DEVELOPMENTAL BIOLOGY **199,** 93–110 (1998)
ARTICLE NO. DB988927

Ventral and Lateral Regions of the Zebrafish Gastrula, Including the Neural Crest Progenitors, Are Established by a *bmp2b*/*swirl* Pathway of Genes

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A bone morphogenetic protein (BMP) signaling pathway is implicated in dorsoventral patterning in *Xenopus*. Here we show that three genes in the zebrafish, *swirl, snailhouse,* and *somitabun,* function as critical components within a BMP pathway to pattern ventral regions of the embryo. The dorsalized mutant phenotypes of these genes can be rescued by overexpression of *bmp4, bmp2b,* an activated BMP type I receptor, and the downstream functioning *Smad1* gene. Consistent with a function as a BMP ligand, *swirl* functions cell nonautonomously to specify ventral cell fates. Chromosomal mapping of *swirl* and cDNA sequence analysis demonstrate that *swirl* is a mutation in the zebrafish *bmp2b* gene. Interestingly, our analysis suggests that the previously described nonneural/neural ectodermal interaction specifying the neural crest occurs through a patterning function of *swirl/bmp2b* during gastrulation. We observe a loss in neural crest progenitors in *swirl/bmp2b* mutant embryos, while *somitabun* mutants display an opposite, dramatic expansion of the prospective neural crest. Examination of dorsally and ventrally restricted markers during gastrulation reveals a successive reduction and reciprocal expansion in nonneural and neural ectoderm, respectively, in *snailhouse, somitabun,* and *swirl* mutant embryos, with *swirl/bmp2b* mutants exhibiting almost no nonneural ectoderm. Based on the alterations in tissue-specific gene expression, we propose a model whereby *swirl/bmp2b* acts as a morphogen to specify different cell types along the dorsoventral axis.

INTRODUCTION

Through a combination of molecular, genetic, and biochemical experiments a BMP signal transduction pathway is being unraveled (reviewed in Baker and Harland, 1997; Massagué, 1996). The BMP ligands (members of the TGF-β superfamily) bind type II and type I transmembrane serine–threonine kinase receptors. The BMP ligand-bound type II receptor phosphorylates and activates a type I receptor. The activated type I receptor phosphorylates an intracellular Smad protein, which then translocates as a heteromeric complex into the nucleus, where it is involved in transcriptional activation. Components of a BMP signaling pathway have been implicated in the specification of ventral cell fates in *Xenopus* (reviewed in Graff, 1997; Hogan, 1996; Thomsen, 1997). Overexpression of BMP2, BMP4, an activated BMP type I receptor (Suzuki *et al.*, 1997), or Smad1 can ventralize wild-type embryos. Dominant negative forms or antisense expression of BMP2, BMP4, BMP7, or the BMP type I receptor have the opposite effect to dorsalize the embryo.

Recent molecular and biochemical experiments reveal a mechanism whereby the Noggin, Chordin, and Follistatin proteins, secreted from the Spemann organizer, bind BMP4 in dorsal regions, thus preventing it from binding its receptor (Fainsod *et al.*, 1997; Piccolo *et al.*, 1996; Zimmerman *et*

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0012-1606/98 $25.00

al., 1996). Different doses of either BMP4 or Noggin can shift the specification of cells to more ventral or dorsal fates, respectively, suggesting that a modulation of BMP4 levels specifies different tissue types along the dorsoventral axis (Dosch *et al.,* 1997; Knecht and Harland, 1997; Neave *et al.,* 1997; Wilson *et al.,* 1997). Further modulation of BMP activity is likely mediated by the metalloprotease Tolloid/Xolloid, which ventralizes the embryo when overexpressed and has recently been shown to cleave Chordin, thus releasing BMP4 to bind its receptor (Blader *et al.,* 1997; Piccolo *et al.,* 1997).

In *Drosophila,* the specification of the dorsoventral axis depends on a set of genes related to those described in *Xenopus* (reviewed in Bier, 1997; Massagué, 1996). These genes include the BMP4/2 counterpart, *dpp;* the *dpp* antagonist *short gastrulation (sog),* related to *chordin; tolloid* (Marqués *et al.,* 1997); the *dpp* receptors *thick veins, punt,* and *saxophone;* and the downstream acting *Mad* gene. These molecular and functional homologies indicate a conservation of mechanisms establishing dorsal cell fates in *Drosophila* and ventral cell fates in vertebrates (De Robertis and Sasai, 1996; Hogan, 1995).

In *Xenopus laevis* it is not currently possible to study loss-of-function mutations. To determine the endogenous roles of several of the genes that show dorsalizing and ventralizing activity in *Xenopus,* null mutations have been produced in their mouse orthologs. These mutations include *BMP2* (Zhang and Bradley, 1996), *BMP4* (Winnier *et al.,* 1995), *BMP7* (Dudley *et al.,* 1995), a BMP type I receptor (Mishina *et al.,* 1995), *goosecoid* (Rivera-Perez *et al.,* 1995; Yamada *et al.,* 1995), and *follistatin* (Matzuk *et al.,* 1995). Surprisingly, none of these mutations reveals a clear role in dorsoventral patterning. The primary defect found in the BMP4 and the BMP receptor mutants is arrest of development at the egg cylinder stage, a stage prior to overt dorsoventral patterning. A small number of BMP4 mutants survive beyond this stage and exhibit defects in ventral and posterior mesoderm (Winnier *et al.,* 1995). It is possible that BMP4 and the BMP type I receptor specify ventral cell fates, but also function at an earlier stage in development, precluding an analysis of their later functions.

In zebrafish at least eight genes when mutated exhibit dorsoventral axis defects. A ventralized mutant, *dino* (Hammerschmidt *et al.,* 1996a), also known as *cerebum* (Fisher *et al.,* 1997) and *captain hook* (Solnica-Krezel *et al.,* 1996), has recently been shown to encode *chordin* and renamed *chordino* (Schulte-Merker *et al.,* 1997). Mutations in six genes exhibit dorsalized mutant phenotypes: *swirl (swr), somitabun (sbn), snailhouse (snh), piggytail, lost-a-fin,* and *mini fin* (Mullins *et al.,* 1996). These mutants display a series of dorsalized phenotypes, which can be divided into five classes where class 5 is the strongest phenotype and class 1 the weakest (Mullins *et al.,* 1996, Figs. 2G–2L). We focused on the analysis of the strongest dorsalized mutants, *swirl, snh,* and *sbn. sbn* is a completely penetrant dominant maternal and zygotic mutation that exhibits both class 5 (the strong *sbn* phenotype) and class 4 (weak *sbn)* mutant embryos. *swirl* and *snh* are zygotically acting genes displaying class 5 and 4 phenotypes, respectively.

Here we present data indicating that *swirl, snh,* and *sbn* function within a BMP signaling pathway to establish ventral regions of the embryo. We demonstrate that *swirl* functions cell nonautonomously and is a mutation in the zebrafish *bmp2b* gene. *swirl, snh,* and *sbn* function to maintain the expression of *bmp2b* and *bmp4* in the zebrafish embryo, but do not establish their initial expression domains. We show that *swirl* and *sbn* specify most or all nonneural ectodermal and ventral mesodermal derivatives, while the *snh* mutation affects a smaller ventral ectodermal and mesodermal domain of the fate map. Specification of the neural crest and placodal tissues, which form at the neural/nonneural ectodermal boundary, also depends on the function of *swirl/bmp2b.* Based on the alterations in gene expression in these three mutants, we propose a model whereby *swirl/bmp2b,* functioning as a morphogen, differentially regulates gene expression along the dorsoventral axis, thus specifying different cell types.

MATERIALS AND METHODS

Fish maintenance and breeding. Maintenance of fish and breeding were done as described by Mullins *et al.* (1994) and Haffter *et al.* (1996).

Whole-mount in situ hybridization. *In situ* hybridizations were performed as described by Schulte-Merker *et al.* (1992), with modifications by C. Houart (personal communication). The following probes were used: *gsc* (Stachel *et al.,* 1993), *gata1* and *gata2* (Detrich *et al.,* 1995), *fkd3* and *fkd6* (J. Odenthal, unpublished), *bmp2b* (*zbmp-2*) (Nikaido *et al.,* 1997), *bmp4* (Chin *et al.,* 1997), *dlx3* (Akimenko *et al.,* 1994), *AP-2* (Fürthauer *et al.,* 1997), *otx2* (Li *et al.,* 1994), *krox 20* (Oxtoby and Jowett, 1993), and *sna2* (Thisse *et al.,* 1995). Embryos were mounted and viewed using Nomarski optics on a Zeiss Axioskop. Images were acquired via a digital (Kontron) camera, saved on a Macintosh computer, and processed with Adobe software.

Embryos were obtained from matings between heterozygous fish. *swirl* and *snh* mutant embryos were determined at bud or later stages of development by their abnormal morphology or at earlier stages by 25% of the embryos exhibiting an altered staining pattern. For *sbn,* all of the progeny of a cross are mutant, due to the dominant maternal effect. Two classes of aberrations were typically observed in *sbn* mutant embryos. The embryos that showed alterations more similar to *swirl* were classified as strong, and those more similar to *snh* were classified as weak.

Assay for apoptotic cells. Detection of cell death in fixed whole-mount embryos was performed using a terminal transferase assay (Apotaq Kit-peroxidase, Oncor, Inc.) according to the manufacturer's instructions, with minor modifications described in Fisher *et al.* (1997). Positive controls consisted of five embryos from each batch that were pretreated with 1 N HCl for 15 min at 37°C and then processed together with untreated sibling embryos.

mRNA injections. Plasmids of p64Tzbmp2, p64Tzbmp4 (Nikaido *et al.,* 1997), and p64TXbmp7 (Nishimatsu *et al.,* 1992) were linearized with *Bam*HI. The activated form of the BMP type I receptor, pCMV5-Alk6/HA (Hoodless *et al.,* 1996), was subcloned into the *Eco*RI and *Xba*I sites of the CS2+ vector (Rupp *et al.,* 1994)

and linearized with *Not*I. p64TEN-Xmad1 (Graff *et al.,* 1996) was linearized with *Xba*I. All linearized constructs were *in vitro* transcribed with the SP6 mMessage mMachine Kit (Ambion). Synthetic mRNA was injected into the yolk of one- to four-cell-stage embryos as described in Westerfield (1995).

