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Cloning and Genetic Mapping of Zebrafish BMP-2

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ABSTRACT The BMP family of polypeptide growth factors has been shown to play diverse roles in establishing embryonic patterning and tissue fates. We report the cloning of the zebrafish homologue of BMP-2, examine its expression during embryogenesis, and find that it is localized to the distal end of the long arm of zebrafish chromosome 20. A missense mutation of the *bmp*2 gene has recently been shown to be responsible for the early dorsalized phenotype of the zebrafish *swirl* mutant [Kishimoto *et al.,* 1997]. Given the dynamic expression of *bmp*2 in the developing embryo and the complex interactions of BMP signaling response in vertebrates, it is possible that other mutant phenotypes, due to altered *bmp*2 gene expression, will eventually map to or interact with this genetic locus. Dev. Genet. 23:97–103, 1998. © 1998 Wiley-Liss, Inc.

Key words: bone morphogenetic protein; zebrafish; genetics

INTRODUCTION

The zebrafish, *Danio rerio,* has emerged as a model system particularly suited to the elucidation of gene programs underlying various developmental processes [Driever *et al.,* 1996]. Through a process of mutagenesis, phenotype screening, and positional cloning, investigators hope to identify novel gene products regulating early axis determination, germ-layer formation, organogenesis, and cell-type specification [Haffter *et al.,* 1996]. Mapping of candidate genes that function in conserved developmental processes may serve as a useful adjunct to these efforts. The bone morphogenetic proteins or BMPs, a sub-family of the TGF-β growth factor family, have been implicated in a broad variety of developmental processes conserved throughout vertebrate species, ranging from mesodermal induction [Dale *et al.,* 1992; Fainsod *et al.,* 1994; Schultheiss *et al.,* 1997; Smith, 1995] to organogenesis [Bellusci *et al.,* 1996; Hogan, 1996], neural crest cell differentiation [Lo *et al.,* 1997], and early embryonic patterning [Dale *et al.,* 1992; DeRobertis and Sasai, 1996; Ferguson, 1996; Graff, 1997; Hemmati-Brivanlou and Thomsen, 1995; Pourquie *et al.,* 1996; Reshef *et al.,* 1998]. Recently, much progress has been made in delineating the signaling pathways by which they influence gene expression and cell phenotype. Similar to other TGF-βs in vertebrates, the various BMPs signal through heterodimeric serine/threonine kinase receptors to phosphorylate intracellular homologues of the *Drosophila mad* gene product, collectively known as Smads [Attisano and Wrana, 1996; Heldin *et al.,* 1997; Hoodless *et al.,* 1996; Josso and DiClemente, 1997; Kretschmar *et al.,* 1997b; Lagna *et al.,* 1996; Liu *et al.,* 1996]. Upon phosphorylation, Smad proteins undergo a conformational change and translocate to the nucleus where they likely participate in the formation of specific transcriptional activation complexes [Lagna *et al.,* 1996; Liu *et al.,* 1996, 1997; Massague, 1997]. Other factors interact with BMPs and BMP signaling pathways to influence their tissue-specific effects during the course of early embryonic development. At the pre-receptor level, soluble anti-BMPs like chordin [Sasai *et al.,* 1994], noggin [Smith and Harland, 1992], or follistatin [Hemmati-Brivanlou *et al.,* 1994] directly antagonize BMP ligand interaction with receptor and may act to regulate the level of BMP signaling and corresponding tissue response [Pourquie *et al.,* 1996; Reshef *et al.,* 1998]. Determining specificity

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of action of the various BMPs in developmental processes will likely prove difficult given the many levels of gene interaction in BMP signaling, particularly in the cases of BMP-2, BMP-4, and BMP-7, which are often co-expressed during development [e.g., Schultheiss *et al.*, 1997]. As in mice, use of loss of function mutants in the zebrafish may provide information regarding the relative function of each of these factors and their signaling molecules [reviewed in Hogan, 1996].

