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Frzb, a Secreted Protein Expressed in the Spemann Organizer, Binds and Inhibits Wnt-8

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Summary

We isolated a Xenopus homolog of Frzb, a newly described protein containing an amino-terminal Frizzled motif. It dorsalized Xenopus embryos and was expressed in the Spemann organizer during early gastrulation. Unlike Frizzled proteins, endogenous Frzb was soluble. Frzb was secretable and could act across cell boundaries. In several functional assays, Frzb antagonized Xwnt-8, a proposed ventralizing factor with an expression pattern complementary to that of Frzb. Furthermore, Frzb blocked induction of MyoD, an action reported recently for a dominant-negative Xwnt-8. Frzb coimmunoprecipitated with Wnt proteins, providing direct biochemical evidence for Frzb-Wnt interactions. These observations implicate Frzb in axial patterning and support the concept that Frzb binds and inactivates Xwnt-8 during gastrulation, preventing inappropriate ventral signaling in developing dorsal tissues.

Introduction

Classical transplantation experiments established that anatomically discrete regions of early vertebrate embryos control patterning of both the developing body axis (Spemann and Mangold, 1924) and limb (Harrison, 1918; Saunders and Gasseling, 1968; Tickle et al., 1975) and suggested that diffusible factors could mediate these effects. Decades later, Urist (1965) reported induction of ectopic skeletal tissue by a protein fraction from demineralized bone and explicitly compared this phenomenon to that of embryonic induction. In the past several years, the number of secreted factors implicated in both limb and axial patterning has increased steadily (Sive, 1993; Dawid, 1994a, 1994b; Kessler and Melton, 1994; Slack, 1994; Hogan, 1996).

Some of these, including noggin, follistatin, chordin, and the nodal-related genes, are expressed in the organizer, the region implicated by Spemann in specification of the dorsal axis (Spemann and Mangold, 1924). In contrast, BMP-4 and Xwnt-8 are expressed in presumptive ventral mesoderm and endoderm early in gastrulation and are thought to act as positive ventral inducers (Christian et al., 1991; Christian and Moon, 1993; De Robertis and Sasai, 1996; Hogan, 1996; Hemmati-Brivanlou and Melton, 1997). Noggin (Zimmerman et al., 1996), chordin (Piccolo et al., 1996), and follistatin (Hemmati-Brivanlou et al., 1994) are thought to produce their dorsalizing effects by binding to BMP-4 or a related TGF-β class signal and inactivating it. No secreted factor with Wnt binding activity has been identified to date.

Another puzzle presented by the Wnt proteins—of great interest in view of their participation in a wide variety of developmental and neoplastic processes (Nusse and Varmus, 1992; Moon et al., 1993; Moon, 1993; Parr and McMahon, 1994)—has been the identity of their receptors. Recently, Frizzled class proteins were proposed as receptors for the Wnt growth factors (Wang et al., 1996). This concept was supported further by the observation that Wingless protein (Wg), the Drosophila prototype of the Wnt family, binds to cells transfected with the Drosophila *frizzled2* gene (*Dfz2*). Moreover, addition of Wg to cells transfected with *Dfz2* causes increased accumulation of Armadillo, a Drosophila homolog of β-catenin; this is an expected consequence of Wg signaling (Bhanot et al., 1996). In Xenopus embryos, overexpression of rat *frizzled-1* (*Rfz-1*) resulted in recruitment of Xwnt-8 and Xenopus Dishevelled, a component of the Wnt signaling pathway, to the plasma membrane (Yang-Snyder et al., 1996), providing additional evidence for interactions between Frizzled and Wnt proteins.

Recently, we identified a novel protein, Frzb, in a highly purified fraction from bovine articular cartilage (Hoang et al., 1996). Though this fraction contained potent chondrogenic/osteogenic activity, extensive peptide sequence analysis failed to reveal known classes of proteins associated with specification of skeletal tissues (M. M., Jr., and F. P. L., unpublished data). However, the amino terminal region of Frzb is quite similar to the putative receptor binding domain of the *frizzled* product (Vinson and Adler, 1987), which has been implicated in polarity determination in Drosophila. This finding, together with its dynamic expression pattern in developing limbs, suggested that Frzb may contribute to pattern formation in vertebrate limb development. The parallels between axial and limb patterning noted earlier prompted us to exploit the experimental advantages afforded by assays of axial patterning in Xenopus embryos to explore the biological activities of Frzb. Since the amino-terminal region of Frzb is highly similar in primary structure to the putative ligand binding domains of the Frizzled proteins, we also investigated the possibility of both protein–protein and functional interactions between Frzb and members of the Wnt family.