Cell transplantations. Cell transplantations were performed as previously described by Ho and Kane (1990) and van Eeden *et al.* (1996), with the following modifications. Donor embryos for the transplants were injected with the vital dye rhodamine–dextran and the fixable tracer biotin–dextran at the one- to four-cell stage. Transplantations were performed between high and dome stages, a time point before which the cells are committed to a cell fate and hence are susceptible to a new environment. Pipets were prepared with a micropipet puller (Sutter Instruments, Model P-87), broken off to a diameter of 40–55 μm. Pipets were attached via tubing to a 1-cc syringe filled with air. Five to 15 labeled cells were transplanted into an unlabeled host embryo. Rhodamine-labeled cells were examined by fluorescence on a Zeiss Axioskop. Twenty-six to 28-h embryos with circulating fluorescent blood were mounted in methylcellulose and anesthetized in Tricaine so that the blood cells stopped circulating. Photographs of fluorescent donor cells and their counterpart bright-field picture were merged using Adobe Photoshop. Some chimeras were examined at shield stage for the dorsoventral position of wild-type donor cells; chimeras containing ventrally located donor cells were processed separately. A subset contained *gata1*-expressing *swirl* mutant cells. Wild-type cells in a *swirl* mutant host were visualized by staining for biotin in embryos fixed at the eight-somite stage. Biotin-labeled cells were detected following *in situ* hybridization by washing embryos in PBS plus 0.1% Tween 20, four times for 10 min, incubating embryos in ABC (Vectastain Kit, used according to manufacturer's instructions) diluted in newborn calf serum/PBST for 1 h, washing four times for 10 min with PBST, and developing with diaminobenzidine.

Southern hybridization. Embryonic DNA was isolated from pools of 50 embryos by standard procedures (Sambrook *et al.,* 1989). Approximately 5 μg of DNA was digested with the restriction enzyme *Bgl*II and run on 0.7% agarose gels. DNA was transferred onto nylon membranes as previously described (Sambrook *et al.,* 1989). Labeling of the *bmp4* probe and hybridization were done as described in Herrmann *et al.* (1986). Blots were analyzed with a Storm 860 (Molecular Dynamics) machine and images were processed with Adobe software.

Chromosomal mapping. Crosses between Tübingen fish carrying the swr^{ta72}, sbn^{dtc24}, or snh^{ty68a} mutations and polymorphic WIK or AB fish were used for mapping. The mapping procedure and the WIK line used are described in Rauch *et al.* (1997) and Knapik *et al.* (1996). We analyzed the SSLP markers by agarose gel electrophoresis analysis (Rauch *et al.,* 1997). The PCR conditions were modified as follows: 94°C for 1 min, 5 cycles of 94°C for 30 s, 54°C for 2 min, and 73°C for 1 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 73°C for 1 min.

Mapping of *bmp2b* was performed using a 698-bp *zbmp-2* fragment [corresponding to positions 1 to 629 in the sequence deposited in Genbank under Accession No. U82232 and extending 69 bp further upstream (Martinez-Barbera *et al.,* 1997; Nikaido *et al.,* 1997)] kindly provided by M. Tada and N. Ueno. It was radiolabeled using the random hexamer procedure and used as a probe to analyze DNA from a collection of zebrafish/mouse somatic cell hybrids (Ekker *et al.,* 1996) by hybridization following Southern transfer. Ten micrograms of DNA from each hybrid was used and equivalent DNA amounts from the zebrafish ZF4 and mouse B78 parental cell lines were used as positive and negative controls, respectively.

Cloning of swr^{tc300} and swr^{ta72}. Mutant embryos from crosses between either swr^{ta72} or swr^{tc300} heterozygous fish were collected at the bud stage, when the mutant phenotype is easily distinguishable from wild type. Wild-type control embryos were collected at bud stage. Total RNA was extracted by shaking 100 embryos with 2 g glass beads (Sigma) in 2 ml guanidinium thiocyanate buffer by standard procedures (Sambrook *et al.,* 1989). First-strand cDNA was synthesized from total RNA with the Superscript Preamplification System for First Strand Synthesis (Life Technologies) according to the manufacturer's instructions. Two to three independent PCR were performed with the primer pairs M11/M14 (amplifying the entire coding region), M11/M12 (amplifying the 5′ coding region), and M13/M14 (amplifying the 3′ coding region) on cDNA of the wild-type, swr^{ta72}, and swr^{tc300} pool. Primers used were M 11, 5′-GAGGAACTTAGGAGACGAC-′3; M 12, 5′-GCGTAAAA-GTCCCTGGTT-3′; M 13, 5′-GCAGAGCAAACACGATACG-3′; and M 14, 5′-CTCGCTGATAAAACCTCC-3′. PCR was done as described by Postlethwait *et al.* (1994). The PCR products were subcloned with the TA-PCR cloning kit (Invitrogen) according to the manufacturer's instructions. We sequenced at least three independent PCR clones to rule out mutations caused by PCR artifacts. The sequence was analyzed using MacMolly software.

RESULTS

Maintenance of bmp2b and bmp4 Expression Depends on swirl, somitabun, and snailhouse

A BMP signaling pathway is implicated in the specification of ventral cell fates in *Xenopus.* In zebrafish three genes related to BMP2/4 have been identified: *bmp4* (Chin *et al.,* 1997; Nikaido *et al.,* 1997), *bmp2a* (Martinez-Barbera *et al.,* 1997), and *bmp2b* (Martinez-Barbera *et al.,* 1997; Nikaido *et al.,* 1997; *bmp2b* is *zbmp-2* in Nikaido *et al.,* 1997).[3] *bmp2b* and *bmp4* are expressed during gastrulation (Chin *et al.,* 1997; Nikaido *et al.,* 1997), while *bmp2a* is not detectable by *in situ* hybridization (Martinez-Barbera *et al.,* 1997). Both *bmp4* and *bmp2b* are expressed in a ventral domain by shield stage in wild-type embryos, but additionally *bmp4* is found dorsally in the shield and *bmp2b* in the dorsal margin at slightly later stages (Chin *et al.,* 1997; Nikaido *et al.,* 1997).

We investigated whether *swirl, sbn,* and *snh* regulate the expression of the *bmp4* or *bmp2b* genes through whole-mount *in situ* hybridization analyses. The expression of *bmp4* and *bmp2b* appears unchanged or slightly reduced at early shield stage in all three mutants (data not shown). By 60% epiboly *bmp2b* expression is reduced in *swirl, sbn,* and *snh* mutants (Figs. 1A–1C, data not shown). Similarly, the ventral expression domain of *bmp4* is no longer visible in *swirl* (as previously reported by Hammerschmidt *et al.,* 1996b) and *sbn* mutants at 60% epiboly or by 65% epiboly in *snh* mutant embryos, while the dorsal domain is normal

[3] Each group abbreviated differently the names of these genes. Here we follow the zebrafish nomenclature conventions (Mullins, 1995) in referring to these genes.

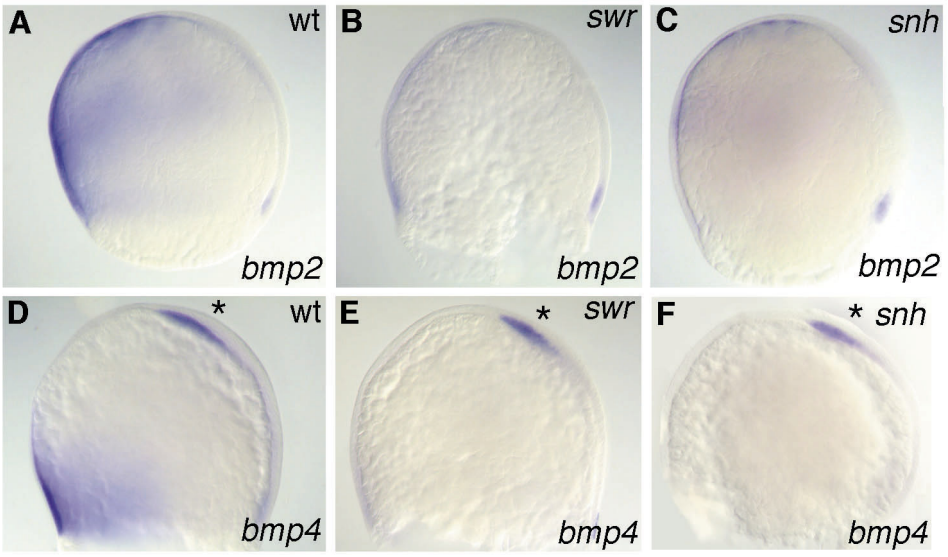

FIG. 1. *swirl* and *snh* are required to maintain wild-type levels of expression of *bmp2b* and *bmp4* in ventral regions of the embryo. (A–C) *bmp2b* expression at 80% epiboly in wild-type, *swirl,* and *snh* embryos. (D–F) Expression of *bmp4* at 70% epiboly in wild-type, *swirl,* and *snh* embryos. Lateral views, with dorsal to the right and animal pole up. Asterisks (*) mark the dorsal domains of *bmp4* expression.

(Figs. 1D–1F, data not shown). Throughout the rest of gastrulation the dorsal expression domains of *bmp2b* and *bmp4* remain normal in these mutants, while the ventral expression domains are strongly reduced and absent, respectively (data not shown). These data indicate that *swirl, sbn,* and *snh* do not establish the initial expression domains of *bmp2b* or *bmp4,* but function to maintain their expression in ventral regions shortly after the onset of gastrulation.

Rescue of swirl, somitabun, and snailhouse by BMP Pathway Components

The fact that *swirl, sbn,* and *snh* regulate the expression of *bmp2b* and *bmp4* suggests a function for these genes within a BMP signaling pathway. To examine whether *swirl, sbn,* and *snh* act via a BMP pathway to specify ventral cell types, we tested the ability of the BMP ligands, an activated Bmp type I receptor, and the *Smad1* gene to rescue the dorsalized mutant phenotypes. We expected BMP pathway components functioning downstream of the mutant gene to rescue the dorsalized mutant phenotype, while upstream-acting genes may not. For each BMP pathway gene, we first assessed the ability of *in vitro* synthesized mRNA to ventralize wild-type zebrafish embryos. We titrated the amount of mRNA injected to partially rescue *swirl* and *snh* mutants in order to unambiguously identify them, since these mutants when rescued to a wild-type or ventralized phenotype cannot be distinguished morphologically from their wild-type siblings.

