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Differential Regulation of *chordin* Expression

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Patterning along the dorsal–ventral (D–V) axis of *Xenopus* and *Drosophila* embryos is believed to occur through a conserved molecular mechanism, with homologous proteins Chordin and Short gastrulation (Sog) antagonizing signaling by bone morphogenetic protein 4 (BMP-4) and Decapentaplegic (Dpp), respectively. We have isolated a zebrafish gene that is highly homologous to *chordin* and *sog* within cysteine-rich domains and exhibits conserved aspects of expression and function. As in *Xenopus* embryos, zebrafish *chordin* is expressed in the organizer region and transiently in axial mesoderm. Injection of zebrafish *chordin* mRNA to the ventral side of *Xenopus* embryos induced secondary axes. Ectopic overexpression in zebrafish resulted in an expansion of paraxial mesoderm and neurectoderm at the expense of more lateral and ventral derivatives, producing a range of defects similar to those of dorsalized zebrafish mutants (Mullins *et al.*, 1996). In accordance with the proposed function of *chordin* in D–V patterning, dorsalized zebrafish mutants showed expanded domains of *chordin* expression by midgastrulation, while some ventralized mutants had reduced expression; however, in all mutants examined, early organizer expression was unaltered. In contrast to *Xenopus*, zebrafish *chordin* is also expressed in paraxial mesoderm and ectoderm and in localized regions of the developing brain, suggesting that there are additional roles for *chordin* in zebrafish embryonic development. Surprisingly, paraxial mesodermal expression of *chordin* appeared unaltered in *spadetail* mutants that later lack trunk muscle (Kimmel *et al.*, 1989), while axial mesodermal expression was affected. This finding reveals an unexpected function for *spadetail* in midline mesoderm and in differential regulation of *chordin* expression during gastrulation.

INTRODUCTION

Dorsal marginal cells of the early amphibian embryo can influence mesodermal differentiation along the dorsal–ventral axis and direct presumptive ectoderm to take on a neural rather than epidermal fate (refer to Lemaire and Kodjabachian, 1996; Thomsen, 1997). Transplantation of this dorsal region, referred to as the Spemann organizer, from one gastrula to the ventral side of another, results in the induction of a second embryonic axis consisting of both donor and host cells (Spemann and Mangold, 1924). It was long-held that the organizer directs patterning of adjacent dorsolateral mesoderm and ectoderm through the action of secreted molecules, and in the absence of these factors, ventral cell fates would be assumed. Candidate proteins for mediating organizer activity *in vivo* include Chordin, Noggin, and Follistatin, all of which dorsalize mesoderm and induce neurectoderm (Smith and Harland, 1992; Hemmati-Brivanlou *et al.*, 1994; Sasai *et al.*, 1994).

More recently, development of ventral derivatives such as blood and epidermis has been found to occur not by default, but by the activity of ventralizing factors such as bone morphogenetic proteins (BMPs). Ectopic expression of BMP-4 in *Xenopus*, for example, causes an expansion of ventral mesoderm at the expense of more lateral and dorsal mesoderm (Jones *et al.*, 1992; Schmidt *et al.*, 1995a) and rescues embryos dorsalized by LiCl (Fainsod *et al.*, 1994). Conversely, disruption of BMP signaling leads to an increase in dorsal mesoderm and neurectoderm (Graff *et al.*, 1994; Hawley *et al.*, 1995; Schmidt *et al.*, 1995a; Steinbeisser *et al.*, 1995). Further studies have shown that both Chordin and Noggin proteins directly bind BMP-4 and prevent it from activating its receptor (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). These findings suggest that dorsally derived proteins function *in vivo* by antagonizing the ventralizing action of BMP-4.

[1] This paper is dedicated in memory of Dr. Jane Oppenheimer (1911–1996) for her pioneering studies on the teleost organizer.

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The role of Chordin in antagonizing BMP signaling is conserved in evolution (Holley *et al.*, 1995; Sasai *et al.*, 1995). In *Drosophila melanogaster*, *short gastrulation* (*sog;* François *et al.*, 1994; Biehs *et al.*, 1996), a *chordin* homologue (François and Bier, 1995; Schmidt *et al.*, 1995b), antagonizes the function of *decapentaplegic* (*dpp*), a *bmp-4* homologue (Padgett *et al.*, 1987). In addition, the proteins themselves can functionally substitute for one another. *Xenopus* embryos ventralized by ultraviolet light can be rescued by injection of *sog* mRNA, whereas injection of *chordin* mRNA into *D. melanogaster* mimics some of the ventralizing effects of ectopic *sog* expression (Holley *et al.*, 1995; Schmidt *et al.*, 1995b).

As in amphibians, gastrulae of other vertebrates possess organizing centers which presumably mediate their effects through similar mechanisms (Kintner and Dodd, 1991; Ho, 1992; Storey *et al.*, 1992; Beddington, 1994; Shih and Fraser, 1996). In developing mouse embryos, BMP-4 is also essential for mesoderm formation; however, a detailed phenotypic analysis of BMP-4 null mutants has been difficult since they are most often early embryonic lethals. Mice deficient for BMP-4 which survive gastrulation, possibly through rescue by diffusion of maternal BMP-4, exhibit selective mesodermal defects, including a lack of blood islands in the visceral yolk sac (Winnier *et al.*, 1995).

In zebrafish and other teleost fishes, the dorsal embryonic shield has properties similar to those of the *Xenopus* organizer. Transplantation of cells from the embryonic shield to the ventral side of another embryo results in the formation of a second axis (Oppenheimer, 1936; Ho, 1992; Shih and Fraser, 1996). Furthermore, the embryonic shield has been found to express many of the same genes expressed in the *Xenopus* organizer region (Stachel *et al.*, 1993; Strähle *et al.*, 1993; Schulte-Merker *et al.*, 1994; Thisse *et al.*, 1994; Talbot *et al.*, 1995, Toyama *et al.*, 1995).

From recent mutagenesis screens in zebrafish, mutations have been isolated that alter specification of the dorsal–ventral axis at gastrulation (Hammerschmidt *et al.*, 1996b; Mullins *et al.*, 1996; Solnika-Krezel, 1996; Fisher *et al.*, 1997), thereby providing a genetic means for exploring the mechanisms underlying vertebrate embryonic patterning. A number of mutations result in a dorsalized phenotype, in which dorsal and dorsolateral derivatives are expanded with a corresponding reduction in more ventral derivatives. In contrast, ventralizing mutations are characterized by an increase in ventral derivatives (blood and epidermis) and a decrease in dorsolateral derivatives (somites and neurectoderm). Analysis of the ventralizing mutation *dino* (Hammerschmidt *et al.*, 1996a,b) strongly corroborates the model for dorsal–ventral pattern formation derived from the amphibian and *Drosophila* studies (Holley and Ferguson, 1997). In fact, recent molecular characterization confirms that the defect of *dino* (renamed *chordino*) is due to a lesion in the zebrafish *chordin* homologue (Schulte-Merker *et al.*, 1997). In the absence of *chordin*, ventralizing signals are inappropriately active in more dorsal regions of the embryo, thereby producing the ventralized *chordino* mutant phenotype.

We have tested the dorsalizing activity of the zebrafish *chordin* homologue and examined expression in early zebrafish development. Zebrafish *chordin* is initially expressed shortly after midblastula transition and, as in *Xenopus*, comes to be strongly expressed in the organizer region at early gastrulation stages. The effect of ectopic *chordin* mRNA in wild-type (WT) embryos and the expanded expression domains that we find in dorsalized zebrafish mutants are consistent with the dorsalizing action of *chordin in vivo*.