We have recently cloned zebrafish *bmp*2, a TGF-β homologue most closely related in sequence and expression to previously cloned vertebrate BMP-2s. *bmp*2 is expressed early in prospective ventral mesoderm of zebrafish embryos, as well as in multiple tissues and organs late in development. We have found in previous studies that a missense mutation resulting in expression of an altered *bmp*2 gene product selectively effects the early induction of ventral mesodermal derivatives [Kishimoto *et al.*, 1997]. We report here the cloning of *bmp*2, its expression through development, and its map position on the distal end of the long arm of chromosome 20.

MATERIALS AND METHODS

Cloning and Characterization of Zebrafish *bmp* Clones

cDNA and genomic fragments representing TGF-β homologues were amplified from zebrafish genomic DNA or cDNA from 18 hours postfertilization (hpf) zebrafish embryos using the degenerate oligonucleotides: Forward: 5′-TGGAATTCTGG (G/A/C)A(G/A/T/C)GA(T/C)TGGAT(A/T/C)(G/A)T(G/A/T/C)GC-3′ and Reverse: 5′-GAGGATCCA(G/A)(G/A/T/C)GT(T/C)TG(G/A/T/C)AC(G/A/T)AT(G/A/T/C)GC(G/A)T G-3′. This encodes the conserved TGF-β peptide motifs W(N/D/Q)DWI(V/I)A and HAIVQTL (amino acids 315–321 and 347–352) of *Xenopus* BMP-2, [for reference, Nishimatsu *et al.*, 1992; Plessow *et al.*, 1991] and 5′ *Eco* R1 and 3′ *Bam* H1 restriction sites. Amplifications used Taq DNA polymerase (Perkin-Elmer, Foster City, CA) using standard buffer adjusted to 1.5 mM Mg^{2+} for 30 cycles at 55°C annealing. Amplified fragments of appropriate size were cloned into the *Eco* R1 and *Bam* H1 sites of pBluescript (Stratagene, LaJolla, CA) for sequencing. cDNA fragments representing various TGF-β homologues were excised by restriction digestion from Bluescript, radiolabelled, and used to screen 1×10^6 recombinants from UNIZAP directional cDNA libraries representing mRNA from 12 and 24 hpf zebrafish embryos (kind gift of K. Zinn) by standard methods [Benton and Davis, 1977]. Hybridization filters were washed at high stringency, i.e., 0.5 × SSC/0.5% SDS up to 55°C, exposed to Kodak XAR film (Kodak, Rochester, NY) from 12–72 hours, and 5′ ends of candidate lambda clones for *bmp*2 were additionally characterized by PCR cloning using an exact sequence oligonucleotide derived from the original PCR fragment (5′GGCCGCGGCCGCGTGGAGTTTAGATGGTCC-3′, containing a Not I cloning site and nt 735–752 of the zebrafish cDNA) and the T7 Bluescript flanking oligonucleotide 5′-AATACGACTCACTATAG-3′ (Stratagene) (50°C annealing, 30 cycles, 1.5 mM $MgCl_2$). Plasmids bearing cDNAs were obtained from purified clonal phage stocks via M13 helper phage and an in vivo excision protocol (Stratagene). Cloned cDNAs were sequenced using the Sanger dideoxynucleotide method and sequential cDNA-specific oligonucleotides with an ABI Model 373A automated sequencer (Perkin Elmer). Homologies to other vertebrate genes were first determined using the BLASTP algorithm on the National Center for Biotechnology Information database [Altschul *et al.*, 1990] on the SwissProt peptide sequence database. Peptide alignment to other vertebrate BMPs was calculated using the Pileup algorithm available on the Genetics Computing Group (GCG) program database, version 8.1 [Devereux *et al.*, 1985].