We examined whether the zebrafish *bmp2b* and *bmp4* genes (Nikaido *et al.,* 1997) and the *Xenopus* BMP7 (XBMP7) gene (Nishimatsu *et al.,* 1992) can rescue *swirl, sbn,* or *snh* mutant embryos. Overexpression of these BMPs in wild-type zebrafish embryos produced a range of ventralized phenotypes (*bmp2b* in Figs. 2A–2E, data not shown) (Nikaido *et al.,* 1997). Injection of equivalent or lower amounts of any one of these BMP mRNAs into *swirl, sbn,* and *snh* mutant embryos partially or completely rescued the dorsalized mutant phenotypes. *swirl* mutants, which exhibit the strongest dorsalized phenotype, die by the 14-somite stage (16 h of age) (Figs. 2F and 2G). In contrast, nearly all *swirl* mutant embryos injected with either *bmp2b* or *bmp4* survive beyond this stage (100 and 99% of the mutants, respectively, Table 1) and were either partially (Figs. 2H and 2I) or fully rescued or slightly ventralized. These results are consistent with previous data showing partial rescue of *swirl* mutants by the *Xenopus* BMP4 gene (Hammerschmidt *et al.,* 1996b). Injection of XBMP7 mRNA also partially rescued *swirl* mutant embryos (76% of the mutants, Table 1). We found that the *Xenopus* BMP2, BMP4, and BMP7 genes were less effective than the zebrafish *bmp2b* or *bmp4* genes at ventralizing wild-type zebrafish embryos or rescuing the dorsalized mutant phenotypes (Table 1, B. Schmid and M. C. Mullins, unpublished observations), with the exception of XBMP7 in *snailhouse* mutants (described below).

sbn is a dominant maternal mutation in which all embryos from crosses between heterozygotes display either a class 4 or 5 mutant phenotype. *sbn* mutants can be rescued by injection of *bmp4* mRNA to a weaker dorsalized phenotype (78% of the mutants) or a wild-type

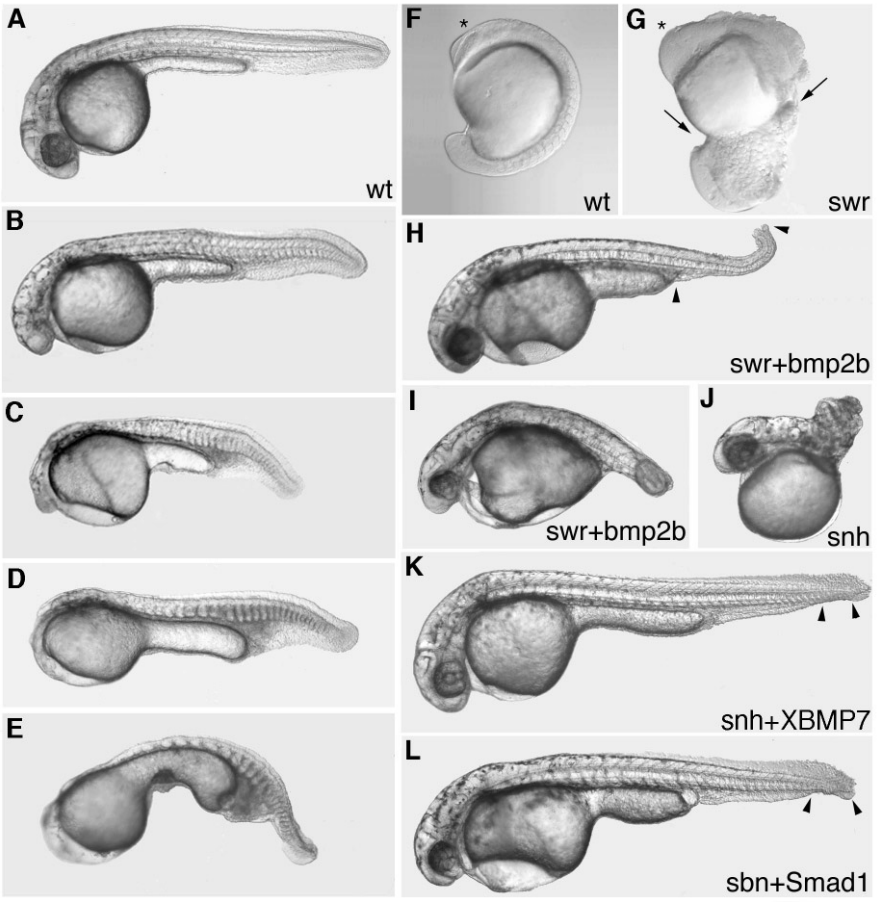

FIG. 2. Overexpression of members of the BMP pathway can ventralize wild-type embryos (A–E) and rescue *swirl*, *sbn*, and *snh* mutant phenotypes (F–L). One and one-half day old wild-type embryo (A). Increasing degrees of ventralization due to overexpression of *bmp2b* are shown in B–E. Mildly ventralized embryos (B) show a reduction in eye size. In more affected embryos (C), the eyes, notochord, and anterior brain are absent. With increasing ventralization, more anterior structures as well as the anterior somites are progressively reduced (C–E). A 14-somite wild-type (F) and *swirl* (class 5) mutant embryo (G), which normally dies at this stage and is shown dying due to the bursting of its yolk (arrows). Asterisks (*) in F and G indicate the prospective anterior head regions. *swirl* embryo rescued to a class 2 phenotype (H) and a class 3 phenotype (I) by injection of *bmp2b* mRNA. Class 4 phenotype (J) of an uninjected *snh* mutant embryo. *sbn* mutant embryos display the same class 4 phenotype as *snh*. *snh* embryo rescued to a class 1 phenotype (K) by overexpression of *bmp7* mRNA. *sbn* embryo (L) rescued to a class 1 phenotype by overexpression of *Smad1* mRNA. Arrowheads in H, K, and L mark the extents of loss of the ventral tail fin.

or slightly ventralized phenotype (3%, Table 1). Overexpression of *bmp2b* similarly rescued *sbn* mutant embryos, while XBMP7 mRNA exhibited only weakly rescuing activity (8.2% to a class 3 phenotype, Table 1), similar to the very poor rescue observed in overexpressing *Xenopus* BMP4 or 2 in *sbn* mutant embryos (B. Schmid and M. C. Mullins, unpublished observations). In contrast, overexpression of XBMP7 in *snh* mutant embryos rescued nearly all mutants (95%, Table 1) and partially(Figs. 2J and 2K), completely, or slightly ventralized them. Injection of either *bmp2b* or *bmp4* mRNA also rescued *snh* mutant embryos and partially, completely, or slightly ventralized them (Table 1). In contrast to *swirl* and *sbn*, the more effective rescue of *snh* mutant embryos by XBMP7 may reflect a different BMP signaling pathway or ligand subclass, more closely related to BMP7 than BMP2/4, being affected in *snailhouse* mutants. Analysis of closely linked molecular markers to the *swirl* and *snh* mutations confirmed that homozygous mutants were partially or completely rescued (data not shown). These results provide evidence for *swirl*, *sbn*, and *snh* functioning within a BMP signaling pathway.

Components functioning downstream of the BMP ligands should also rescue these mutants, if *swirl*, *sbn*, and *snh* function within a BMP signaling pathway. We tested a downstream-acting BMP type I receptor (mAlk6)

TABLE 1

Percentage of Rescued *swirl*, *snailhouse*, and *somitabun* Mutant Embryos by Expression of Members of the Bmp Pathway

	RNA (amount injected)	% rescued mutants[a]	% Normal and ventralized (includes class 1[b])	% Weaker dorsalized phenotypes				% Mutant phenotype		Total No. injected
				% class 2	% class 3	% class 4	Σ[c]	(% class 5)	% dead	
swirl	*bmp2b* (40 pg)	100	94	2.5	0	1.5	4	0	2.5	203
	bmp4 (35 pg)	99	89	1.9	3.7	4.3	9.9	0.3	0.5	376
	XBMP7 (175 pg)	82	78	5.0	5.0	7.7	18	4.6	0.5	220
	mAlk6act (14 pg)	76	79	8.6	2.6	2.3	13.5	5.9	2.0	304
	Smad1 (2.5 ng)	66	80	2.5	2.9	3.8	9.1	8.5	0.8	235
	lac-Z (200 pg)	—	77	0	0	0	0	23	0	420
	Uninjected	—	72	0	0	0	0	28	0	598
				% class 1	% class 2	% class 3	Σ[c]	(% class 4 and 5[d])		
snailhouse	*bmp2b* (40 pg)	36	76	0	1.3	7.2	8.5	16	[e]	319
	bmp4 (35 pg)	64	78	2.2	2.0	8.2	13	9.1	[e]	404
	XBMP7 (40–175 pg)	95	94	4.6	0.1	0	4.7	1.3	[e]	691
	mAlk6act (14 pg)	60	83	1.7	1.1	3.4	6.2	10	[e]	179
	Smad1 (2.5 ng)	65	79	6.9	3.5	2.3	13	8.7	[e]	173
	lac-Z (200 pg)	—	78	0	0	0	0	22	[e]	291
	Uninjected	—	76	0	0	0	0	24	[e]	606
				% class 1	% class 2	% class 3	Σ[c]	(% class 4 and 5)		
somitabun[f]	*bmp2b* (40 pg)	68	0	3.5	9.2	55	68	32	[e]	260
	bmp4 (35 pg)	81	3.1	5.9	21	51	78	19	[e]	390
	XBMP7 (300 pg)	8	0	0	0	8.2	8.2	92	[e]	552
	mAlk6act (14 pg)	61	26	8.7	11	15	35	39	[e]	184
	Smad1 (2.5 ng)	56	11	10	13	23	45	44	[e]	1003
	lac-Z (200 pg)	1	0	0	0	0.7	0.7	99	[e]	286
	Uninjected	—	0	0	0	0	0	100	[e]	1507

[a] Percentage rescued mutants is the percentage of mutants which exhibit weaker dorsalized phenotypes or normal or ventralized phenotypes. It is calculated as 100% (1-% mutant phenotype/% expected mutants). The percentage expected mutant embryos is 25% for *swirl* and *snailhouse* and 100% for *somitabun*.

[b] Normal and class 1 phenotypes are combined in *swirl*, since we could not distinguish between a rescued class 1 and the dominant class 1 *swirl* phenotype.

[c] Σ, sum of all weaker dorsalized phenotypes.

[d] Occasionally a small percentage of *snailhouse* mutants displays a class 5 phenotype.

[e] Embryos were not sorted at somite stages into class 4 and class 5 pools. Dead embryos at 1 day of age were therefore considered to be class 5 embryos.

[f] *somitabun* is a dominant maternal mutation. All embryos from matings of heterozygous fish show a class 4 or class 5 phenotype.

that possesses a mutation rendering the kinase constitutively active (Hoodless *et al.*, 1996; Wieser *et al.*, 1995). This activated receptor was able to rescue 76% of *swirl*, 61% of *sbn*, and 60% of *snh* mutant embryos to weaker dorsalized or wild-type or ventralized phenotypes (Table 1).

We tested *Smad1*, a gene acting further downstream in a BMP signaling pathway, for its ability to rescue *swirl*, *sbn*, and *snh* mutant embryos. Smad1 is phosphorylated by a BMP type I receptor and then translocates into the nucleus where it is involved in transcriptional activation (reviewed in Baker and Harland, 1997). In overexpression experiments, however, the necessity for phosphorylation by the type I receptor is alleviated causing ectopic Smad1 to ventralize the embryo (Graff *et al.*, 1996; Thomsen, 1996). We also found *Smad1* when overexpressed to weakly ventralize wild-type embryos (phenotypes similar to Fig. 2B). Expression of *Smad1* in the dorsalized mutants rescued 66% of *swirl*, 56% of *sbn*, and 65% of *snh* mutant embryos (Table 1, Fig. 2L).