In contrast to *Xenopus chordin*, expression of the zebrafish gene is not restricted to the organizer and its derivatives, but is also transiently expressed in the developing brain and in paraxial mesoderm and ectoderm. To probe the functions of these additional *chordin* domains, we have examined the effect of other zebrafish mutations on *chordin* expression. In *spadetail* mutants, which lack *myoD* expression at gastrulation (Weinberg *et al.*, 1996), and later lack trunk somitic mesoderm (Kimmel *et al.*, 1989; Ho and Kane, 1990), expression of *chordin* in paraxial mesoderm appears normal. However, expression of *chordin* is not maintained in the mutant midline, revealing a function for *spadetail* in axial mesoderm and in the differential regulation of *chordin*.

MATERIALS AND METHODS

Embryo Culture and Staging

Techniques for the care and breeding of zebrafish were followed as described (refer to Westerfield, 1993). Embryos were collected from single pair matings, maintained in embryo medium (15 mM NaCl, 0.5 mM KCl, 1mM $CaCl_2$, 1mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 0.7 mM $NaHCO_3$) at 28.5°C, and staged according to hours (h) postfertilization and morphological criteria (Kimmel *et al.*, 1995).

Zebrafish Mutants

All studies on WT fish were carried out using the AB line from Oregon (Fritz *et al.*, 1996; provided by C. Walker). The gamma ray-induced mutations *cyclops*^{b16} (Hatta *et al.*, 1991), *no tail*^{b160} (Halpern *et al.*, 1993), and *short tail*^{b180} (C. Kimmel, personal communication); the spontaneous mutations *floating head*^{n1} (Halpern *et al.*, 1995; Talbot *et al.*, 1995), *spadetail*^{b104} (Kimmel *et al.*, 1989), and *ogon*^{m60} (Solnika-Krezel *et al.*, 1996); and the ethylnitrosourea (ENU)-induced mutations *swirl*^{ta72}, *somitabun*^{dtc24}, *snailhouse*^{ty68a} (Mullins *et al.*, 1996), and *mercedes*^{tm305} (Hammerschmidt *et al.*, 1996b) were used for *chordin* expression analyses. Intercrosses of heterozygous fish produced approximately 25% homozygous mutant progeny, with the exception of *swr*^{ta72} and *sbn*^{dtc24} which are zygotic semidominant alleles (refer to Mullins *et al.*, 1996). All mutations were maintained in their original genetic background through intercrosses of heterozygous parents, except for *sbn* which is also a maternal dominant mutation and was maintained by outcrossing heterozygous males.

Isolation of a Zebrafish chordin Gene

Degenerate oligonucleotides, 5′-TT(C/T)GG(A/G/C/T)GT(A/G/C/T)ATG(C/T)A (C/T)TG-3′ and 5′-GG(A/G)CA(A/G/C/T)GT(C/

T)TT(A/G)CA(A/G)CA-3′, were prepared from DNA sequences in the first cysteine-rich (CR) repeat of *Xenopus chordin* and *Drosophila sog* and used for polymerase chain reaction (PCR) amplification from a zebrafish gastrula stage cDNA library (provided by M. Rebagliati and I. Dawid). Reaction conditions were as follows: initial denaturation at 96°C for 2 min, 72°C for 10 min, followed by 36 cycles of 94°C for 1 min, 50°C for 1.5 min, 72°C for 15 s, and a final incubation of 72°C for 10 min. The resulting 161-bp fragment was gel purified using the Qiaex II gel extraction kit (Qiagen), reamplified, and subcloned using the Original TA cloning kit (Invitrogen). The zebrafish fragment was used as a probe to screen a gastrula stage cDNA library prepared in λZAPII (provided by S. Ekker), and two clones, 1.12S and 11.13, were obtained.

Northern Analysis

Total RNA was isolated from staged zebrafish embryos using Trizol (Gibco BRL), and aliquots (10 μg) from each stage were separated by formaldehyde–agarose gel electrophoresis and transferred to Magnagraph nylon membrane (Micron Separations, Inc.). High specific activity probes ($>10^9$ dpm/μg) were prepared using the PrimeIt II system (Stratagene) from a *Hin*fII restriction fragment (601 bp) encompassing the *chordin* CR3 and CR4 repeats. After hybridization, the blot was washed to a stringency of 0.1× SSC, 0.5% SDS at 65°C and visualized with a Storm Fluorimager system (Molecular Dynamics).

RNA in Situ Hybridization

Zebrafish *chordin* (clone 11.13 in pBluescript) was linearized with *Spe*I for synthesis of antisense mRNA by T7 RNA polymerase (Promega), in the presence of digoxigenin-UTP (Boehringer-Mannheim). Whole-mount *in situ* hybridization was performed according to Thisse *et al.* (1993). Double-labeling *in situ* hybridization techniques using fluorescein-UTP (Boehringer-Mannheim) for a *chordin* probe and digoxigenin-UTP for a *krox-20* probe (Oxtoby and Jowett, 1993) were performed as previously described (Hauptmann and Gerster, 1994).

Embedding and Sectioning of Zebrafish Embryos

Following *in situ* hybridization, embryos were postfixed in 4% paraformaldehyde, dehydrated through a graded ethanol series, and infiltrated overnight with London Resin Gold (Ted Pella, Inc.), then transferred to fresh resin. The resin was polymerized by adding benzoin methyl ether (0.5%) and UV irradiating overnight. Sections (1.5 μm) were cut using a Reichert ultramicrotome equipped with a diamond knife.

RNA Synthesis and Microinjection

To produce an expression vector for *in vitro* synthesis of full-length *chordin* RNA, the 2.8-kb coding region of clone 1.12S was amplified by long-range PCR using *Pfu* DNA polymerase (Stratagene). Oligonucleotide primers included *Bam*HI restriction sites for ligation of the partially digested PCR product into linearized pCS2+ vector (Turner *et al.*, 1994). Capped *chordin* mRNA was synthesized using the mMessage mMachine kit (Ambion).

Xenopus laevis oocytes were fertilized *in vitro*, and following cortical rotation, viable embryos were dejellied in 2% *L*-cysteine. A mixture (5–10 nl) of *chordin* mRNA and green fluorescent protein (GFP) mRNA (20 ng/μl each) was injected into the ventral side of one- to two-cell embryos. Following injection, embryos were incubated in 3% Ficoll for approximately 2 h and transferred to dechlorinated water. Embryos were examined for the appearance of the dorsal blastopore lip and, at various times, were scored for abnormal blastopores and the development of secondary axes.

Zebrafish embryos (one- to four-cell stage) were pressure injected (8 nl) with a mixture of *chordin* mRNA and GFP mRNA (25–100 and 100 ng/μl, respectively). At gastrulation, injected embryos were examined using a stereomicroscope (MZ12, Leica) with fluorescence and GFP filter attachments (Kramer Scientific). At the four- to six-somite stage, the degree of dorsalization was assessed using the morphological criteria described by Mullins *et al.* (1996). The most severely dorsalized embryos (classes 4 and 5) were fixed in 4% paraformaldehyde and processed for whole-mount *in situ* hybridization using either *pax2* (Krauss *et al.*, 1991) and *myoD* (Weinberg *et al.*, 1996) or *pax2* and *ntl* (Schulte-Mercker *et al.*, 1992) antisense RNA probes. Less severely affected embryos (classes 1–3) were allowed to develop and were reexamined at 24 h.