In Situ Hybridization

In situ mRNA analysis was performed largely as described by Schulte-Merker *et al.* [1994], with some modifications. Following hybridization of digoxigenin labeled RNA probes and washing, embryos were blocked in 10% heat-treated lamb serum and 2% BMB block (Boerhinger Mannheim, Indianapolis, IN) in 100 mM maleic acid (pH 7.5), 150 mM NaCl and 0.01% Tween20 (Sigma, St. Louis, MO) prior to secondary antibody detection with alkaline phosphatase-conjugated antidigoxigenin Fab. Embryos were mounted in 70% glycerol/30% PBS for photography with Kodak SP64T tungsten film.

Chromosomal Localization

Primers 5′ GTCCAGCCCTATCTCACTGC-3′ (forward) and 5-TTTCTTCCTCCAAAATAGCTCG-3′ (reverse) were designed to amplify a region corresponding to a 920 bp portion of the *bmp*2 gene (nt 753–1673) that was polymorphic for single-strand conformation polymorphisms (SSCPs) between zebrafish strains C32 and SJD. These were used to analyze DNA prepared from a haploid mapping panel [Johnson *et al.*, 1996] as previously described [Beier *et al.*, 1993; Brady *et al.*, 1997]. Briefly, oligonucleotides were radiolabeled with ^{32}P-ATP using polynucleotide kinase and used to amplify genomic DNA fragments from a series of zebrafish strains using standard PCR protocols (95°C denaturation for 1 min, 55°C annealing for 1 min, 72°C extension for 2 min, final extension at 72°C; 40 cycles with standard PCR buffer and 1.5 mM Mg^{2+}). Of this reaction, 2 μl was added to 8.5 μl formamide stop solution (USB, Cleveland, OH), denatured at 94°C for 5 min, and immediately chilled on ice. Of the resulting samples, 2 μl were analyzed on a 5% nondenaturing polyacrylamide TBE sequencing gel (electrophoresis at 40 watts constant power at 4°C). Segregation of this SSCP polymorphism was compared to those of informa-

tive RAPD [Johnson *et al.*, 1996; Postlethwaite *et al.*, 1997] and microsatellite [Knapik *et al.*, 1996] markers and mapped mutations [Postlethwaite *et al.*, 1997]. Recombination between loci analyzed by SSCP and closely linked loci was calculated using the program Map Manager Classic repeated at the 99.9% confidence limit and the resulting analysis exported into Map-Maker for drawing [Lander *et al.*, 1987; Manley and Cudmore].

RESULTS AND DISCUSSION

Initial PCR amplification yielded four independent 116 bp (128 bp including restriction sites) fragments with highest homology to *Xenopus* BMP-2 and BMP-4, mouse GDF-7, and human BMP-5 [Celeste *et al.*, 1990; Nishimatsu *et al.*, 1992; Storm *et al.*, 1994]. Initial screening of 12 and 24 hpf zebrafish cDNA libraries with radiolabelled probes derived from these sequences yielded positive results only for the fragment with homology to *Xenopus* BMP-2, and these were further pursued. Of 16 primary positives, five were found by PCR and secondary hybridization to contain *Xenopus* BMP-2-related cDNA fragments of varying lengths. The two longest were further cloned and characterized and found to contain overlapping cDNA fragments encoding a 411 amino acid protein with high homology to vertebrate BMP-2 and BMP-4 (Fig. 1a). Whereas *bmp*2 is more similar in sequence conservation to previously characterized BMP-2 genes than BMP-4 genes, it is also apparent that the fish gene is fairly divergent from that of other vertebrate BMP-2/4s (see esp. residues 234–239 and 301–304) (Fig. 1b).