Our finding that three components in a BMP pathway can rescue *swirl*, *sbn*, and *snh* mutants indicates that these genes function within a BMP pathway to specify ventral

regions of the zebrafish embryo. The fact that the most upstream-acting genes tested in the pathway, the BMP ligands, rescued mutant embryos of all three genes may indicate that *swirl*, *sbn*, and *snh* function as BMP ligands or upstream of the ligands. However, since the alleles may not be null mutations, it is possible that both upstream- and downstream-acting BMP components could rescue the mutant phenotypes. Overexpression of an upstream gene could function through the residual activity of the mutated gene. Since *sbn* is a dominant maternal mutation, all mutant embryos are expected to receive at least 50% wild-type *sbn* gene product from their mother (in addition to three-quarters of the embryos also producing the wild-type *sbn* gene product zygotically). Due to the presence of this wild-type *sbn* gene product, upstream-acting genes may rescue *sbn* mutant embryos.

snailhouse and swirl Are Not Essential Beyond Embryogenesis

We raised 20 homozygous mutant *snh* embryos to adulthood that had been rescued to a class 1 phenotype by XBMP7 or *Smad1*. The homozygous mutant genotype of these fish was confirmed in crosses to heterozygous *snh* males. Fifty percent of the progeny (262/519) displayed a dorsalized mutant phenotype, the percentage expected in a cross between a homozygote and a heterozygote. No maternal-effect phenotype was detected in the progeny of crosses between homozygous *snh* females and wild-type males. We also rescued two *swirl*^{tc300} and two *swirl*^{ta72} homozygous fish to fertile viable adults by expression of either *bmp2b* or *bmp4* (186/368 dorsalized mutant embryos in crosses to *swirl* heterozygotes). Because the injected mRNA likely functions early in embryogenesis, our ability to raise homozygous *snh* and *swirl* fish indicates that these mutations do not disrupt an essential function outside of embryogenesis.

swirl Functions Cell Nonautonomously

We examined the cell autonomy of *swirl* to determine more precisely where it may function in a BMP pathway. We reasoned that if *swirl* functions as a BMP ligand or in the generation of a ligand, then it would act cell nonautonomously. Alternatively, if *swirl* encodes a BMP receptor or a downstream signal transducer, then it would function cell autonomously in the specification of ventral cell fates.

We tested this hypothesis by transplanting fluorescently labeled homozygous mutant *swirl* cells into wild-type embryos or vice versa and determining whether the mutant cells could form blood cells, a ventral cell type normally absent in *swirl* mutant embryos (Mullins *et al.*, 1996). *swirl* mutant cells when transplanted into a wild-type embryo were able to form blood cells (Figs. 3A and 3B). Likewise, when wild-type cells were transplanted into *swirl* mutant embryos, homozygous *swirl* cells could be specified to the blood progenitor cell fate, as evidenced by their expression

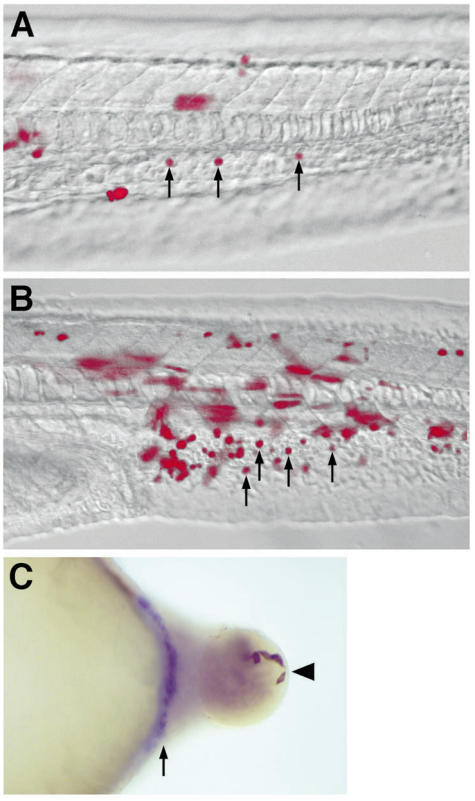

FIG. 3. Swirl functions cell nonautonomously. (A and B) Fluorescently labeled *swirl* mutant cells exhibited the round morphology of blood cells and could be visualized moving through the vasculature in live embryos. Fluorescently labeled *swirl* mutant cells (red) are visible in the ventral tail vein of wild-type hosts, among other tissues. Arrows point to a subset of labeled blood cells present in the chimera. Lateral views, with anterior to the left and dorsal up. (C) Ventral view (anterior to the left) of an eight-somite *swirl* host embryo that expresses *gata1* (arrow), normally not expressed in the mutant, when wild-type donor cells are transplanted into the mutant. Some labeled donor cells are shown (arrowhead).

of *gata1* (Fig. 3C) (Detrich *et al.*, 1995). These results demonstrate the ability of *swirl* mutant cells to form blood cells when placed in the vicinity of wild-type cells. Thus, *swirl* functions cell nonautonomously in the specification of blood cells, consistent with *swirl* acting upstream of or as a BMP ligand.

bmp4 Is Not Linked to swirl

The cell nonautonomous function of *swirl* suggests that *swirl* may encode a BMP ligand. Since BMP4 is the most strongly implicated BMP in dorsoventral patterning in both *Xenopus* and mouse, we first examined the linkage of *bmp4* to *swirl*. In a mapping cross line, we identified a restriction fragment length polymorphism (RFLP) in the *bmp4* gene between the founder *swirl* heterozygote and the polymor-

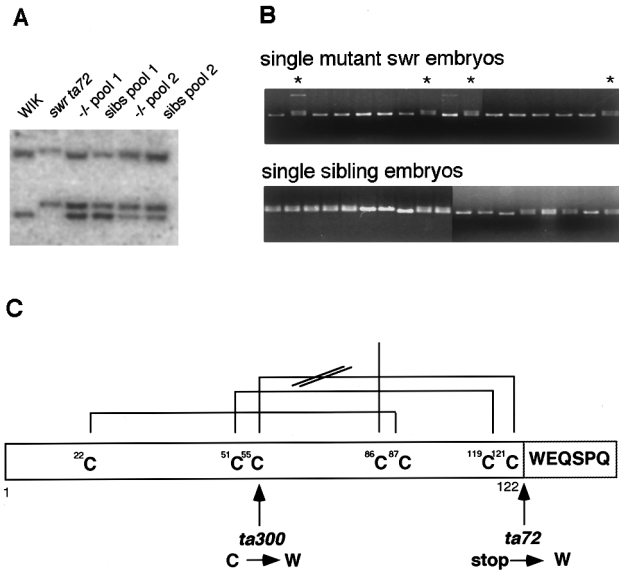

FIG. 4. Genomic Southern blot hybridization reveals nonlinkage between *bmp4* and the *swirl* locus (A). A *Bgl*II RFLP in the *bmp4* gene between the swr^{ta72}/+ and WIK mapping cross grandparents. Lane 1, WIK grandparent. Lane 2, Tü grandparent carrying the swr^{ta72} mutation. DNA from two independently collected pools of F2 mutant (lanes 3 and 5) and sibling (lanes 4 and 6) embryos show both the WIK and Tü bands, indicating that *swirl* and *bmp4* are not linked. Single swr^{ta72}/swr^{ta72} embryos (B) show predominantly the *swirl*-specific lower migrating band. Four of 16 embryos have both bands present, representing recombinant embryos between the Z536 WIK allele and swr^{ta72} (marked with asterisks). In the single-sibling embryos, the WIK- and Tü-specific bands are frequently found together. (C) Schematic representation of the mature 122-amino-acid region of the Bmp2 protein shown with its conserved seven cysteines. The cysteines form intra- as well as intermolecular bonds as indicated by the black lines connecting the cysteines and the open line from cysteine 86, respectively.

phic WIK strain (Fig. 4A, lanes 1 and 2). We examined segregation of the *bmp4* RFLP in the F2 mutant and wild-type sibling progeny. As seen in Fig. 4A (lanes 3–6), we found the Tü and WIK RFLPs in the F2 homozygous *swirl* mutant DNA, indicating the independent segregation of the *swirl* mutation and the *bmp4* gene. Thus, *swirl* is not a mutation in *bmp4*.

Chromosomal Positions of swirl and bmp2b

We next examined whether *swirl* corresponds to the *bmp2b* gene by mapping *swirl* and *bmp2b* to chromosomal positions. We mapped *swirl* using simple sequence length polymorphic (SSLP) markers (Knapik *et al.*, 1996). We tested linkage of the SSLPs to the $swirl^{ta72}$ mutation by examining DNA from pools of mutants and pools of wild-type siblings (genotypic +/− and +/+) of our mapping crosses. SSLPs linked to the mutation are detected by a predominance of the Tü SSLP in the pooled mutant DNA. We identified the linkage between *swirl* and SSLP Z536 on linkage group 20 (Zbf22, E. Knapik and M. Fishman, personal communication; Postlethwait *et al.*, 1994). In examining 144 chromosomes from single F2 mutant embryos, we found 13 recombinants placing *swirl* about 9 cM from Z536 (Fig. 4B).

We determined the linkage group on which *bmp2b* is located by testing for the presence of the zebrafish *bmp2b* gene in a panel of somatic cell hybrids between a mouse and zebrafish cell line (Ekker *et al.*, 1996). These cell lines have previously been typed with SSLP markers to determine the zebrafish linkage groups present in each line. We found that the *bmp2b* gene cosegregates with Z536, the SSLP linked to *swirl*, strongly implicating *swirl* as a mutation in the *bmp2b* gene.

swirl Is a Mutation in the bmp2b Gene

To determine if *swirl* corresponds to the *bmp2b* gene, we cloned and sequenced a *bmp2b* cDNA from the $swirl^{tc300}$ and $swirl^{ta72}$ alleles by reverse-transcriptase PCR. We identified a single base pair alteration in the *bmp2b* cDNA from $swirl^{tc300}$, changing amino acid 344 from a cysteine to a tryptophan (Fig. 4C). This missense mutation is the third of seven highly conserved cysteine residues, known to form disulfide bonds in other TGF-β molecules (McDonald and Hendrickson, 1993). The absence of this intramolecular disulfide bond in $swirl^{tc300}$ is likely to impair severely its function. Alteration of this same cysteine residue in activin A, another TGF-β family member, results in the absence of dimer formation and a near complete loss of function of the protein (Mason, 1994), similar to that found when this amino acid is altered in TGF-β1 (Brunner *et al.*, 1992). A mutation in this residue was also found in a loss-of-function allele of one of the mouse *short ear* mutants, which corresponds to the BMP5 gene (Marker *et al.*, 1996).