In the present work, we demonstrate that Frzb shows dorsalizing activity in Xenopus embryos. It is expressed in the Spemann organizer, a region critical to patterning of the amphibian embryo. This pattern is complementary to that of Xwnt-8, a secreted protein thought to ventralize mesoderm during gastrulation (Christian and Moon, 1993). Naturally occurring Frzb protein can be identified biochemically during gastrulation. It is soluble and secretable and can act across cell boundaries. Of

particular interest, Frzb binds to Xwnt-8 directly and blocks its activity in several functional assays in vivo. Furthermore, Frzb blocks induction of MyoD, an action reported recently for a dominant-negative Xwnt-8 (Hoppler et al., 1996). These lines of evidence lead us to propose that Frzb can act as a functional inhibitor of Wnt signaling through direct extracellular binding. It could thus play an important role in dorsoventral patterning of the mesoderm, as well as other processes modulated by Wnt signaling.

Results

Frzb Can Dorsalize Embryos but Not Animal Cap Explants

As an initial test of the possibility that Frzb may play a role in patterning of the vertebrate embryo, we evaluated the effects of overexpression and ectopic expression in developing embryos. Injection of bovine *frzb* (*Bfrzb*) mRNA into single ventral blastomeres at the four cell stage generated duplicated partial posterior dorsal axes reproducibly (Figure 1C). Muscle and neural tissues were apparent in frontal sections taken from these embryos (Figure 1D), but notochord was absent. The frequency of axis duplication was approximately 15% (24/159; four independent experiments) with bovine Frzb; it was somewhat less with the Xenopus gene. This difference may be due to the presence of a consensus translation initiation site (Kozak, 1991) in the bovine, but not the amphibian sequence. The phenotypes were identical in either case. When *frzb* mRNA was injected into UV-irradiated embryos, dorsal axes were partially rescued in approximately 56% (37/66; three independent experiments; Figure 1G). The rescued axes contained muscle and neural tube, but no notochord (Figure 1H). Nevertheless, overexpression of *frzb* in animal cap explants did not induce markers for mesoderm (*Brachyury* [*Xbra*]), neural tube (*NCAM*), or somites (*muscle actin*) (not shown).