RESULTS

Isolation of a Zebrafish chordin Homologue

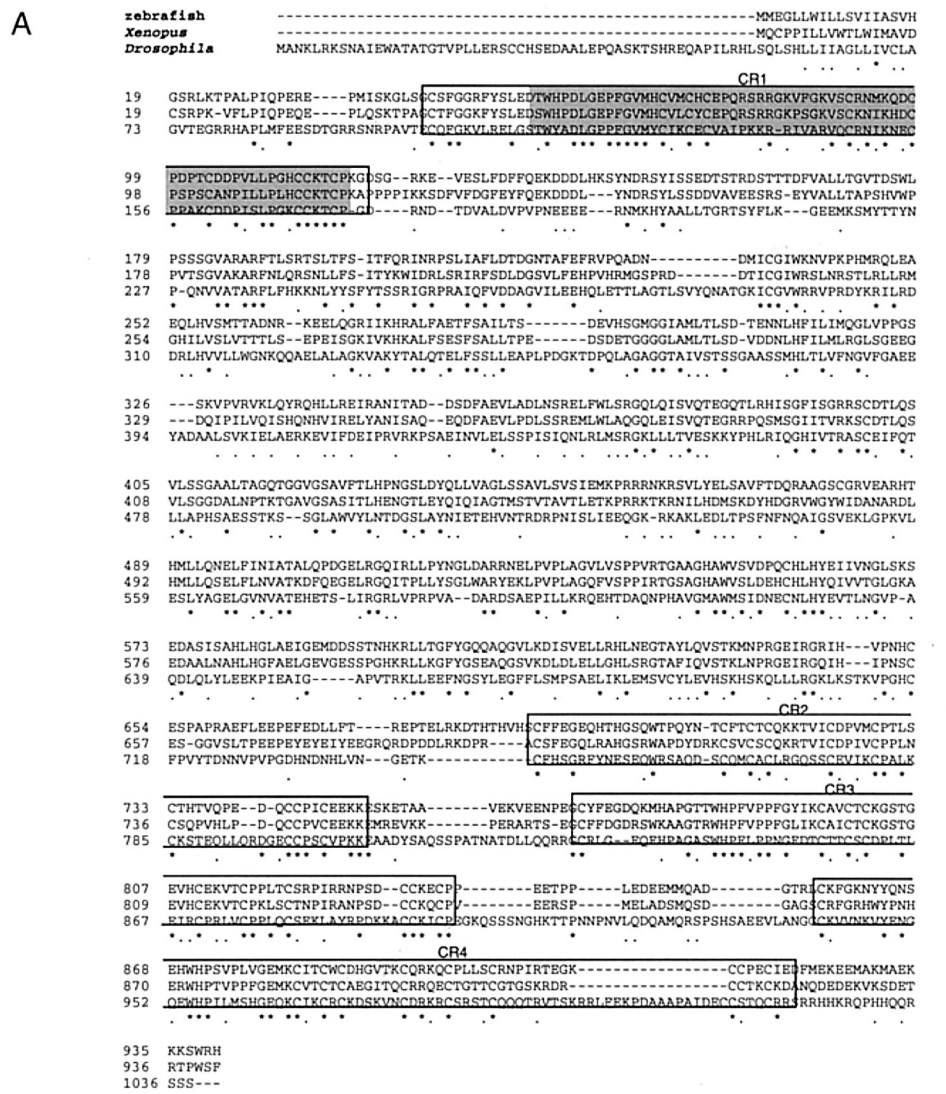
Degenerate DNA primers were designed within four highly conserved CR regions (CR1–CR4) of the *Xenopus chordin* and *Drosophila sog* coding sequences and used in PCR amplifications with a zebrafish cDNA library as template. A resulting amplification product (161 bp) was found to be 45% homologous to *Xenopus* CR1 at the nucleotide level (Fig. 1A). A full-length coding sequence of the zebrafish gene (Fig. 1A) was derived from two cDNA clones (1.12S and 11.13) that were obtained by screening a gastrula-stage cDNA library. Translation of the single open reading frame generates a predicted protein of 940 amino acids which is 54 and 29% identical to the *Xenopus* and *Drosophila* proteins, respectively (Fig. 1B). Proteins of all three species contain the four highly conserved and similarly spaced CR repeat regions.

A single *chordin* transcript of approximately 4 kb was detected by Northern blot analysis prior to gastrulation and a maximal level of expression was reached at 70% epiboly (Fig. 1C). Expression persisted throughout early somitogenesis but was greatly decreased by the 20-somite stage (19 h) and not detected at 24 h.

Dynamic Pattern of chordin Expression during Gastrulation

In *Xenopus* embryos, *chordin* is first expressed in the dorsal lip and subsequently in tissues derived from the organizer, including the prechordal plate, notochord, and chordoneural hinge (Sasai *et al.*, 1994). As in *Xenopus*, the expression pattern of zebrafish *chordin* is highly dynamic and is initially confined to the organizer region, the dorsal embryonic shield.

Expression of zebrafish *chordin* was first detected by *in situ* hybridization approximately 1 h following the midblastula transition (MBT) and 1 2/3 h prior to the onset of gastru-


A
zebrafish
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CR1
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CR3
CR4
Amino Acid Identity (%)
B
Xenopus
Zebrafish
Drosophila
CR1
CR2
CR3
CR4
40 77 52 59 24 76 39 42 22
16 51 24 36 23 41 26 36 6
mat sph 30% shld 70% 6s 20s 24h
28 s
18 s

lation in a region presumed to form the future dorsal one-third of the embryo (oblong stage; data not shown). By sphere stage (4 h postfertilization), the *chordin*-expressing domain encompassed approximately one-quarter of the circumference of the embryo (Figs. 2A and 2B) and by the onset of gastrulation coincided with the dorsal embryonic shield (Figs. 2C and 2D). As gastrulation proceeded *chordin* was strongly expressed in the midline of the newly forming axis and in new bilateral domains flanking the dorsal shield (Figs. 2E–2H).

Sectioning of gastrulas (75% epiboly) revealed that the strong midline expression was confined to deep cells (Figs. 2D and 2H) which likely corresponded to mesoderm of the prechordal plate and presumptive notochord, based on partial overlap between *chordin* and *goosecoid* expression (data not shown). By late epiboly stages, *chordin* was no longer expressed in anterior axial mesoderm but was transiently expressed in adaxial cells (Figs. 2I and 2J). In contrast to the midline, *chordin* expression in lateral domains encompassed all ectodermal and mesodermal cell layers, with the exception of the cell layer closest to the yolk which did not express *chordin* (Fig. 2H). These domains spread in a lateral and vegetal direction during epiboly and were largely confined to the tailbud by somitogenesis stages (Figs. 2J and 2K).

In the central nervous system (CNS), *chordin* expression was first detected at 100% epiboly in discrete regions of the forebrain, midbrain, and hindbrain (Fig. 2L). Double *in situ* hybridization for *chordin* and *krox-20,* which is expressed in rhombomeres 3 (r3) and 5 (r5), demonstrated that expression of *chordin* was dynamic in the hindbrain. Although there was considerable variability between embryos of the same stage, *chordin* was generally expressed in r2 through r5 at 100% epiboly and only in r2 and r4 at the 3- to 5-somite stage. By the 8-somite stage, *chordin* expression was greatly diminished in the brain and was barely detectable in 12- to 15-somite stage embryos (data not shown).

Dorsalizing Activity of Zebrafish chordin in Frog and Fish Embryos

To test whether zebrafish Chordin possessed activities similar to the *Xenopus* and *Drosophila* proteins, full-length zebrafish *chordin* mRNA was injected into the ventral side of one- to two-cell stage *Xenopus* embryos. Morphologically recognizable secondary axes were formed in 45% of injected embryos ($n = 89$; Table 1). In no case were supernumerary dorsal blastopore lips or secondary axes seen in uninjected or GFP mRNA-injected control embryos.