The cDNA sequence of $swirl^{ta72}$ revealed a point mutation in an unusual position, in the stop codon, changing it to a tryptophan residue (Fig. 4C). The next stop codon lies 15 base pairs downstream of the mutation resulting in an extension of the $swirl^{ta72}$ open reading frame by six amino acids, Trp-Glu-Gln-Ser-Pro-Gln. The carboxy terminus of nearly all TGF-β molecules lies one amino acid following the most terminal cysteine residue [an exception is Xnr3, a nodal related gene (Hansen *et al.*, 1997)]. The crystal structure of a TGF-β dimer indicates that the carboxy-terminal residue of the ligand lies at the interface between the two monomers (Daopin *et al.*, 1992; Griffith *et al.*, 1996; Schlunegger and Grütter, 1992). An additional six amino acids at the carboxy terminus may disrupt the conformation of the dimer, its stability, or receptor–ligand interaction. The mutations identified in the *bmp2b* gene in two *swirl* alleles demonstrate that *swirl* encodes Bmp2b.

somitabun and snailhouse Are Independent Genes from swirl

Due to strong genetic interactions between *swirl* and *sbn*, we previously were unable to determine whether *swirl* and *sbn* are mutations in the same or independent genes, and we tentatively assigned them separate gene names (Mullins *et al.*, 1996). To directly address this issue and confirm that *snh* and *swirl* are mutations in different genes, we examined the segregation of *snh* and *sbn* to Z536, the SSLP linked to *swirl*. In F2 mutant embryos from mapping crosses between these mutations and the WIK or AB polymorphic strains, both mutations segregated independently from Z536 (data not shown), thus establishing that *snh* and *sbn* are separate genes from *swirl*.

An Expansion of Dorsal Tissue

From work in *Xenopus*, mutants of genes in a BMP2/4 signaling pathway are predicted to exhibit a loss and reciprocal expansion of nonneural and neural ectoderm, respectively, in addition to ventral and dorsal mesoderm (reviewed in Sasai and De Robertis, 1997; Thomsen, 1997). One expects these alterations to be visible prior to stages of cell commitment. Our previous analysis of the zebrafish dorsalized mutants showed an expansion of dorsolateral mesodermal tissue at the expense of ventral mesodermal derivatives during early somitogenesis (Mullins *et al.*, 1996). Furthermore, we observed an expansion of presumptive neural tissue during gastrulation and somitogenesis. Here we investigate whether an expansion of neural tissue is apparent prior to the point of cell commitment, which occurs between shield stage and 80% epiboly (Ho and Kimmel, 1993), and if it is associated with a reciprocal loss in nonneural ectoderm during gastrulation. In addition, we examined whether cell death could account for the loss of gene expression observed during gastrulation.

We examined a marker of dorsal tissue, *fkd3* (J. Odenthal, unpublished observations), prior to the onset of gastrulation, a time point when cells are not committed to a particular cell fate (Ho and Kimmel, 1993; Woo and Fraser, 1997). At sphere and dome stages *fkd3* expression is restricted to dorsal regions in *swirl, sbn,* and *snh* mutant embryos as in wild type (data not shown). By 45% epiboly, a stage prior to the onset of gastrulation, the expression domain encircles both *swirl* and strong *sbn* mutant embryos (Figs. 5A and 5B, data not shown) and expands laterally, fading in the most ventral regions in weak *sbn* embryos (Fig. 5C). *otx2*, a marker of prospective anterior neural tissue first expressed at 65% epiboly (Li *et al.*, 1994), similarly encircles *swirl* and strong *sbn* (Figs. 5D and 5E, data not shown) and expands to ventrolateral regions in weak *sbn* mutant embryos (Fig. 5F). Thus, an expansion of dorsal and prospective neural tissue is observed in *swirl* and *sbn* at a stage before cells are committed to a cell fate, consistent with a model where dorsoventral patterning is affected in these mutants.

Reduction of Nonneural Ectoderm

We investigated whether the expansion of neural progenitors is associated with a reduction in nonneural ectoderm by examining the expression of *AP-2* (Fürthauer *et al.*, 1997), *dlx3* (Akimenko *et al.*, 1994), and *gata2* (Detrich *et al.*, 1995), all of which are first expressed in the ventral ectoderm between 65 and 75% epiboly. In *swirl* mutant embryos at 65% epiboly *AP-2* is absent or severely reduced, while in strong and weak *sbn* and *snh* mutants *AP-2* is moderately to weakly reduced (Figs. 5G–5I, data not shown). Expression of *dlx3* and *gata2* at 75% epiboly is absent or severely reduced in *swirl* and strong *sbn* mutants (Figs. 5J and 5K, data not shown). In *snh* and weak *sbn* mutants, *dlx3* is moderately reduced and *gata2* is severely reduced to absent (Fig. 5L, data not shown). These results suggest that there is some ventral ectodermal character in *sbn* and *snh* mutants, while in *swirl* mutants almost no ventral character is apparent.

Rather than reflecting defects in dorsoventral patterning, the dorsalized mutant phenotypes could be caused by ventral-specific cell death and an overproliferation and ventral migration of dorsally specified cells. To determine whether ventral-specific cell death could account for the reduction in ventral ectodermal markers, we examined cell death in *swirl, snh,* and *sbn* using an assay for apoptosis in whole-mount embryos. From sphere stage (1 1/2 h prior to gastrulation) to 80% epiboly we detected almost no cell death in either wild-type or mutant embryos (Figs. 5M and 5N). At 85% epiboly, cell death was observed sporadically in both the mutant and wild-type sibling embryos (Fig. 5O). Thus, cell death cannot account for the loss of ventral gene expression observed in these mutants during gastrulation.

Examination of Lateral Regions of the Fate Map

We have shown a loss or reduction in cell types derived from ventral regions of the embryo in *swirl, sbn,* and *snh* mutants, indicating that ventral portions of the fate map are absent or reduced in these mutants. To determine the portion of the fate map that remains, we examined three cell types derived from lateral regions of the blastoderm: the ventrolaterally derived otic and olfactory placodes (Kozlowski *et al.*, 1997), the laterally derived neural crest (Woo *et al.*, 1995), and the dorsal–lateral hindbrain progenitors (Kimmel *et al.*, 1990; Woo and Fraser, 1995).

Placodal Tissues Are Absent in swirl

To investigate the otic and olfactory placodal tissues we analyzed the expression of *dlx3* in these placodal progenitor cells at the 13- to 14-somite stage. In *swirl* mutant embryos, no otic or olfactory placodal expression was observed (data not shown). Strong *sbn* mutants exhibit very weak to normal olfactory placodal expression, but no otic placodal expression (Figs. 6A and 6B).

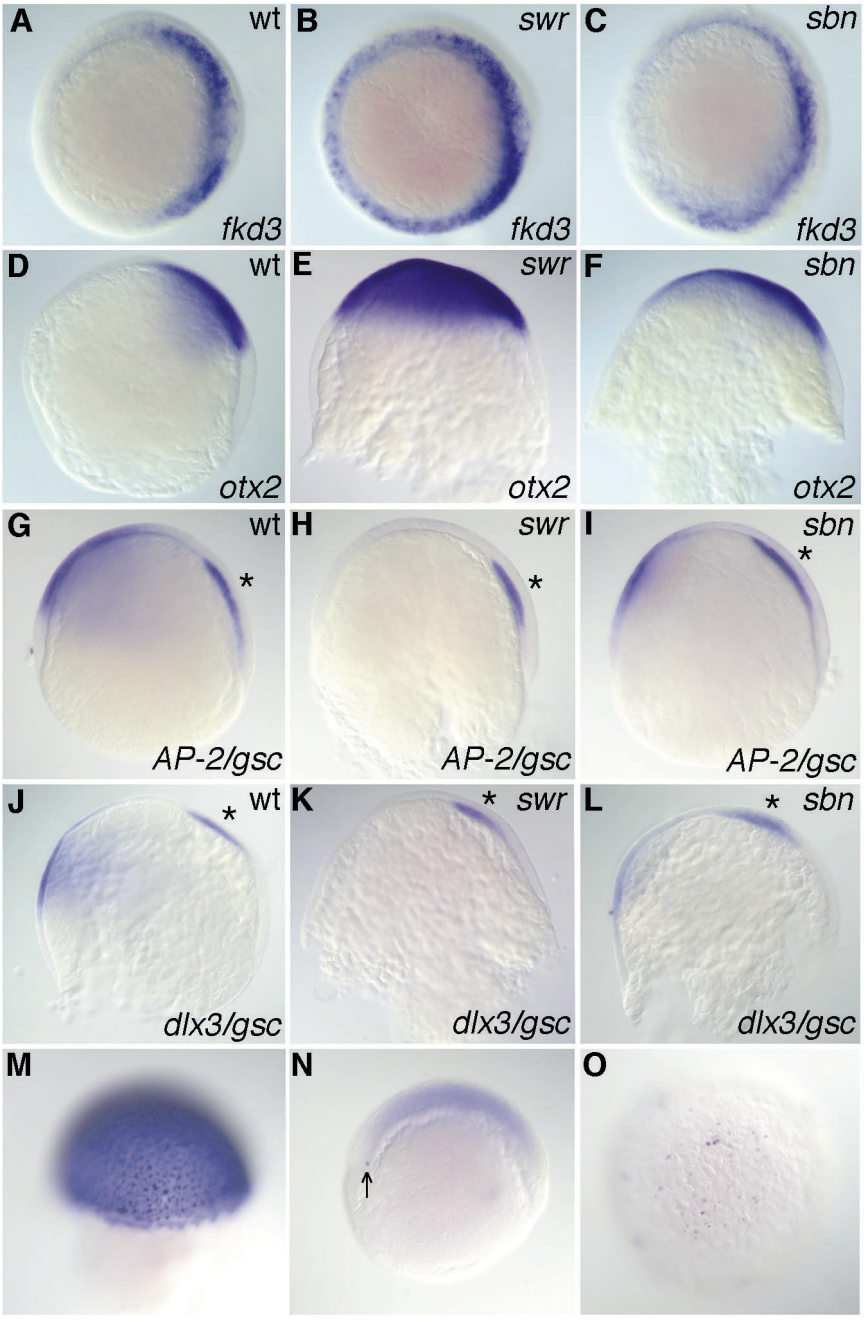

FIG. 5. Whole-mount *in situ* hybridizations show that the expansion of neuroectoderm (A–F) in *swirl* (*swr*) and *sbn* mutants is accompanied by a reduction in nonneural ectoderm (G–L), which cannot be attributed to early death of ventral cells (M–O). (A–C) *fkd3* expression at 45% epiboly in wild-type (A), *swirl* (B), and *sbn* (C) mutant embryos (animal pole views, with dorsal to the right). D–L are lateral views, with dorsal to the right and animal pole up. (D–F) Expression of *otx2* at 65% epiboly in wild-type (D), *swirl* (E), and weak *sbn* (F) mutant embryos. (G–I) Double *in situ* hybridizations showing the expression of *gsc* (*) in the dorsal midline mesoderm, which serves as a positive control, and *AP-2* in wild-type (G), *swirl* (H), and *sbn* (I) embryos at 65% epiboly. (J–L) *dlx3* and *gsc* expression, where *gsc* (*) is again a positive control, in wild-type (J), *swirl* (K), and weak *sbn* (L) embryos at 75% epiboly. (M–O) Detection of cell death in *swirl* mutants. M and N are 50% epiboly sibling embryos from a cross of two heterozygous *swirl* fish. Note that the embryos have been overstained to ensure that all apoptotic cells can be visualized. M serves as a positive control for the assay (see Materials and Methods). (N) All embryos from this brood show a similar staining pattern to that shown. The arrow marks one labeled cell. (O) An embryo at 85% epiboly from a cross of heterozygous *swirl* fish. Sibling embryos exhibited similar staining.