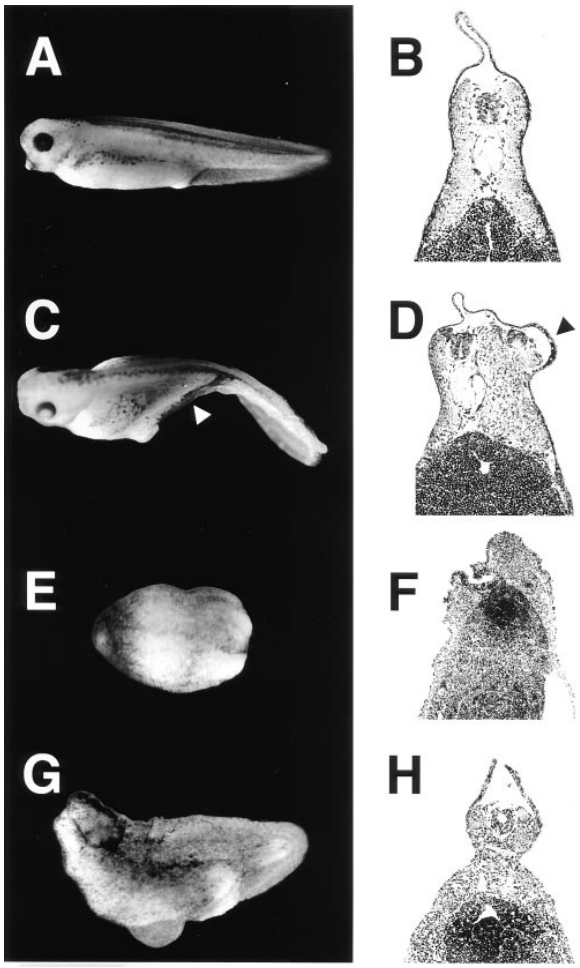

Figure 1. Partial Axis Duplication and Rescue by *frzb*

Control embryos are shown in (A) and (B). Of the embryos injected with 1 ng of *Bfrzb* mRNA into single ventral blastomeres at the 4–8 cell stage, 15% developed partial secondary axes (C and D), as indicated by arrows. Nearly all of the UV-irradiated embryos (E and F) were ventralized completely. 56% of the UV-irradiated embryos injected with 1 ng of *frzb* mRNA showed partial rescue of a dorsal axis as shown (G and H); the other embryos remained completely ventralized. (B), (D), (F), and (H) are frontal sections. The partial secondary axis in (C) and (D) and the rescued axis in (G) and (H) contain muscle and neural tissue, but no notochord. Nevertheless, *frzb* overexpression in animal cap explants did not induce mesoderm or dorsal markers (see text for details).

Xenopus *frzb* Is a Zygotic Transcript Expressed in the Dorsal Blastopore Lip

The results of our initial functional assays prompted us to isolate a Xenopus homolog of *frzb* (*Xfrzb*). We isolated several clones with similar sequences from a Xenopus neurula cDNA library by conventional low-stringency hybridization. The clone selected for analysis was designated *Xfrzb-1b*. A closely related gene with similar characteristics, *Xfrzb-1a*, which probably corresponds to an alternative allele, has also been identified (Leyns et al., 1997 [this issue of *Cell*]). For simplicity, we refer to *Xfrzb-1b* as *Xfrzb* in the remainder of this report. The amino-terminal domain of Xfrzb was 92% identical in amino acid sequence to the mammalian orthologs; the overall identity was 72%. The regions of lowest similarity correspond to the putative signal sequences and the exon–intron boundaries of mammalian *frzb* genes (J. Terrig Thomas and F. P. L., unpublished data), which characteristically are not conserved across species. Xfrzb shares several features common to the mammalian genes described earlier (Hoang et al., 1996), including a consensus site for asparagine-linked glycosylation, a conserved cysteine-rich domain characteristic of Frizzled proteins, and a carboxyl-terminal motif (amino acids 244–293) that appears to be homologous to the netrin-specific carboxyl-terminal domain of C. elegans unc-6 (Wadsworth et al., 1996).

Xfrzb expression first became apparent in the late blastula (stage 9) by hybridization in situ (Figure 2A, 9V). In early gastrulas (stage 10), mRNA expression was most apparent in the Spemann organizer. In later gastrulas (stage 10.5–11), there was expression in the blastopore lip that extended beyond the organizer as the blastopore lip progressed ventrally. At about stage 11, *Xfrzb* expression appeared in the dorsal midline. Examination of cleared embryos and corresponding histological sections revealed that this expression was in the involuted