In most *chordin*-injected embryos, an incomplete secondary axis emerged from the primary axis (compare Figs. 3A and 3B); however, partial secondary axes were occasionally formed on the ventral side (Fig. 3C). Somites and the dorsal tail fin were easily distinguished in embryos where the second axis formed opposite the primary axis. Sectioning ($n = 6$) revealed the presence of neural tissue (data not shown); however, notochord cells were not detected. Formation of anterior structures, such as the eyes or cement glands, was never observed.

The dorsalizing activity of zebrafish Chordin was also tested by ectopic overexpression in zebrafish embryos. Full-length *chordin* mRNA (200–800 pg) was coinjected with GFP mRNA into the yolk of one- to four-cell stage embryos. At early somite stages, injected embryos were examined for morphological phenotypes. The most severely dorsalized embryos (equivalent to classes 4–5 of Mullins *et al.,* 1996) were elongated in shape and had laterally expanded somites (Figs. 3E and 3F). Less severely affected embryos were characterized at later stages of development (refer to Table 2) by the lack of the ventral tail fin (classes 1–3), slightly enlarged tailbud (classes 2 and 3), and fused posterior somites (class 3).

In strongly dorsalized embryos ($n = 20$), expression patterns of *pax2* (Krauss *et al.,* 1991), *myoD* (Weinberg *et al.,* 1996), and *ntl* (Schulte-Merker *et al.,* 1992) were considerably altered compared to mock-injected embryos (Figs. 3D–3I). Somite expression of *myoD* typically extended around the entire embryonic circumference (Figs. 3E and 3F), as did expression of *pax 2* in the midbrain. Expression of *pax2* in the pronephric ducts (Fig. 3G) was also ventrally displaced or entirely lacking ($n = 10$; Figs. 3H and 3I). Analysis of *ntl* expression in the presumptive notochord revealed varying, but limited, degrees of lateral expansion (Fig. 3H). Together, the results indicate that *chordin* overexpression promotes differentiation of dorsolateral cell fates at the expense of ventral or ventrolateral fates, but is not sufficient to induce ectopic development of the most dorsal mesodermal derivatives.

Expanded and Reduced chordin Expression in Dorsalized and Ventralized Zebrafish Mutants

To explore the action of *chordin* further, we examined the relationship between *chordin* expression and zebrafish mutations previously implicated in dorsal–ventral pattern

FIG. 1. Sequence conservation and expression of zebrafish *chordin.* (A) The alignment compares the amino acid sequence of zebrafish Chordin with *Xenopus* Chordin and *Drosophila* Short gastrulation. Stars and dots indicate identical amino acids and conservative amino acid substitutions, respectively. Cysteine-rich repeats are outlined in boxes and the region corresponding to the CR1 PCR amplification product is shown in gray. (B) The numbers indicate the percentages of amino acid identity within and outside the CR repeats (shaded) in the three proteins (proteins are not drawn to scale). (C) Northern blot analysis demonstrates that a single *chordin* transcript is present at low levels in the late blastula, increases during gastrulation, and persists through early somitogenesis. RNA was collected from embryos prior to midblastula transition (mat) and at sphere stage (sph), 30% epiboly (30%), 50% epiboly (shld), 70% epiboly (70%), 6 somites (6s), 20 somites (20s), and 24 h postfertilization (24h). For this blot, the probe was a *Hin*fII restriction fragment (601 bp) that included CR3 and CR4.

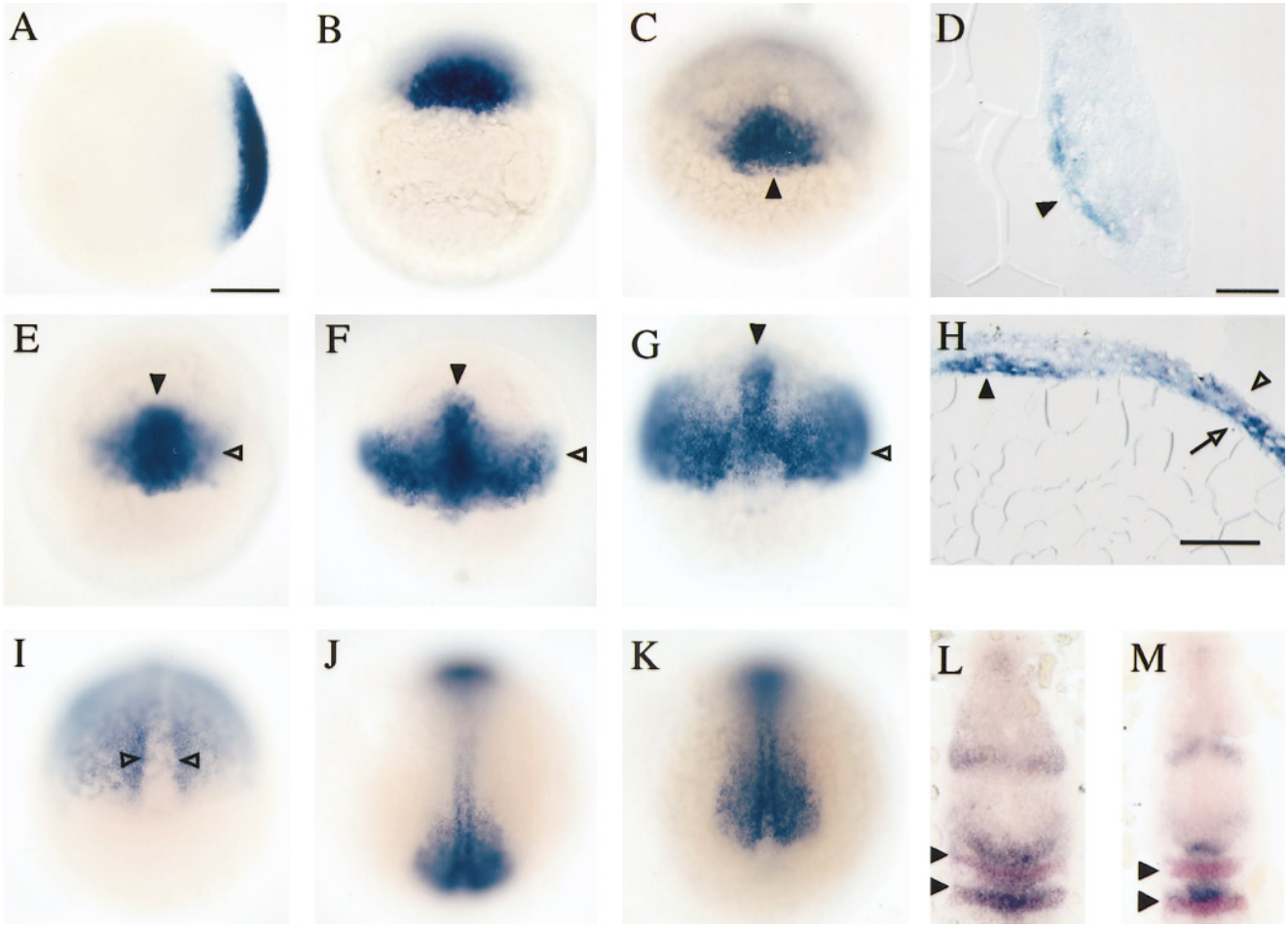

FIG. 2. Dynamic expression of *chordin* in the developing zebrafish. (A, B) Zebrafish *chordin* was detected at the future dorsal side of the late blastula (sphere stage: A, animal pole view; B, dorsal view). (C, D) By early gastrulation, expression was confined to deep cells in the embryonic shield (arrowhead). D is a sagittal section through the shield at 50% epiboly. (E–H) As gastrulation proceeded, expression was found transiently in the axis (arrowhead) and new bilateral domains of expression appeared (open arrowhead) in all superficial (ectoderm) and deep (mesoderm) cell layers, except the layer closest to the yolk (open arrow, H). E is shield stage at 50% epiboly, F is 30 min after E, and G and H are at 70% epiboly. H is a transverse section through the axis and flanking regions. (I–K) In late gastrulae, *chordin* was expressed in cells adjacent to the notochord and downregulated in the axis (open arrowheads). Bilateral expression domains persisted in the most posterior region only. I is at 90% epiboly and J and K are at 100% epiboly. (L, M) At late gastrulation, *chordin* was also expressed in discrete domains in the forebrain, midbrain, and hindbrain (in blue). Double labeling with a probe for *krox-20*, which is expressed in rhombomeres 3 and 5 (in pink), indicated that *chordin* expression in the hindbrain is very dynamic, present in different rhombomeres over time. Scale bars = 150 μm (A–C, E–G, and I–M), 25 μm (D), and 50 μm (H).