This cDNA is identical in sequence to that obtained by Nikaido *et al.* [1997] and in situ hybridization data obtained with this cDNA are generally in good agreement with their data (Fig. 2). At 24 hpf, we similarly find *bmp*2 to be expressed in the developing eye and otic vesicles and tailbud or caudal fin regions. In addition to these expression patterns, we also detect expression of *bmp*2 in the anterior lateral mesendoderm at stages intermediate to those examined by Nikaido *et al.* [1997], from which will develop both cardiac and pharyngeal endodermal structures [Chen and Fishman, 1996; Lee *et al.*, 1996; Stanier *et al.*, 1993]; we subsequently also detect *bmp*2 in anterior mesendoderm including pectoral fin primordia at 24 hpf. Nikaido *et al.* [1997] detect only *bmp*4 in heart and fin primordia near this region; discrepancies may be due to our omission of RNAse (see Materials and Methods) in the in situ hybridizations, which affords greater sensitivity in the detection of *bmp*2 mRNA. BMP-2 in the chick appears to be a specific inducer of cardiac mesoderm [Schultheiss *et al.*, 1997], and cardiac and anterior mesendoderm appear to be lost in *swirl* mutants [Kishimoto *et al.*, 1997]. However, whether this is due to loss of a specific induction following gastrulation as found in chick, or whether this is due to a broader lack of ventral mesoderm and endoderm arising from an earlier step in development remains unclear.

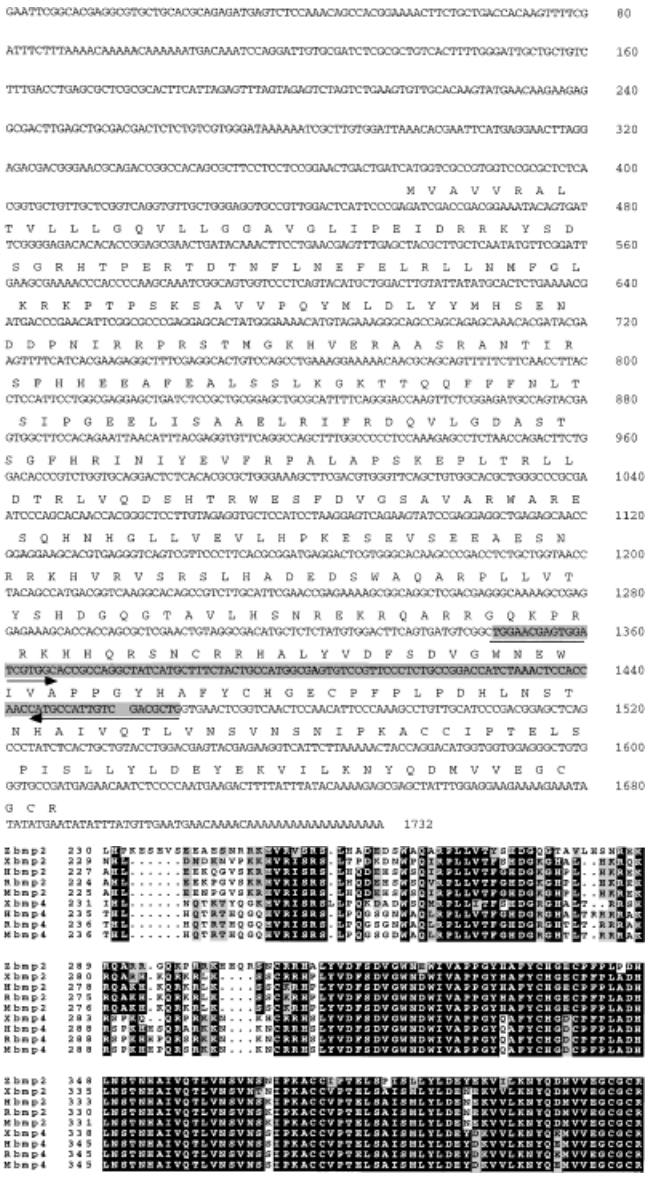


Fig. 1. (a) Nucleotide sequence and conceptual translation of *bmp*2 cDNA. Translation is shown in single letter amino acid abbreviation underneath corresponding nucleotide sequence. An initiating methionine codon with good upstream Kozak [1987] consensus sequence is found at nucleotide position 376 at the start of the longest open reading frame. The location of the 116 bp degenerate PCR product amplified by the original degenerate primers is shown in the gray box; sequences corresponding to priming sites for degenerate nucleotide primers are shown underlined with arrows. Conceptual translation predicts a 411aa polypeptide of 46.9 kD molecular weight. **(b)** Alignment of conserved c-terminal 178 aa of *bmp*2 product with corresponding regions of known vertebrate BMP-2 and BMP-4 gene products. Amino acid identities are boxed in black; conservative changes are boxed in gray [Celeste *et al.*, 1990; Chen *et al.*, 1993; Dickinson *et al.*, 1990; Feng *et al.*, 1994; Kurihara *et al.*, 1993; Wozney *et al.*, 1988].