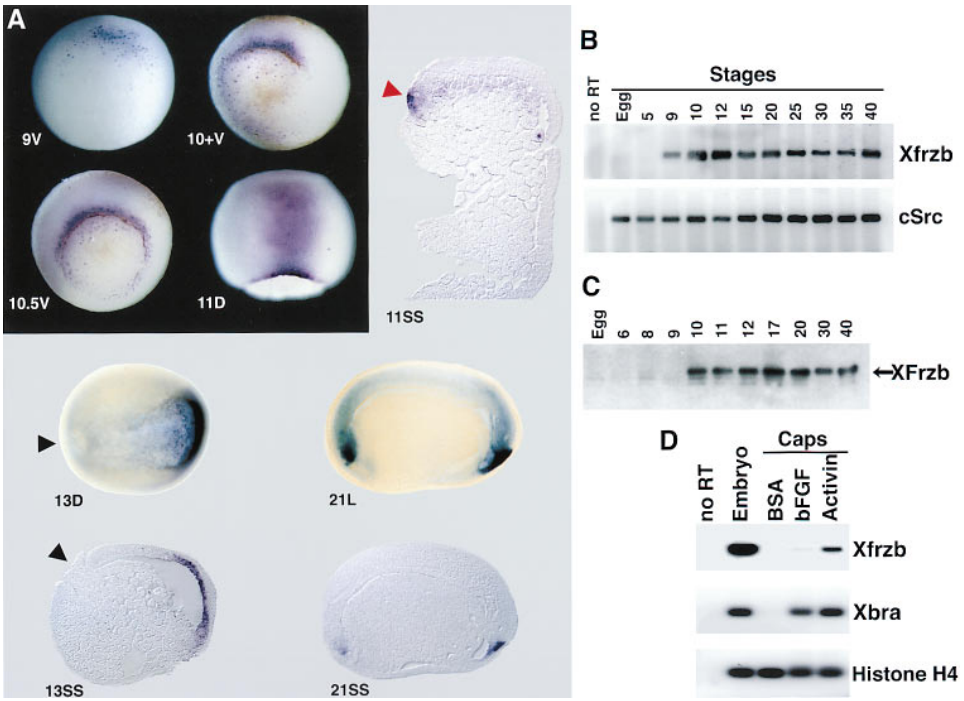

Figure 2. Expression of Frzb during Xenopus Development

(A) Localization of *Xfrzb* mRNA during early development by whole-mount hybridization in situ. Numbers indicate embryonic stages. (V), vegetal view; (D), dorsal view; (SS), sagittal section; (L), lateral view. All embryos except the dorsal views (11D and 13D) are shown with dorsal on top; the stage 13 and 21 embryos are presented with anterior to the right. High levels of *Xfrzb* expression are identified by dark purple-blue staining. In the late blastula (stage 9) and early gastrula (stage 10), expression is most prominent in the region of the Spemann organizer. The red arrowhead (stage 11SS) indicates the dorsal lip of the blastopore; this section demonstrates hybridization in the involuted dorsal mesoderm. Black arrowheads identify the yolk plug; the cleared early neurula (stage 13D) and corresponding section (13SS) both show prominent staining in anterior mesoderm. By the neural tube stage (21L, 21SS), *Frzb* is expressed primarily in anterior and posterior mesoderm.
(B) Expression levels of *Xfrzb* mRNA. RT–PCR with total RNA isolated from the indicated stages was performed for *Xfrzb*; *cSrc* was used to confirm similar amounts of input cDNA between samples.
(C) Immunoblot analysis of endogenous Frzb protein. Each lane represents 0.5 embryo. Specific staining is indicated by the arrow. These analyses were performed twice with similar results, and they confirm the presence of endogenous Frzb protein in developing embryos.
(D) Induction of *Xfrzb* by activin but not bFGF. Animal caps were explanted at stage 8 and cultured until sibling embryos reached stage 11. RT–PCR analysis of explants incubated with BSA, bFGF (20 ng/mL), or activin (5 U/mL) indicated that *Xfrzb* behaves as a dorsal marker. cDNA from whole embryos (Embryo) was used as a positive control template. *Brachyury* (*Xbra*), a general mesodermal marker known to be induced by both growth factors, is used as a positive control for mesoderm induction. *Histone H4* was used to confirm similar amounts of input cDNA between samples. For both RT–PCR assays, reactions were done with template from which reverse transcriptase was omitted (no RT) to control contamination.

mesoderm (Figure 2A, 11SS). Near the onset of neurulation, posterior expression was markedly reduced, and expression in the prechordal plate became apparent (Figure 2A; 13D, 13SS). The field of expression was then restricted progressively, stabilizing in the putative pituitary, and posteriorly in the vicinity of the proctodeum (Figure 2A, 21L; 21SS). These results are consistent with RT–PCR analysis (Figure 2B).

Endogenous Xenopus Frzb protein could be detected in early gastrulas (stage 10, Figure 2C) and in all subsequent stages analyzed by immunoblot analysis. *Xfrzb* expression was unaffected by bFGF, enhanced by activin (Figure 2D) or lithium (not shown), and suppressed by UV irradiation (not shown), as has been described for other genes expressed in the organizer (Kao and Elinson, 1988; Slack, 1994).