formation at gastrulation. The mutations we focused on fell into two classes: those that produce dorsalized phenotypes (*swirl, snailhouse, somitabun,* Mullins *et al.,* 1996) and those that produce ventralized phenotypes (*mercedes,* Hammerschmidt *et al.,* 1996b; *ogon,* Solnika-Krezel *et al.,* 1996; and *short tail,* V. Miller-Bertoglio and M. E. Halpern unpublished observations).

Embryos mutant for *swirl* (*swr*), *somitabun* (*sbn*), or *snailhouse* (*snh*) are characterized by the expansion of dorsally derived tissues such as neurectoderm and somitic mesoderm, as well as a decrease in more ventral derivatives such as the pronephric ducts and blood (Mullins *et al.,* 1996). In all of the dorsalized mutants that we examined, *chordin* expression appeared normal in the dorsal organizer (through shield stage; Fig. 4G and data not shown) and in the axial midline at later stages (Fig. 4B). However, as epiboly proceeded, the lateral domains of *chordin* expression were more ventrally expanded than in WT, which was most noticeable in animal or vegetal pole views (Figs. 4C–4F and 4H–4L). By 70% epiboly, *chordin* expression extended around the entire embryonic margin of *swr* mutants (23%, 9/39) and of more severely affected *sbn* mutants (33%, 16/45; Figs. 4D and 4J). In *snh* mutants (24%, 31/127), and in less severely affected *sbn* embryos (67%, 29/45), *chordin*

TABLE 1
Induction of Secondary Axis in *Xenopus* Embryos

chordin RNA[a] (pg)	Total number[b]	Number abnormal[c]	Number normal[d]	Number with second axis[e]	(%)
0	>150	0	>150	0	0
100	34	2	12	20	59
200	55	7	28	20	36

[a] All embryos were coinjected with GFP mRNA.
[b] Total number of injected embryos surviving gastrulation and neurulation.
[c] Number of embryos which did not neurulate properly.
[d] Number of injected embryos which developed a single, morphologically normal axis.
[e] Number of injected embryos which developed a secondary axis.

expression encompassed approximately 75% of the circumference of the margin (Figs. 4I and 4L). By 95% epiboly, however, this expression expanded around the entire margin of *snh* embryos, equivalent to the pattern observed in *swr* mutants (Fig. 4F and data not shown).

Ventralized mutants develop a phenotypic syndrome opposite to that of dorsalized embryos, with a narrower axis and reduced rather than expanded expression of neurectodermal and dorsal mesodermal markers. A variable increase in ventral derivatives such as blood and ectoderm, including extra fin folds in the tail, is also observed (Hammerschmidt *et al.,* 1996b, Solnica-Krezel *et al.,* 1996; V. Miller-Bertoglio and M. E. Halpern, unpublished observations).

In *mercedes* (*mes*) mutants that exhibit a weakly ventralized phenotype, *chordin* expression was indistinguishable from WT (Fig. 4M). In *ogon* (*ogo*) mutants, expression was normal in the embryonic shield; however, an overall decrease in the axial and lateral *chordin* expression domains was observed by 75% epiboly (25%, 7/28) and was more pronounced by 90% epiboly (25%, 22/88; Fig. 4O) compared to WT (Fig. 4N). In *short tail* (*stl*) mutants, alterations in the *chordin* expression pattern were not observed until 80% epiboly, when the lateral *chordin* expression domains flanking the axis were found to be less broad and thus more dorsally restricted (Figs. 4P and 4Q).

Selective Regulation of chordin Expression

The involvement of *chordin* in tissue patterning was examined further using zebrafish mutations that perturb the development of specific dorsal or dorsolateral derivatives such as the ventral neural tube, notochord, or somites.

In *cyclops* (*cyc*) mutants that display reduced axial mesoderm and neurectoderm and notably lack the CNS floor plate later in development (Hatta *et al.,* 1991; Thisse *et al.,* 1993; Yan *et al.,* 1995), *chordin* expression appeared normal throughout gastrulation (data not shown). Although *no tail* (*ntl*) mutant embryos fail to form notochords, expression of *chordin* was also relatively normal in progeny from *ntl*/+ intercrosses. Variably diffuse and wider expression domains were found at the midline of some embryos (data not shown), consistent with defects in cellular convergence previously observed in *ntl* mutant gastrulas (Melby *et al.,* 1997).

In *floating head* (*flh*) and *spadetail* (*spt*) mutants, *chordin* expression was unchanged in early gastrulation, but was noticeably altered by late gastrula stages (Fig. 5). At 90% epiboly, *chordin* was strongly expressed in the posterior region of the *flh* mutant midline (25%, 11/44), a region where expression was downregulated in WT embryos of the same age (Figs. 5A and 5B). The high level of expression that persisted in the midline of *flh* mutants likely resulted from the respecification of axial mesodermal cells to paraxial mesoderm that has been proposed to be the basis of the *flh* mutant phenotype (Halpern *et al.,* 1995; Melby *et al.,* 1996). Thus, as with the fused somites that later form in *flh* mutants, the lateral *chordin* expression domains appeared continuous across the midline.

The *spadetail* (*spt*) mutation is thought to cause the misspecification of trunk mesoderm, resulting in defective cellular convergence toward the dorsal side of the embryo, and an increase in cells in the tailbud at the expense of trunk somites (Kimmel *et al.,* 1989; Ho and Kane, 1990). We expected that the paraxial domains of *chordin* expression (Fig. 5C) would be altered in *spt* mutants, possibly contributing to the defect in trunk somite formation; however, this did not prove to be the case (Fig. 5D). Rather, axial mesodermal expression of *chordin,* which was normal at early gastrulation, was lost by 80% epiboly in *spt* mutants (26%, 24/92; compare Figs. 5E and 5F), revealing an unexpected role for spt^{+} in the gastrula midline.