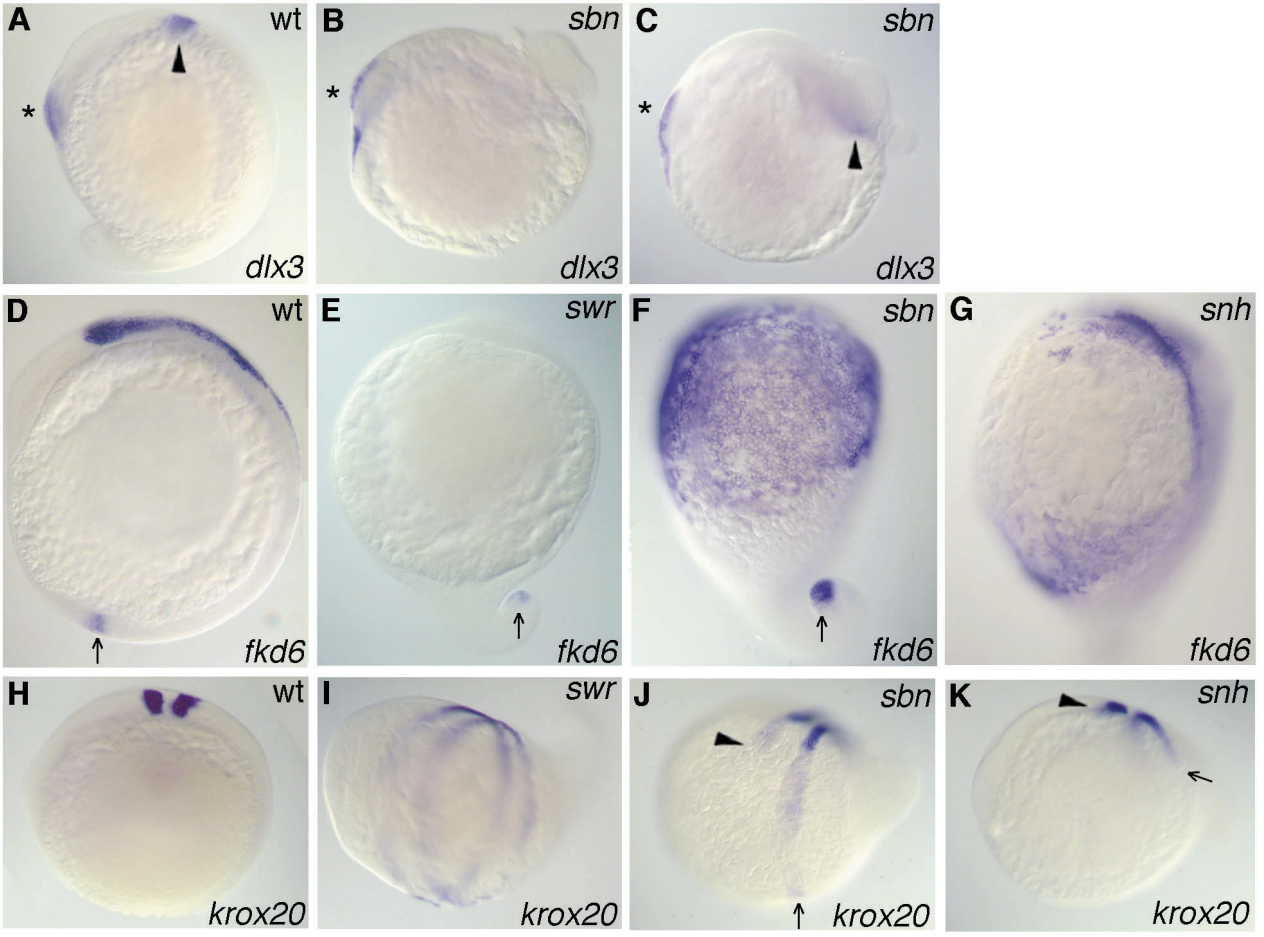

FIG. 6. Whole-mount *in situ* hybridizations to examine laterally derived cell types show altered expression of *dlx3* (A–C), *fkd6* (D–G), and *krox20* (H–K) in *swirl*, *sbn*, and *snh*. (A–C) *dlx3* expression in a 14-somite-stage wild-type embryo (A) positioned laterally, with dorsal to the right; strong *sbn* (B) and weak *sbn* (C) embryos, lateral views, with dorsal up. The asterisks label the expressions of *dlx3* in the presumptive olfactory placodal tissue. In A the arrowhead marks the expression domain in the presumptive otic placodal cells; in C it is directed at expression at the posterior end of the axis of the mutant (because the axis of the embryo is severely twisted at this stage, this expression domain is not readily apparent in this orientation). *fkd6* expression in five-somite-stage wild-type (D), *swirl* (E), *sbn* (F), and *snh* (G) embryos, shown as lateral views, with anterior up and dorsal to the right. Only one of the dorsal bilateral stripes of cells expressing *fkd6* is visible in D. Some *sbn* embryos exhibit a less strong expansion of fkd6, stronger than, but more similar to that of the *snh* embryo (G). The *snh* mutant shown in G displays a moderate expansion in the number of cells expressing *fkd6*. The *fkd6* expression domain in the tailbud (arrows) is present in all embryos (but is not shown in G). (H–K) Expression of *krox20* at the 5-somite stage in wild-type (H), *swirl* (I), weak *sbn* (J), and *snh* (K) embryos. In J and K, the arrowheads mark presumptive rhombomere 3 and the arrows presumptive rhombomere 5. Lateral view (H) or oblique lateral view (I–K), all with dorsal up.

Weak *sbn* and all *snh* mutants display normal olfactory placode expression and a second domain of expression behind the twisted axis of the embryo, which likely corresponds to the otic placode precursors (Fig. 6C, data not shown). Thus, the ventrolaterally derived otic and olfactory placodes are absent in *swirl* mutants. The olfactory but not the otic placodal precursors are present in strong *sbn* mutants, and both placodal cell types are present in weak *sbn* and *snh* mutants.

Neural Crest Progenitors Are Absent in swirl, but Expanded in somitabun and snailhouse

We tested three markers of the prospective neural crest, *fkd6* (J. Odenthal, unpublished observations), *AP-2* (Fürthauer *et al.*, 1997), and *snail2* (Thisse *et al.*, 1995), all of which at the five-somite stage in wild-type embryos are expressed in two stripes along the dorsal neural tube (*fkd6* is shown in Fig. 6D). We summarize the data for *fkd6;* however, similar

results were found for all three markers in the mutants. In *swirl* mutant embryos *fkd6* expression is greatly reduced to absent (Fig. 6E). In striking contrast, *sbn* mutant embryos exhibit an expansion in this expression domain that extends laterally to the most ventral region of the embryo (Fig. 6F). This domain is slightly to moderately enlarged in *snh* mutant embryos (Fig. 6G). These results indicate that in *swirl* mutants the laterally derived neural crest is severely reduced, while in *sbn* and to a lesser degree in *snh* it is expanded.

Progressive Expansion of the Prospective Hindbrain in snh, sbn, and swirl

We examined the presumptive hindbrain, a tissue derived from a more dorsal–lateral position relative to the neural crest, through analysis of *krox20* expression in rhombomeres 3 and 5 (Oxtoby and Jowett, 1993). In *swirl* and strong *sbn* mutants both rhombomeres 3 and 5 circle the dorsoventral axis of the embryo (Fig. 6I, data not shown). In some weak *sbn* mutants, the third rhombomere is enlarged medial laterally, while the fifth rhombomere encircles the axis of the embryo (Fig. 6J). In *snh* and other weak *sbn* mutant embryos neither rhombomere circles the embryo; however, both are enlarged with rhombomere 5 consistently extending further medial laterally than that of rhombomere 3 (Fig. 6K, data not shown).

These data, together with our previous results, show a progressive loss of cell types of ventral [blood and pronephros (Mullins *et al.*, 1996)] and lateral (placodes and neural crest) origin in the fate map accompanied by a complementary expansion in dorsolaterally derived neural and mesodermal (Mullins *et al.*, 1996) tissue of *snh*, *sbn*, and *swirl* mutants. The laterally derived structures reveal the point at which *swirl* and *sbn* differ in their alterations in the fate map. In addition to dorsolateral neural tissue, the laterally derived neural crest is expanded in *sbn* mutants, while it is greatly reduced to absent in *swirl* mutant embryos.

DISCUSSION

A bmp2 Pathway Establishes Ventral Positional Information in the Zebrafish Embryo

Here we show that three zebrafish genes, *swirl*, *sbn*, and *snh*, likely function within a BMP signaling pathway to establish ventral cell fates of the embryo. Dorsalized mutants of all three genes can be rescued by overexpression of several components within a BMP pathway. While we cannot exclude the possibility that overexpression of the BMP pathway components rescues *swirl*, *sbn*, and *snh* mutants defective in a parallel pathway, further evidence, as discussed below, reveals that *swirl* encodes a BMP family member and supports the involvement of *sbn* and *snh* in a BMP signaling pathway.

Through chromosomal mapping and cDNA sequence analysis of two *swirl* alleles, we identified *swirl* as a mutation in the zebrafish *bmp2b* gene. Our analysis of gene expression patterns during gastrulation and early somitogenesis demonstrates a requirement for *swirl/bmp2b* in the establishment of all ventrally and ventrolaterally derived tissues of the zebrafish embryo.