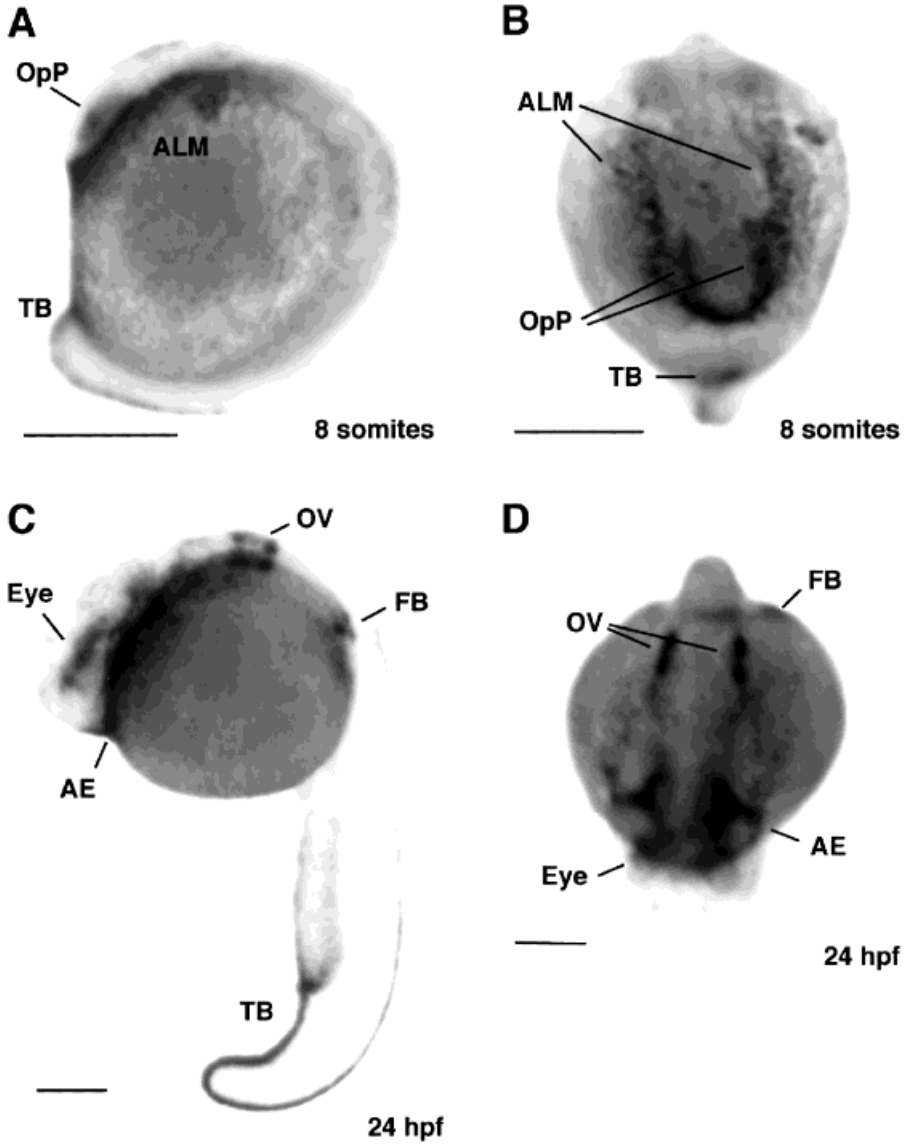
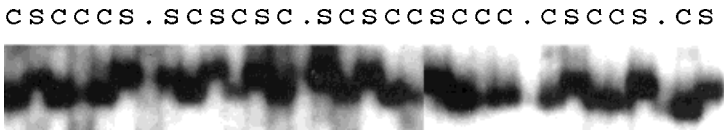