DISCUSSION

We have cloned and characterized a zebrafish gene that encodes a protein highly homologous to Chordin and Sog in four similarly spaced cysteine-rich repeat regions. The cysteine-rich domains characterize a superfamily of extracellular matrix or cell-surface proteins, members of which have been shown to bind TGF-β (Paralkar *et al.,* 1991; Murphy-Ullrich *et al.,* 1992), and more recently BMP homo- and heterodimers (Piccolo *et al.,* 1996). The amino acid identity between the fish, fly, and frog proteins is relatively low

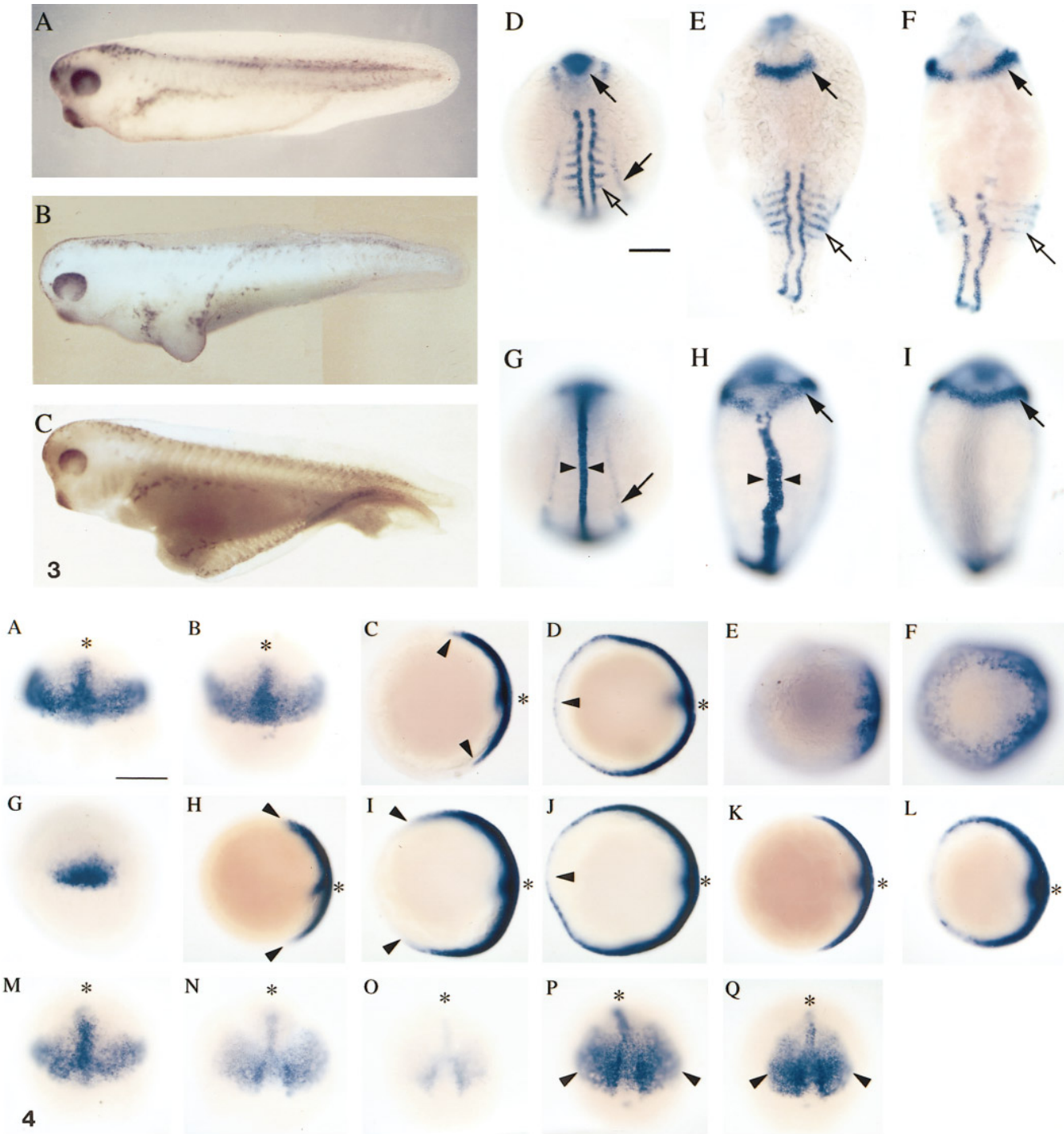

FIG. 3. Dorsalizing activity of zebrafish Chordin in *Xenopus* and zebrafish embryos. (A) Uninjected *Xenopus* embryo (stage 37/38); (B) *Xenopus* embryo coinjected with 10 nl of zebrafish *chordin* and GFP RNA (20 ng/μl) on the ventral side at the two-cell stage; (C) *Xenopus* embryo coinjected ventrally at the one-cell stage with 5 nl of zebrafish *chordin* and GFP RNA (20 ng/μl). (D–I) *In situ* hybridization using *pax2* and *myoD* probes (D–F) or *pax2* and *ntl* probes (G–I) of uninjected zebrafish embryos (D and G) or embryos injected with *chordin* RNA (E, F, H, and I) at the one- to four-cell stage during segmentation. All injected embryos shown correspond to class d of Table 2. (E, F) Severely dorsalized embryos were characterized by their elongated shape, laterally expanded domain of *pax2* expression at the midbrain–hindbrain junction (arrow), and ventrally extending somites (open arrows). (H) In some injected embryos, the *ntl* expression domain (arrowheads) in the notochord was widened relative to controls (G). D–H are dorsal views. (I) A ventral view of the same embryo as H shows the expansion of *pax2* expression in the brain around the entire circumference of the embryo (arrow). Pronephric duct expression of *pax2* (arrow, G) was often missing in injected embryos.

TABLE 2
Ectopic *chordin* Expression Dorsalizes Wild-Type Zebrafish Embryos

chordin RNA[a] (pg)	Total number of embryos[b]	Number morphologically wild-type[c]	Number mildly dorsalized[d]	Number strongly dorsalized[e]	Dorsalized (%)
0	98	—	—	—	0
200	62	25	20	17	60
400	64	11	24	29	83
800	99	20	42	37	80

[a] All embryos were coinjected with GFP mRNA.
[b] Total number of injected embryos which survived gastrulation.
[c] Embryos with a ventral tail fin at 24 h.
[d] Embryos classified as C1 and C2 after Mullins *et al.* (1996).
[e] Embryos classified as C3–C5.

outside of the cysteine-rich regions, yet all three proteins are involved in establishing cell identity along the dorsal–ventral axis of developing embryos.

Several lines of evidence support a conserved function for zebrafish Chordin as an antagonist of ventralizing signals at gastrulation. These include the timing and location of *chordin* expression, the dorsalizing activity resulting from ectopic expression of the zebrafish gene in frog and fish embryos, the alteration of expression in ventralized and dorsalized zebrafish mutants, and the embryonic phenotypes produced by deletion or point mutations of *chordin* (Fisher *et al.*, 1997, and unpublished observations; Schulte-Merker *et al.*, 1997).

Organizer Activity of Zebrafish Chordin

The amphibian organizer is believed to be composed of three domains which have distinct inductive capacities in the head, trunk, and tail (Gont *et al.*, 1993; refer to Lemaire and Kodjabachian, 1996). In *Xenopus, chordin* is initially expressed in the organizer and later in derivatives of each of these domains, including the prechordal plate, notochord, and chordoneural hinge (Sasai *et al.*, 1994). The zebrafish homologue is first expressed in the late blastula in a restricted domain of cells that give rise to the dorsal embryonic shield, the region presumed to be the teleost equivalent of the amphibian organizer (Oppenheimer, 1936; Ho, 1992; Shih and Fraser, 1996).

As with injections of *sog* RNA (Schmidt *et al.*, 1995b), injection of zebrafish *chordin* RNA to the ventral side of *Xenopus* embryos produced a partial secondary axis. While somites and dorsal fins were visible in some induced axes, notochords and anterior structures, such as eyes and cement glands, were not observed. Ectopic expression of *Xenopus chordin,* however, often resulted in the formation of notochord in secondary axes (Sasai *et al.*, 1994), suggesting that the frog protein is capable of inducing notochord. The zebrafish and fly proteins may have a lower overall activity in *Xenopus,* which could account for the absence of notochord in the ectopic secondary axis, or have different properties than the frog protein.