In addition to our ability to rescue *sbn* by BMP pathway components, genetic evidence supports a role for *sbn* in this pathway. *somitabun* interacts genetically with *swirl* (Mullins *et al.*, 1996). Yet, we found these mutations to map to different chromosomal positions showing that *swirl* and *somitabun* are mutations in different genes. The strong genetic interaction between these two genes suggests that they function within the same pathway. Since *swirl* encodes *bmp2b*, the data together implicate *somitabun* as a component of a BMP pathway that establishes dorsoventral patterning in the zebrafish embryo.

BMP2 versus BMP4 and BMP7 in the Frog, Fish, and Mouse

Our results support the work in *Xenopus* that implicates a BMP signaling pathway in dorsoventral patterning of the frog embryo. Although BMP4 is the most studied of the BMP ligands with respect to early frog patterning, BMP2 exhibits many of the same functional properties as BMP4 (Clement *et al.*, 1995; Hemmati-Brivanlou and Thomsen, 1995; Suzuki *et al.*, 1997). Interestingly, Nikaido *et al.* (1997) postulated that zebrafish *bmp2b* is functionally more similar to *Xenopus* BMP4, based on the more similar expression patterns of *bmp2b* and *Xenopus* BMP4, than zebrafish *bmp4* and *Xenopus* BMP4.

In the mouse where it is possible to study loss-of-function mutants, the role of the BMP2, BMP4, and BMP7 ligands has been studied. The primary defect in mice with null mutations in the BMP4 gene (Winnier *et al.*, 1995) or the BMP type I receptor (Mishina *et al.*, 1995) is an absence of all mesoderm. A small fraction of BMP4 mutant embryos develop further and display losses in posterior and ventral tissue, which may reflect a dorsoventral patterning defect. Functions in mesoderm formation may mask the roles of BMP4 and the BMP type I receptor in dorsoventral patterning at later stages.

Mutants of BMP2 (Zhang and Bradley, 1996) and BMP7 (Dudley *et al.*, 1995) in the mouse do not exhibit defects in dorsoventral pattern formation. Hence, our finding that *swirl* encodes *bmp2b* was unexpected. The mouse BMP2 mutant fails to close the proamniotic canal and heart development proceeds abnormally. The absence of a defect in dorsoventral patterning reveals a nonconserved essential function of the BMP2 homologues in the mouse and the zebrafish.

BMP2 and BMP4 are equally related to *dpp*, the *Drosophila* counterpart to these genes. Based on a recent model to explain the maintenance of redundant functioning genes (Cooke *et al.*, 1997), BMP2 and BMP4 may represent a gene duplication event, and as such these genes functioned redundantly in dorsoventral patterning in an ancestral ver-

tebrate. In the hundreds of millions of years separating the mouse from the zebrafish, the roles of these genes may have diverged. The zebrafish may have acquired an essential function for *bmp2b* in dorsoventral patterning, while in the mouse BMP4 may fulfill this function. BMP2 in the mouse and *bmp4* in zebrafish may play roles in dorsoventral patterning; however, it is clear that BMP2 in the mouse does not afford an essential role in this process.

In the zebrafish there are two genes closely related to BMP2, *bmp2a* and *bmp2b* (Martinez-Barbera *et al.*, 1997), *swirl* corresponding to the *bmp2b* gene. Thus, an additional gene duplication event likely occurred since the separation of tetrapods and teleosts. The very strong dorsalized phenotype of *swirl* suggests that *bmp2a* does not function redundantly to *bmp2b* in dorsoventral patterning, although these two genes could have overlapping functions later in development. Transient expression of *bmp2b* during embryogenesis can rescue homozygous *swirl* embryos to fertile adults, indicating that *bmp2b* is essential only during embryogenesis.

Overexpression of related BMPs can functionally substitute for *bmp2b/swirl* in the zebrafish. The rescuing abilities of *bmp4* and XBMP7 may reflect nonspecific actions of these ligands due to their overexpression. Alternatively, these BMPs may have the ability to function redundantly to *bmp2b*. The later onset of expression of *bmp4* compared to *bmp2b* (Nikaido *et al.*, 1997) and, as discussed below, the fact that *bmp4* expression in ventral regions is dependent on *bmp2b/swirl* during gastrulation may preclude endogenous *bmp4* from performing this role in *swirl* mutant embryos.

Conservation of Dorsoventral Patterning in Vertebrates and Invertebrates

The recent identification of *dino* as *chordin* (Fisher *et al.*, 1997; Schulte-Merker *et al.*, 1997) along with our results showing that *swirl* encodes *bmp2b* supports the hypothesis that the genes and mechanisms establishing dorsoventral patterning have been conserved between vertebrates and invertebrates. The *dpp* null phenotype displays a complete loss of dorsal structures, similar to the near absence of all ventral tissues in *swirl* mutants. *dpp* displays a weak haplo-insufficient ventralized phenotype, while heterozygous *swirl*^{ta72} fish frequently exhibit weakly dorsalized defects (Mullins *et al.*, 1996). Similar to *dpp*, our analysis of *swirl*, *sbn*, and *snh* indicates the function of a morphogen, likely *swirl/bmp2b*, in establishing the dorsoventral pattern. Both *swirl* and *dpp* are epistatic in double-mutant *swirl*, *chordino*, and *dpp* and *sog* double mutants (Biehs *et al.*, 1996; Hammerschmidt *et al.*, 1996b; Holley *et al.*, 1996). However, we also identified nonconserved features of the pathway (discussed below). The autoregulation of *bmp2b/swirl* is not conserved in dorsoventral patterning in *Drosophila*, i.e., *dpp* is not required for maintenance of its own expression in the early embryo (Ray *et al.*, 1991). Furthermore, we show a function for this *bmp2b* pathway in the establishment of the vertebrate-specific cell types of the neural crest and placodal tissues.

swirl, somitabun, and snailhouse Maintain bmp2b and bmp4 Expression

swirl is required to maintain its own expression. It is unlikely that the *swirl* mutations cause instability of the *bmp2b* mRNA, since the dorsal marginal expression of *bmp2b* is normal in *swirl* mutants (Fig. 1B) and an identical loss of *bmp2b* expression is observed in mutant embryos of both *swirl* alleles (data not shown). In ectopic expression experiments in *Xenopus*, BMP4 can activate its own expression (Jones *et al.*, 1992), which may be mediated through a *Vox/Xvent-2/Xom* (Ladher *et al.*, 1996; Onichtchouk *et al.*, 1996; Schmidt *et al.*, 1996) and *Xvent-1* (Gawantka *et al.*, 1995) regulatory loop. *swirl/bmp2b* maintains, but does not initiate, the ventral expression of *bmp4*, which appears an hour after that of *bmp2b* in wild-type embryos. Similarly, *snh* and *sbn* are required to maintain high levels of expression of *bmp4* and *swirl/bmp2b*, consistent with our proposal that they function within a BMP pathway.

In *swirl* mutant embryos, the dorsal expression of *fkd3* is expanded to ventral regions prior to the onset of gastrulation. Interestingly, the expression of *chordin*, a BMP antagonist and neural inducer, is unaltered in *swirl* mutants at this stage, while it is expanded at early gastrulation stages (Miller-Bertoglio *et al.*, 1997). Thus, the absence of *bmp2b* activity at 45% epiboly is reflected in the regulation of *fkd3*, but not *chordin* expression, suggesting that different regulatory mechanisms may be at work. Since *bmp2b* and *bmp4* expression is also not affected at 45% epiboly, but is reduced by early gastrulation, similar mechanisms may regulate *chordin* and the ventral *bmp2b* and *bmp4* expression domains, but in a reciprocal manner. Such reciprocal regulation in the generation of dorsally and ventrally restricted gene expression domains has also been proposed based on ectopic expression experiments in the frog (reviewed in Lemaire and Kodjabachian, 1996).

swirl May Function as a Morphogen

Models for dorsoventral pattern formation in *Xenopus* have hypothesized the function of a morphogen in establishing different cell types along this axis. Recently, it has been shown that BMP4 and *noggin* can function in a dose-dependent manner to specify different tissues in *Xenopus* and zebrafish (Dosch *et al.*, 1997; Knecht and Harland, 1997; Neave *et al.*, 1997; Wilson *et al.*, 1997). Smad1 has been implicated as a direct mediator of the BMP activity gradient in the activation of gene expression (Wilson *et al.*, 1997). We previously hypothesized that the zebrafish dorsalized mutants affect a pathway in which a morphogen functions based on the graded series of dorsalized phenotypes observed (Mullins *et al.*, 1996). As shown here, the progressive expansion of presumptive rhombomeres 3 and 5, and successive loss of expression of *gata2*, *dlx3*, and *AP-2*

(as discussed below) in *snh*, *sbn*, and *swirl* mutant embryos (Figs. 5 and 6), exemplifies this point. Our findings that *swirl* specifies all ventral mesodermal and ectodermal cell types, acts cell nonautonomously, and encodes *bmp2b* suggest that *swirl*/*bmp2b* may be the morphogen, possibly together with one or more other BMPs. However, further tests are required to demonstrate such a function for *swirl*.

swirl and sbn Specify Placodal Tissues

swirl specifies the otic and olfactory placodes, nonneural ectodermal tissues forming adjacent to the neural plate. The mechanism by which these tissues are specified is likely different from that proposed below for the neural crest, as revealed by the differential specification of these tissues in *swirl*, *snh*, and *sbn* mutants. In *swirl* mutants the presumptive otic and olfactory placodal cells are absent, while in strong *sbn* mutants only the olfactory tissue is present, and in *snh* mutants both cell types are present. The range of effects observed suggests that in *sbn* mutants Bmp activity is at the threshold necessary to specify these placodal tissues. Since an expansion is not observed, it may be that a particular Bmp activity renders a tissue competent to respond to secondary interactions that determine these placodes, consistent with models for placodal tissue specification (Gallagher *et al.*, 1996; Grainger, 1992).

Specification of the Neural Crest

Most features of the *swirl* and strong *sbn* mutant phenotypes are indistinguishable from each other. However, opposite effects are seen in the specification of the neural crest progenitors in these two mutants. The prospective neural crest is severely reduced to absent in *swirl* mutant embryos, while this cell population is greatly expanded in *sbn* mutant embryos (Figs. 6E and 6F). A correlation can be made between the presence of AP2 in the ventral ectoderm during gastrulation and the presence or absence of the neural crest progenitors. During gastrulation the ventral *AP-2* expression domain is absent or severely reduced in *swirl* mutants, while in *sbn* mutant gastrula only a moderate reduction in *AP-2* is observed (Figs. 5H and 5I). All other aspects of these mutant phenotypes are identical, including the absence or severe reduction during gastrulation of the ventrally expressed *dlx3*, *gata2*, and *bmp4* genes and the expansion to ventral regions of *fkd3*, *otx2*, and *chordin* (Miller-Bertoglio *et al.*, 1997) expression. In *snh* mutant embryos, where only a weak to moderate expansion of the neural crest progenitors is seen, *AP-2* in addition to *dlx3* expression is present at reduced levels, while *gata2* is nearly absent.

