself may also be expressed normally in *bon* mutants and account for the residual endodermal development.

bon *encodes a zebrafish Mix-like protein*

We present several lines of evidence that *bon* encodes a Mix-like protein. First, we found no recombination between the *bon* phenotype and the *Mix*-like gene in 751 meiotic events. Second, we found a premature stop codon at the beginning of the homeodomain in *bon*^{m425} mutants and a single amino acid substitution in a highly conserved residue in the DNA recognition helix of the homeodomain in *bon*^{s9} mutants. Third, we were able to rescue *sox17* expression and gut tube formation in both *bon*^{m425} and *bon*^{s9} mutants by overexpressing wild-type *bon* mRNA. Together, these data convincingly demonstrate that *bon* encodes a Mix-like protein.

Bon appears to be a novel member of the Mix family

When comparing homeodomains, Bon is related more closely to chick CMIX (Peale et al. 1998; Stein et al. 1998) and mouse Mml (Pearce and Evans 1999) (70% identical) than to either *Xenopus* Mix.1 (Rosa 1989) (62% identical) or Mixer (Henry and Melton 1998) (55% identical) (Fig. 3C). The entire Bon protein, therefore, shows low identity with CMIX (24%), Mml (25%), Mix.1 (25%), and Mixer (24%). *bon*, *CMIX*, *Mml*, and *Mix.1* appear to be expressed in progenitors of both the endoderm and mesoderm, in contrast to *Mixer*, which is expressed exclusively in the prospective endoderm. At the functional level, bon and Mixer, but not Mix.1, are able to induce *sox17* expression, whereas the activities of the chick and mouse genes have not been reported. These sequence, expression pattern, and functional considerations suggest that *bon* is not the zebrafish homolog of a specific *Xenopus* Mix family member. As further information on human and mouse *Mix* genes is becoming available from genome sequencing data, the relationship between *bon* and mammalian *Mix* genes should become clearer.

A valine to alanine substitution in the DNA recognition helix of the homeodomain creates a dominant interfering form of Bon

The *bon*^{s9} mutation leads to a valine-to-alanine substitution in the third (DNA recognition) helix of the homeodomain. The amino acid sequence of the third helix is highly conserved throughout the Mix and Bix family of proteins (Fig. 3C). Functional analyses have shown that paired-class homeodomains can bind cooperatively as homo- or heterodimers to the palindromic DNA sequence TAATYNRATTA (Y, C, or T; R, A, or G; N, any nucleotide) (Wilson et al. 1993). A high resolution crystal structure of a paired-class homeodomain bound to DNA demonstrates that the third helix is inserted into the major groove of the DNA, allowing two highly conserved amino acid residues (valine and asparagine) to make direct contacts with the DNA via hydrophobic/van der Waals interactions (Wilson et al. 1995). By changing this valine to an alanine, the *bon*^{s9} mutation could affect the DNA binding ability or specificity of the third helix. Whereas Bon^{s9} may have a reduced ability to bind DNA at its normal sites, it may still be able to interact with other factors of the transcriptional complex. Our results indicate that Bon^{s9} acts in a dominant interfering fashion when overexpressed, consistent with a model whereby it sequesters other factors important for endoderm development. Such a model may also explain why the *bon*^{s9} phenotype is stronger than the *bon*^{m425} one.

Mutations in the human paired-class homeodomain gene *PAX3* can cause Waardenburg syndrome type I (DeStefano et al. 1998). In a specific family, a valine-to-phenylalanine substitution in the PAX3 homeodomain segregates with the syndrome (Lalwani et al. 1995). This mutation occurs in the same position of the homeodomain as that in Bon^{s9}, further supporting the hypothesis that the valine in the third helix is critical for the proper function of paired-class homeodomain proteins.

bon *functions exclusively downstream of TGF-β signaling*

bon expression is affected by mutations in *nodal*-related genes; it is reduced along the animal-vegetal axis and absent dorsally in *sqt* mutants, and barely detectable in *cyc;sqt* double mutants (Alexander and Stainier 1999). In addition, overexpression of a constitutively active form of the type I TGF-β receptor TARAM-A (Peyriéras et al. 1998) promotes widespread expression of *bon* (Alexander and Stainier 1999). Furthermore, overexpression of *antivin/lefty1*, a feedback inhibitor of Nodal signaling (Bisgrove et al. 1999; Meno et al. 1999; Thisse and Thisse 1999), eliminates *bon* expression (Alexander and Stainier

1999). These data indicate that Nodal signaling regulates *bon* expression. We have found that *bon* overexpression does not lead to ectopic *cyc* or *sqt* expression in wild-type embryos, and that *cyc* and *sqt* expression is normal in *bon* mutants. Together, these data demonstrate that *bon* functions exclusively downstream of Nodal signaling in the molecular pathway leading to endoderm formation in zebrafish.

Additional factors besides Bon regulate sox17 *expression*

Overexpression of *bon* increases the number of *sox17*- and *axial*-expressing endodermal precursors in zygotic *oep* mutants (Alexander and Stainier 1999), indicating that *bon*, by itself or in combination with putative co-factors, has the ability to activate endodermal gene expression in zygotic *oep* mutants. Here, we have shown that *bon* can induce the formation of some *sox17*-expressing endodermal precursors in *cyc;sqt* double mutants. Our results therefore suggest that Bon can induce endoderm formation in the absence of Nodal-related signaling, but that additional factors downstream of *cyc* and *sqt* are needed to generate the full complement of endodermal precursors. These factors may include other paired-class homeodomain proteins that heterodimerize with Bon.