A Role for chordin in the Dorsolateral Territory of the Zebrafish Gastrula

Ectopic expression of *chordin* in the zebrafish embryo, through yolk injections of RNA, resulted in an expansion of dorsolateral mesodermal and neurectodermal derivatives at the expense of more ventral derivatives. In the most severely dorsalized embryos, expression of neural plate and somite markers was fully expanded to the ventral side of the embryo and *pax2*-expressing pronephric ducts derived

FIG. 4. Altered dorsolateral *chordin* expression domains in dorsalized and ventralized zebrafish mutants. (A–F) Expression of *chordin* in dorsalized *swr* mutants (B, D, F) was expanded ventrally (arrowheads) compared to WT sibs (A, C, E). A and B are animal pole views and C and D are dorsal views at 75% epiboly. E and F are vegetal pole views at 90% epiboly. (G) At shield stage, dorsal organizer expression of *chordin* was indistinguishable in *sbn* mutants and WT siblings (dorsal view) and in embryos from intercrosses of all other dorsalizing mutations examined (not shown). (H–L) Animal pole views. Expression was ventrally expanded (arrowheads) compared to WT (H, K) in both *sbn* (I and J) and *snh* (L) mutants at 75% epiboly. As described in the text, all embryos from *sbn* intercrosses had altered expression patterns which fell into two classes (I was the pattern of two-thirds and J was the pattern of one-third of embryos). (M–Q) Dorsal views. (M) *chordin* expression was indistinguishable in weakly ventralized *mes* mutants and WT siblings at 75% epiboly. (N, O) However, expression was decreased overall in ventralized *ogo* mutant embryos (O) compared to WT sibs (N) at 90% epiboly. (P, Q) Dorsolateral domains of *chordin* expression (arrowheads) were also more dorsally restricted in *stl* mutants (Q) compared to WT siblings (P) at 80% epiboly. Asterisks indicate the dorsal midline. Scale bar = 20 μm.

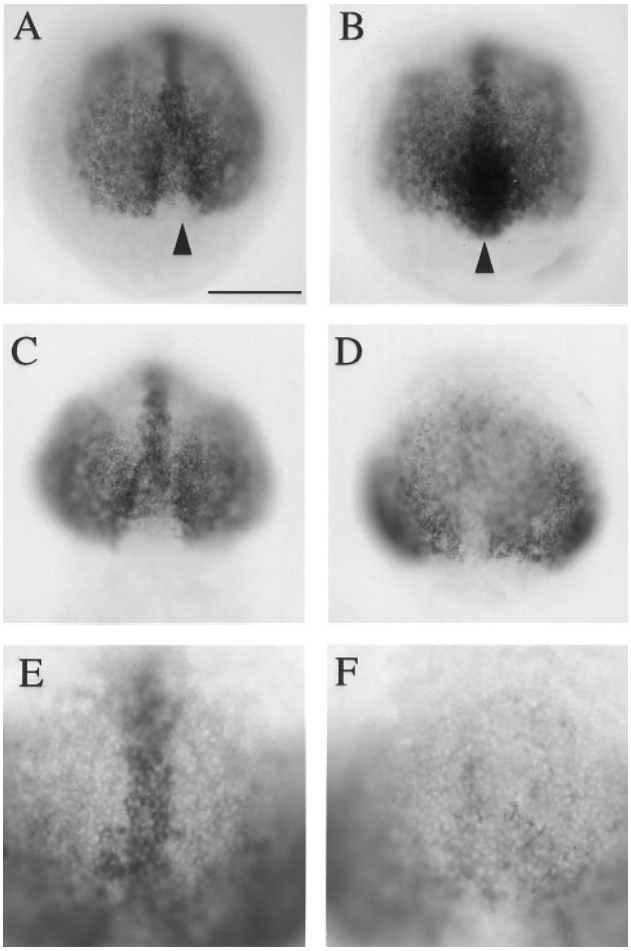

FIG. 5. Domains of *chordin* expression are selectively affected in *flh* and *spt* mutants. (A, B) At 90% epiboly, *chordin* expression decreases in the posterior midline of WT embryos (A), but is increased in this region in *flh* mutants (B). (C–F) At 80% epiboly, *chordin* expression is not maintained in the axis of *spt* mutant embryos (D, F), compared to WT sibs (C, E). Scale bar = 20 μm for A–D and 40 μm for E and F.

from lateral mesoderm were absent. More mildly dorsalized embryos had laterally expanded somites and small, ventrally displaced domains of *pax2* pronephric duct expression. Injected embryos often had wider notochords and an elongated shape, indicative of abnormal cell movements at gastrulation.

The observed classes of effects from *chordin* overexpression closely paralleled the phenotypes of zebrafish dorsalized mutants identified in recent mutagenesis screens (Mullins *et al.*, 1996). Embryos mutant for *swirl, somitabun,* and *snailhouse* have variably expanded somites and neurectoderm and wider notochords. However, as in the most severely dorsalized mutants, *chordin* overexpression alone was insufficient to promote a complete expansion of the most dorsal derivatives such as notochord. A similar phenomenon is found in *Xenopus* embryos, in which elimination of BMP-4 signaling by a dominant negative receptor expands the lateral mesoderm and neural plate without an increase of dorsal midline derivatives (Schmidt *et al.*, 1995a). One interpretation is that there is a lateral limit beyond which axial mesoderm cannot form, either because genes necessary for notochord specification are not expressed outside of the dorsal-most region or because genes expressed lateral to the midline limit the extent to which axial mesoderm can be induced.

Analysis of *chordin* expression in dorsalized and ventralized zebrafish mutants further supports the hypothesis that the dorsolateral region of ectoderm and mesoderm is the principal territory where *chordin* exerts its effects in the zebrafish gastrula. In the dorsalized mutants *swr, sbn,* and *snh, chordin* expression in the embryonic shield was unchanged at the onset of gastrulation, and only later was an expansion of the lateral expression domains observed. Conversely, at late gastrulation, the ventralized mutants *ogo* and *stl* showed an overall decrease in intensity and size of the lateral domains of expression, respectively. Thus, none of the mutations we examined affected the early expression of *chordin* in the organizer region. Rather, changes in the later dorsolateral expression domains correlated well with the phenotypic classes: expanded in dorsalized and decreased in ventralized mutants.

The most compelling evidence for the dorsolateral territory of the gastrula being the focus of *chordin* action comes from the characterization of embryonic phenotypes that result from deletions and point mutations of *chordin* (Hammerschmidt *et al.*, 1996a,b; Fisher *et al.*, 1997, and unpublished observations; Schulte-Merker *et al.*, 1997). Embryos mutant for *chordin* show a pronounced reduction in tissues derived from the dorsolateral region of the gastrula and a corresponding increase in ventral derivatives, while the formation of the organizer and its subsequent differentiation are less affected. We conclude, that at least for primary gastrulation, zebrafish *chordin* does not play a major role in defining the organizer region itself or in promoting the development of organizer derivatives, but is required for correct patterning of adjacent dorsolateral derivatives, by specifically antagonizing ventralizing signals in this region of the embryo.

The Molecular Basis for chordin Action

In *Xenopus* embryos, Chordin has been shown to bind BMPs and prevent them from activating their receptors (Piccolo *et al.*, 1996), and most likely exerts its effect in the zebrafish gastrula by a similar mechanism. To date, there has been no direct measurement of Chordin diffusion in the embryo, although it has been shown to be a secreted protein (Piccolo *et al.*, 1996). Recent work in *Xenopus* and zebrafish supports a model whereby BMP activity is present as a gradient in mesoderm and ectoderm and that the level of activity conveys positional information and sets the limits for development of specific cell types (Dosch *et al.*, 1997; Neave *et al.*, 1997; Wilson *et al.*, 1997). The gradient of BMP activity in the zebrafish has also been proposed to arise through

interactions with Chordin (Hammerschmidt *et al.*, 1996a); thus, in *chordin* mutants, BMP activity would expand unopposed and the gradient would be altered to favor development of ventral derivatives. Conversely, in the presence of increased Chordin, as with overexpression, the gradient would be altered in the opposite direction, favoring development of dorsal derivatives.