Fig. 2. In situ mRNA localization analysis of *bmp*2. Lateral **(A)** and dorsal **(B)** views of in situ hybridization of *bmp*2-derived digoxigenin RNA probes to 8 somite (13 hpf) wild-type AB zebrafish embryos. Lateral **(C)** and dorsal **(D)** views of in situ hybridization of *bmp*2 probe to 24 hpf zebrafish embryos. Abbreviations: AE: anterior endoderm; ALM: anterior lateral mesendoderm; FB: fin bud; OpP: Optic placode; OV: Otic vesicle; TB: tail bud. Scale bar = 250 μm. Staging is as in Westerfield [1995].

The chromosomal location of *bmp*2 was established via single-stranded conformational polymorphism (SSCP) analysis using a strategy similarly employed for the analysis of mouse interspecific crosses [Beier *et al.*, 1993; Brady *et al.*, 1997]. PCR products amplified from genomic DNA using primers corresponding to coding sequences and 3′UTR of *bmp*2 identified an SSCP between C32 and SJD, as shown in Figure 3a. C32 and SJD are the parental strains used to create a haploid zebrafish mapping panel contributing to the characterization of 650 genetic markers [Johnson *et al.*, 1996; Postlethwaite *et al.*, 1997]. This panel was genotyped with respect to the *bmp*2 SSCPs and the allele distribution pattern analyzed using the Map Manager program (Manley and Cudmore). Backcross statistics at the 95% and 99% confidence limits for the three closest markers are shown in Table 1 and relative positions displayed graphically in Figure 3b. *bmp*2 was found to map to the distal end of linkage group 20 [Postlethwaite *et al.*, 1997] with a LOD likelihood score of 9.6, with 13 recombinants found between *bmp*2 and 14P1500 (a RAPD marker) in 83 scored progeny. The position of *bmp*2 with respect to flanking markers is 14p1500 − 15.7 ± 3.99 cM − *bmp*2 − ter.

Mapping of *bmp*2 also positions the zebrafish *swirl* mutation. Early work in *Xenopus* has implicated BMP-4 or a BMP-like activity as a key factor for dorsal-ventral patterning and the generation of ventral mesoderm [Dale *et al.*, 1992; Fainsod *et al.*, 1994]. The zebrafish *swirl* mutant lacks ventral mesoderm and as a result appears relatively dorsalized [Hammerschmidt *et al.*, 1996; Mullins *et al.*, 1996]. Our recent studies have demonstrated close linkage (<1.3 cM) between *bmp*2 and the dorsalizing swr^{ta72} mutation and have shown



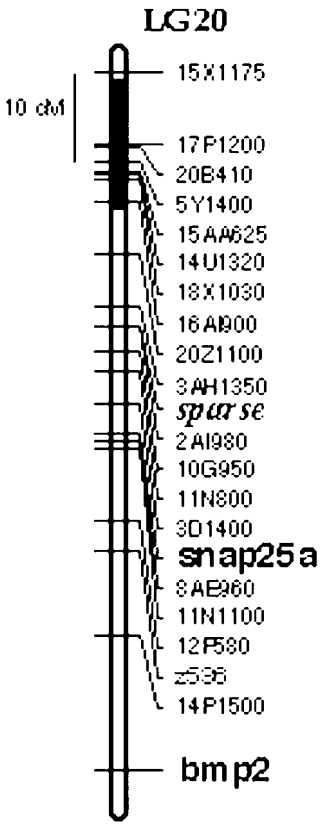


Fig. 3. Localization of *bmp*2. **(a)** Representative sample SSCP gel showing distinct migration of single-stranded PCR products from C32 (C) and SJD (S) alleles for a subset of analyzed genomic DNA samples. **(b)** Graphical representation of *bmp*2 relative to zebrafish linkage group 20. *bmp*2 is shown on distal end of chromosome 20, in reference to relative positions of mapped mutations, RAPD and microsatellite markers. Centromere is at top, telomere at bottom. Mapped zebrafish mutations are shown in color; anonymous markers are shown in black.