Xenopus Mix.1 can bind to the palindromic DNA sequence (TAATTGAATTA) as a homodimer, and injection of *Mix.1* mRNA transforms dorsal mesoderm to a ventral fate (Mead et al. 1996). Mix.1 can also form stable heterodimers with the paired-class homeodomain protein Siamois, and coinjection of *Mix.1* and *siamois* mRNAs induces endodermal gene expression in animal caps (Mead et al. 1996; Tada et al. 1998). Therefore, cofactors of Mix and Bix proteins can have a critical role in specifying their function, raising the possibility that the identification of Bon cofactors will help to elucidate the molecular mechanisms that lead to vertebrate endoderm development.

Molecular events regulating endoderm formation in zebrafish

Previous work has demonstrated that Nodal-related signals encoded by the *cyc* and *sqt* genes, together with the putative extracellular Nodal cofactor Oep, are necessary for zebrafish endoderm formation (Feldman et al. 1998; Zhang et al. 1998a; Alexander and Stainier 1999; Gritsman et al. 1999). *bon* expression is dependent on Nodal signaling (Alexander and Stainier 1999), and in this report we have shown that *bon* works exclusively downstream of Nodal signaling, where it is necessary for the generation of the normal complement of endodermal precursors. *bon* expression closely parallels that of *cyc* and *sqt* temporally and spatially (Alexander and Stainier 1999), raising the possibility that *bon* expression is an immediate early response to Nodal signaling. *sox17* expression in turn closely follows that of *bon* temporally. Only a subset of the *bon*-expressing cells go on to express *sox17*, however, and it remains an important challenge to understand how these cells are selected to become endoderm.

Materials and methods

Zebrafish strains

Adult zebrafish and embryos were maintained and staged as described in Westerfield (1995). Heterozygous carriers were intercrossed to obtain mutant embryos. Our mapping strain was constructed by crossing a *bon* heterozygote male to a WIK female. The following mutant alleles were used: cyc^{b16} (Hatta et al. 1991), sqt^{cz35} (Feldman et al. 1998), sih^{tc300b} (Chen et al. 1996).

Wholemount in situ hybridization and immunohistochemistry

Wholemount in situ hybridization and preparation of RNA probes were performed as described previously (Alexander et al. 1998).

Immunohistochemistry using the antibodies MF20 and S46 was performed as previously described (Alexander et al. 1998). MF20 was obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Biological Studies, University of Iowa under contract NO1-HD-2-3144 from the National Institute of Child Health and Development (NICHD).

Genetic mapping and single-strand conformational polymorphism (SSCP) analysis

Zebrafish *bon* was mapped on the Goodfellow zebrafish radiation hybrid panel (Research Genetics) by PCR with primers 5′-TACAAAACATGTCGGTGC-3′ and 5′-TCAAAGACTAAATGAAGC-3′ and the data were submitted for inclusion on the Tübingen map (see http://wwwmap.tuebingen.mpg.de/).

To extract genomic DNA from single embryos, 30 hpf $bon^{m425/m425}$ and $bon^{s9/s9}$ embryos were digested overnight in 10 mM Tris-HCl at pH 7.5, 1 mM EDTA, 0.1% SDS, 100 mM NaCl, 20 µg/ml of proteinase K at 55°C. Twenty diploid mutant and 11 haploid mutant embryos were analyzed by SSCP (Orita et al. 1989) with primers from the 3′ untranslated region of *bon*: 5′-GAAAGAGACGCCATGTCTGG-3′ and 5′-TATTGAGATTTCGATGCTGG-3′.

Sequence analyses of bon^{m425} *and* bon^{s9}

Mutant genomic DNA was purified from 10 30 hpf embryos using the Nucleon ST Kits (Scotlab Bioscience). For sequencing the genomic locus, we performed three independent PCR reactions for each of six primer pairs designed to cover the *bon* locus, cloned the PCR fragments into pGEM-T (Promega), picked two independent clones from each PCR reaction, and sequenced the inserts using an ABI 377 DNA sequencer.

Linkage analysis

bon DNA fragments were amplified by PCR (for bon^{m425} 5′-CTTCAGGACAGGATGAAGTTCC-3′ and 5′-GAATTCTCGATTCTGGCAAGCC-3′ and for bon^{s9} 5′-CAGCTCTGATCATTTCTGCAGC-3′ and 5′-CTTTGTAAGTTCTCCAGGCCGC-3′). The PCR conditions were 94°C for 5 min (1 cycle), then 94°C for 30 sec, 61°C for bon^{m425} and 64°C for bon^{s9} for 30 sec and 72°C for 1 min (40 cycles), followed by 72°C for 5 min (1

cycle). Amplified products were digested by *Mse*I for bon^{m425} and *Stu*I for bon^{s9} and resolved on 2% agarose gels. The bon^{m425} mutation creates a new *Mse*I site and the bon^{s9} mutation a new *Stu*I site. Following in situ hybridization, embryos were genotyped as described above. Genotyping of *cyc;sqt* double mutants was done using published information (Feldman et al. 1998; Rebagliati et al. 1998).

mRNA injections

To introduce the *s9* mutation in *bon*, we performed site-directed mutagenesis using QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions and using the primer pair 5′-CCAGAATCGAGAATTCAGGC*CTG-GTTCCAGAACCGAAGG-3′ and 5′-CCTTCGGTTCTGGAAC-CAGG*CCTGAATTCTCGATTCTGG-3′ (* indicates the site of the mutation). The coding region of bon^{s9} was verified by sequencing both strands. Capped mRNA was transcribed in vitro from pCS2–*bon* or pCS2–bon^{s9} using the mMESSAGE mMACHINE system (Ambion). For overexpression experiments, 100 pg or 200 pg of mRNA was microinjected at the 1–4 cell stage.

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