Homologues of *bmp-4* and *bmp-2* have been cloned and their expression has been examined in the zebrafish embryo (Chin *et al.*, 1997; Nikaido *et al.*, 1997). Both genes are expressed in ventral and ventrolateral regions of the gastrula and have ventralizing activity when ectopically expressed. However, in contrast to *Xenopus*, zebrafish *bmp-4* is also expressed dorsally, in the embryonic shield and its derivative, the prechordal plate. *bmp-2* is expressed earlier and is not expressed dorsally, leading to the suggestion that it is more of a functional equivalent to *bmp-4* in *Xenopus* (Nikaido *et al.*, 1997). Regions of *bmp-2/4* coexpression could also be sites of heterodimer formation. While the identity of the active BMPs in the zebrafish gastrula remains unknown, the recent discovery that *swr* mutations are lesions in the *bmp-2* gene (M. Mullins, unpublished observations) confirms the importance of BMP-2 in dorsal–ventral pattern formation.

Ectopic overexpression of either *bmp-2* or *bmp-4* in zebrafish embryos eliminates notochord (Nikaido *et al.*, 1997), suggesting that in *chordin* mutants that develop trunk notochord, another BMP antagonist must be present to prevent total ventralization of the embryo. Support for the presence of additional factors that antagonize ventral signals comes from the isolation of other zebrafish mutations that produce ventralized phenotypes (Hammerschmidt *et al.*, 1996b; Solnica-Krezel *et al.*, 1996; V. Miller-Bertoglio and M. E. Halpern, unpublished observations). Noggin and Follistatin (Smith and Harland, 1992; Hemmati-Brivanlou *et al.*, 1994) are candidates for this function in the zebrafish gastrula.

In addition to BMPs, other signaling pathways have been implicated in dorsal–ventral patterning of the zebrafish gastrula, including those involving Wnt (Kelly *et al.*, 1995a,b) and FGF (Fürthauer *et al.*, 1997) family members. At early gastrulation, *fgf-8* is expressed in a gradient emanating from the dorsal side of the embryo and upon overexpression, *fgf-8* promotes development of dorsolateral mesoderm and neurectoderm in a manner similar to *chordin*, most likely through the inhibition of *bmp-2* and *bmp-4* expression (Fürthauer *et al.*, 1997). This suggests that *fgf-8* functions in a common pathway; however, it remains to be shown whether it mediates its effects on BMPs directly or indirectly through regulation of *chordin* or by other mechanisms.

Identification of a number of loci that when mutated dorsalize zebrafish embryos also indicates that there are other genes involved in the pathway and reveals a role for maternal components (Mullins *et al.*, 1996). In particular, the observation of two types of abnormal *chordin* expression patterns in embryos from *sbn*/+ intercrosses correlates well with the dominant maternal effect described for this mutation (Mullins *et al.*, 1996) and further supports the involvement of maternally derived sbn^{+} in dorsal–ventral patterning.

Additional Sites for chordin Activity in the Zebrafish

BMP family members are involved in tissue patterning beyond gastrulation, influencing differentiation of the neural tube and somites, organogenesis of numerous organ systems, and skeletal morphogenesis (Dudley *et al.*, 1995; Luo *et al.*, 1995; Pourquié *et al.*, 1996; Reissmann *et al.*, 1996; Storm and Kingsley, 1996; Zhang and Bradley, 1996; Zou and Niswander, 1996; Macias *et al.*, 1997; Schultheiss *et al.*, 1997; and refer to Hogan, 1996). In addition to expression in the dorsal shield and midline mesoderm of the zebrafish gastrula, similar to that described for *Xenopus* (Sasai *et al.*, 1994), *chordin* transcripts were detected in restricted regions of the presumptive forebrain and midbrain–hindbrain junction and in a highly dynamic pattern in developing hindbrain rhombomeres. Although neither *bmp-4* nor *bmp-2* expression has been detected in the CNS of tailbud and early somite stage zebrafish embryos (Chin *et al.*, 1997; our unpublished observations), Chordin could function in patterning the brain by antagonizing other BMPs. For example, BMP-7 has been shown to influence both dorsal–ventral polarity and growth in the mouse hindbrain at a comparable stage in embryonic development (Arkell and Beddington, 1997). As in the early gastrula, binding to Chordin would be a rapid mechanism for limiting the range of BMP action in the developing zebrafish brain.

Another distinctive feature of *chordin* expression in the zebrafish were the lateral domains that arise secondarily to the dorsal embryonic shield or organizer expression. These domains persisted throughout gastrulation and tailbud stages to the late somite stage, corresponding to a period when elongation of the body axis and secondary neurulation occur (refer to Kimmel *et al.*, 1995). Expression in segmenting embryos was highest in the caudal region of the embryo, where dorsally and ventrally derived cells were shown to exhibit different migratory behaviors and give rise to a different complement of tissue fates (Kanki and Ho, 1997). We hypothesize that the role of the lateral *chordin* expression domains is equivalent to that of the initial organizer expression, to function within a tail organizing center to maintain correct dorsal–ventral polarity of the extending caudal trunk and tail. The lateral expression domains may therefore reflect one of the key differences between zebrafish and *Xenopus* gastrulation, namely the migration of cells over a single yolk mass that occurs in teleost epiboly which results in the eventual fusion of the dorsal and ventral sides of the blastoderm margin (Kimmel *et al.*, 1995; Kanki and Ho, 1997). Indeed, *chordin* may fulfill an expanded role in the zebrafish tailbud, since in contrast to the trunk, *chordino* (Hammerschmidt *et al.*, 1996b) as well as other mutant alleles (S. Fisher, unpublished observations) sometimes cause a loss of tail notochord.

Selective Regulation of chordin Expression

Through examination of *chordin* expression with respect to zebrafish mutations that perturb formation of specific dorsal or dorsolateral derivatives, we have begun to explore whether *chordin* functions in other aspects of tissue patterning. In *flh* mutants, dorsal axial mesoderm is believed to assume paraxial mesodermal identity and muscle forms in the midline in place of notochord (Halpern *et al.*, 1995; Melby *et al.*, 1996). Consistent with having an altered identity, the most posterior axial mesoderm in *flh* mutants expressed *chordin* at a high level more comparable to adjacent paraxial mesoderm, at a stage when expression is decreased in the WT midline. In *flh* mutants, the prolonged and intense *chordin* expression in the posterior midline may account for the patterning defects or loss of tissues that do not themselves express *flh*, such as the caudal blood vessels (Talbot *et al.*, 1995).

In *spt* mutants, cells that would normally form trunk somitic mesoderm are misspecified and accumulate in the tail (Kimmel *et al.*, 1989; Ho and Kane, 1990). At gastrulation, *spt* mutants also fail to express *myoD*, a marker of paraxial mesoderm (Weinberg *et al.*, 1996). We examined whether defects in the earlier expression of *chordin* in paraxial mesodermal domains that flank the gastrula midline contributed to the *spt* mutant phenotype. Expression of *chordin* was normal in the early *spt* embryo, but as gastrulation proceeded, paraxial expression domains remained normal while expression in axial mesoderm was not maintained as in WT. This result reveals an unexpected function in the gastrula midline for *spt*, a gene previously implicated in development of trunk paraxial mesoderm. Maintenance of *chordin* expression in axial mesoderm may be a direct function of *spt* or, alternatively, may require signals provided by paraxial mesoderm which is lacking in *spt* mutants. Analysis of *chordin* expression in mutants such as *flh* and *spt* illustrates the ways in which the pathway for dorsal–ventral patterning at gastrulation could converge with genetic pathways for tissue specification.

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