TABLE 1. Backcross Statistics for Haploid Progeny of C32/SJD Hybrid Female*

	Mat	Pat	X	N	Map	SE	95%	99%	LOD
12F.580	38	45	3	75	4.0	2.26	0.8–11.2	0.5–13.9	17.1
9E.550	37	48	10	82	12.19	3.61	6.0–21.3	4.7–24.3	11.5
14p.1500	44	48	12	81	14.81	3.95	7.9–24.4	6.3–27.6	9.6
BMP2	40	45							

*Recombination between loci analyzed by SSCP and closely linked markers as analyzed by Map Maker Classic [Lander *et al.*, 1987; Manley and Cudmore]. Markers shown to left. Mat: Maternal (C32) allele; Pat: Paternal (SJD) allele; X: crossovers; N: number of progeny analyzed; Map: recombination distance; SE: standard error; 95%: 95% confidence interval; 99%: 99% confidence interval; LOD: Log odds distribution score.

that *bmp*2 contains a base substitution converting the termination codon into a tryptophan codon (TGA → TGG) and extending the open reading frame an additional 18 nucleotides. A second *swirl* allele swr^{tc300} similarly contains a base substitution resulting in a cysteine to tryptophan substitution (TGT → TGG) in BMP2. Consistent with the ability of ectopic BMP2 to ventralize wild-type zebrafish embryos [Hammerschmidt *et al.*, 1996; Nikaido *et al.*, 1997], relatively dorsalized *swirl* mutants can be rescued by early injection of synthetic wild-type, but not mutant *bmp*2 RNA. In fact, injection of synthetic mRNA bearing the *swirl* mutation results in dorsalized embryos, indicating that the mutant BMP2 acts in a dominant negative fashion in early dorsoventral patterning [Kishimoto *et al.*, 1997].

As previously discussed, potential exists for diverse interactions between coexpressed TGF-β signaling elements at multiple levels. Receptors appear to have overlapping specificities for multiple ligands [Attisano and Wrana, 1996; Massague, 1997], and Smad molecules can either be restricted to particular pathways, or form a common component of multiple TGF-β signaling complexes, in both stimulatory and inhibitory roles [Hata *et al.*, 1998; Hayashi *et al.*, 1997; Lagna *et al.*, 1996; Liu *et al.*, 1996; Liu *et al.*, 1997; Nakao *et al.*,

1997; Nakayama *et al.*, 1998]. Interestingly, Smads also appear capable of interpreting signals from other phosphorylation pathways as well, including mitogenic MAP kinase pathways [Kretschmar *et al.*, 1997a]. Thus cell decisions regarding the proliferation or differentiation of a particular cell in response to BMP stimulation could be highly dependent upon both the molecular context of BMP signals, and the modulated level of BMP signaling present [Massague, 1997; Pourquie *et al.*, 1996; Reshef *et al.*, 1998].

In this vein, it is of interest that despite the spatiotemporally complex expression of *bmp*2 in the developing embryo, early ectopic expression of normal BMP2 allows for the development of otherwise healthy and fertile adult fish, implying that early dorsoventral patterning is especially sensitive to the *swirl* mutation of *bmp*2 [Kishimoto *et al.*, 1997]. It remains to be seen whether other distinct mutations of BMP molecules themselves underlie similarly discrete phenotypes due to interactions with pathways sensitive to particular aspects of BMP signaling or kinetics. Given the diversity of gene products involved in or affecting BMP signaling in vertebrate cells, a number of distinct mutations may well manifest similar phenotypes. As other zebrafish developmental mutants are genetically characterized, knowledge of the characteristics and map locations of candidate genes like *bmp*2 may help to more quickly define mutant or interacting loci.

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Sequence of the *bmp*2 cDNA clone referenced here is available through GenBank Accession Number AF072456.

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