The simplest model we found to explain both the absence of neural crest progenitors in *swirl* and the expansion of prospective neural crest in *sbn* and *snh* and which also incorporates the observed changes in gene expression in each of these mutants is a model in which a morphogen acts. We propose that different levels of BMP activity induce differential gene expression along the dorsoventral axis, resulting in the specification of different cell types, including the neural crest. Smad1 could be the direct transcriptional mediator of BMP signaling levels, as proposed by Wilson *et al.* (1997). A related model has been proposed in the zebrafish based on overexpression experiments (Neave *et al.*, 1997).

First, we discuss the model in terms of pattern formation in the wild type and then how this fits with the observed mutant phenotypes. *AP-2*, *dlx3*, and *gata2* are expressed in broad ventral domains during gastrulation. We propose that a low threshold of BMP activity induces *AP-2* expression, while progressively higher thresholds are required to induce *dlx3* and *gata2* expression. Consequently, *AP-2* would be expressed in the broadest ventral domain and *dlx3* and *gata2* in progressively smaller overlapping ventral domains (Fig. 7A). By early somitogenesis, *AP-2* and *dlx3* are expressed in lateral stripes flanking the embryonic axis (Akimenko *et al.*, 1994; Fürthauer *et al.*, 1997). We hypothesize that these stripes arise from repression caused by genes expressed in smaller overlapping ventral domains.

In *swirl*, *sbn*, and *snh* mutants there are likely different levels of BMP activity present in the embryo, leading to different effects in the specification of ventrally and laterally derived tissues. In *swirl* we hypothesize no Bmp activity, while low and moderate levels are present in strong *sbn* and *snh* mutants, respectively. Since in *swirl* there is no Bmp activity, *AP-2*, *dlx3*, and *gata2* are not expressed (Fig. 7D). In *sbn* mutants, where low Bmp activity is present, *AP-2* expression is induced, but the threshold of Bmp activity is not sufficient to induce *dlx3* or *gata2* expression (Fig. 7C). Since the repressive action of genes restricted to more ventral expression domains (possibly *dlx3*) is absent in *sbn*, the broad ventral *AP-2* expression domain is not resolved into the lateral stripes of the presumptive neural crest during early somitogenesis. Consequently, *AP-2* remains expressed in a broad ventral domain resulting in an expansion of the neural crest progenitors (Fig. 7C). In *snh* the moderate levels of Bmp activity present are sufficient to induce *AP-2* and *dlx3*, but not *gata2*. The steeper Bmp activity gradient in *snh* mutant embryos compared to *sbn* mutants results in a less pronounced expansion in neural crest, due to the presence of more ventrally expressed genes (e.g., *dlx3*), which can repress *AP-2* in these regions (Fig. 7B).

How does this model fit with previous studies showing that an interaction between nonneural and neural ectoderm results in the specification of the neural crest (Dickinson *et al.*, 1995; Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995)? A signal, possibly BMP4 and/or BMP7, from the nonneural tissue has been hypothesized to induce neural ectoderm to form neural crest at the border between these two tissues (Liem *et al.*, 1995; Mayor *et al.*, 1995). This model can explain the absence of neural crest progenitors in *swirl*, since there is no apparent nonneural ectoderm in *swirl* mutant embryos. However, the large expansion of neural crest in *sbn* mutant embryos is not easily explained by

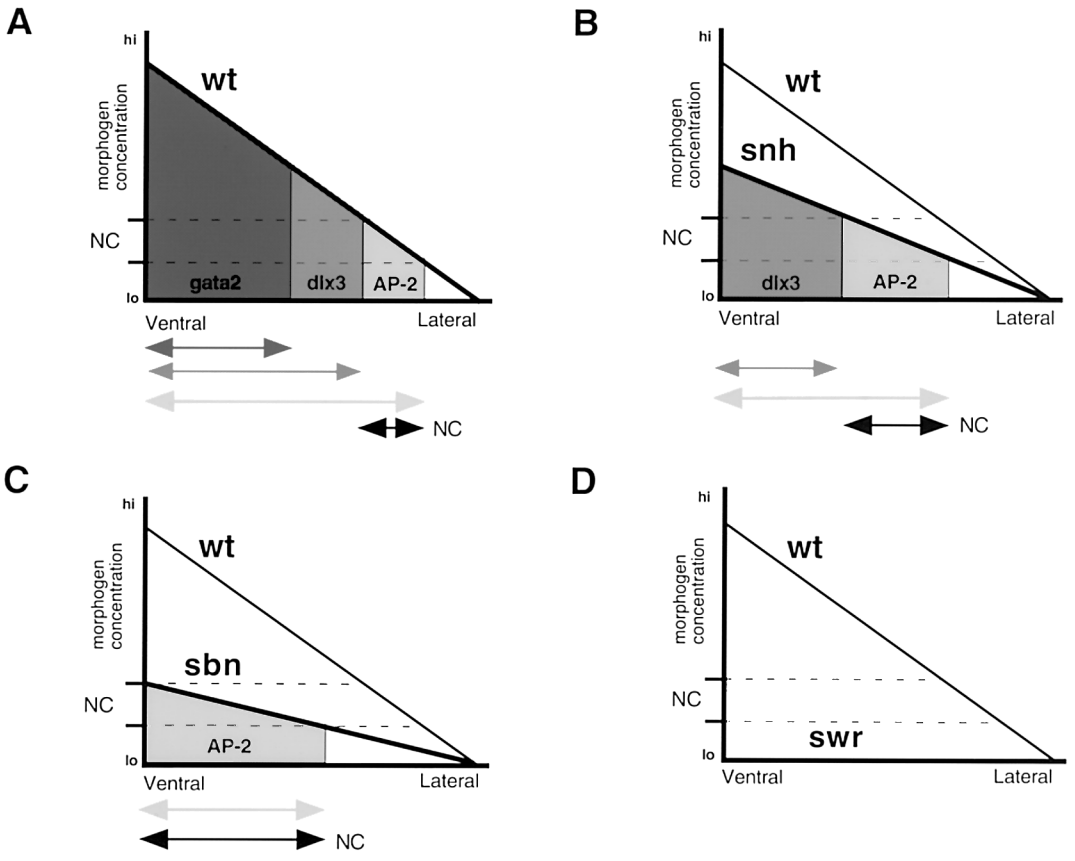

FIG. 7. A ventral morphogen model can account for the patterns of *AP-2, dlx3,* and *gata2* gene expression and the neural crest phenotypes in *swirl, sbn,* and *snh* embryos. (A) In a wild-type early gastrula, the expression of *AP-2, dlx3,* and *gata2* is represented by arrows under the graph. By the end of gastrulation, a gene expressed in a smaller ventral domain than *AP-2* (e.g., *dlx3*) represses the expression of *AP-2* resulting in bilateral stripes of *AP-2* expression. These bilateral stripes correspond to those cells that were present in areas of low morphogen activity, as denoted by NC on the *y* axis, in the early gastrula. Later in development, these cells will differentiate into neural crest cells. The dorsoventral position and quantity of the neural crest progenitors at the end of gastrulation are represented by the location and length, respectively, of the arrow under the graph. Compared to wild-type embryos, a lower gradient of morphogen activity is present during gastrulation in *snh* mutants (B), resulting in the aberrant gene expression patterns and the slight to moderate expansion of neural crest progenitors observed. In *sbn* (C), a very low gradient is present, resulting in a large expansion in the number of neural crest progenitors in a domain that extends to the ventral midline of the embryo. In *swirl* (D), no morphogen activity is present, leading to the absence of neural crest progenitors.

this model, unless both neural and nonneural ectodermal cells are intermixed in ventral and lateral regions in *sbn* mutants. An interaction between these two cell types could then result in the specification of the neural crest throughout the ventrolateral region of *sbn* embryos.

We propose that the role of the neural tissue in the specification of the neural crest may be in the generation of low levels of BMP activity at the neural plate/nonneural ectodermal border. This could be mediated through the action of Chordin, Noggin, and/or Follistatin present in dorsal tissue by directly binding and inhibiting BMP activity. In zebrafish, *chordin* alone does not fulfill this role, since *chordino* mutants display relatively normal amounts of presumptive neural crest (V. H. Nguyen and M. C. Mullins, unpublished observations). In explant studies in *Xenopus,* low doses of Noggin can induce dorsal brain markers suggesting a role for *noggin* in establishing dorsal neural tube cell types via the generation of low levels of BMP activity (Knecht and Harland, 1997). Furthermore, overexpression of *noggin* in ventral regions of the frog embryo can induce ectopic neural crest, suggesting a function for *noggin* in this process as well (Mancilla and Mayor, 1996; Mayor *et al.*, 1997). Although low BMP levels may be necessary for specification of the neural crest, low BMP activity may not be sufficient (Wilson *et al.,* 1997) and other factors, possibly FGF (Mayor *et al.,* 1997), may also be required.

ACKNOWLEDGMENTS

We thank Ela Knapik, Howard Jacob, and Mark Fishman for providing the SSLP markers prior to publication. We thank the following colleagues for generously providing cDNA clones: Jörg Odenthal for *fkd3* and *fkd6;* Alvin Chin for *bmp4;* Naoto Ueno and Masazumi Tada for *bmp2b*, *bmp4*, and *Xbmp7;* Christine and Bernard Thisse for *AP-2* and *sna2;* Eric Weinberg for *otx2;* Douglas Melton for *Xmad1;* and Liliana Attisano and Jeffrey Wrana for the constitutively activated *Alk6*. We also thank Ralph Rupp for providing us with the CS2+ vector; Michael Pack for the Apoptag reagents as well as suggestions about the assay; members of the Weinberg lab, Matt Deardorff, and Peter Klein for helpful discussions about RNA injections; Corinne Houart for her modifications of the *in situ* protocol; Michael Granato, Nadine Peyrieras, Daniel Wagner, Patricia Labosky, and Daniel Kessler for helpful comments on the manuscript; Eric Weinberg for temporary use of his fish facility; and special thanks to Andrea Payne and Rita Kolchinsky for care of the fish and facility. This work was supported by an NIH predoctoral fellowship to V.H.N., by the DAAD organization to B.S., and by NIH Grant RO1-GM56326 and Penn-Hughes start-up funds to M.C.M.

Note added in proof. Kishimoto *et al.* (1997) also recently identified *swirl* to be a mutation in the *bmp2b* gene.

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Received for publication December 31, 1997
Accepted April 6, 1998