Frzb Blocks Wnt-8 Signaling In Vivo

The complementary relationship between the expression patterns of *Xwnt-8* and *Xfrzb* (Christian et al., 1991; Smith and Harland, 1991; Christian and Moon, 1993; Figure 7) resembled those observed for *BMP-4* and its functional antagonists, *chordin* (Piccolo et al., 1996) and *noggin* (Zimmerman et al., 1996). We therefore evaluated the possibility of an analogous functional interaction between Frzb and Xwnt-8. When *Xwnt-8* mRNA is injected during early embryogenesis, secondary dorsal axes with complete head structures are induced reliably (Smith and Harland, 1991; Sokol et al., 1991). This phenomenon can thus be used as an in vivo assay for Xwnt-8 activity. Accordingly, we tested whether coinjection of *Xfrzb* mRNA could influence formation of secondary axes (Figures 3A–3C). When *prolactin* mRNA was coinjected with *Xwnt-8* message, 71% of the embryos (in the experiment shown, 27/38) developed secondary axes. In contrast, when the *prolactin* mRNA was replaced by an identical amount of *frzb* message, axis duplications were suppressed (0/32 for *Xfrzb*; 1/36 for *Bfrzb*). Uninjected embryos did not display axial abnormalities (0/59).

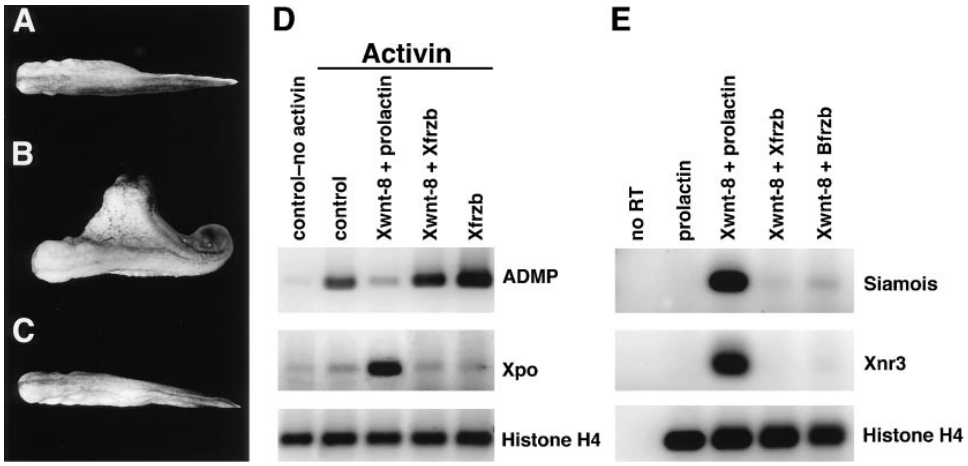

Figure 3. Frzb Blocks Wnt-8 Signaling In Vivo

(A–C) *Xfrzb* blocks induction of secondary axes by *Xwnt-8*. Embryos injected with 25 pg *preprolactin* mRNA (A), 5 pg *Xwnt-8*^{myc} mRNA, and 25 pg *preprolactin* mRNA (B), or 5 pg *Xwnt-8*^{myc} mRNA and 25 pg *Xfrzb* mRNA (C). Dorsal views are shown, with anterior to the left. All injections were into a single ventral vegetal blastomere at the eight cell stage. This experiment was done twice with *Xfrzb* and twice with *Bfrzb* with identical results. In the experiment shown, the group corresponding to panel (A) showed 0/56 secondary axes; to panel (B), 27/38 secondary axes; and to panel (C), 0/32 secondary axes.

(D) Frzb blocks ventralization of animal cap explants by CSKA-*Xwnt-8* plasmid. Embryos were injected with the indicated combinations of *prolactin* or *Xfrzb* mRNA (1 ng) and CSKA-*Xwnt-8* expression plasmid (100 pg) into both blastomeres at the two cell stage. Caps were explanted at stage 8 and incubated in the absence and presence of activin (5 U/mL) until stage 11. The dorsal marker *ADMP* but not the ventral marker *Xpo* was induced in animal caps cultured in the presence of activin. Post-MBT overexpression of Xwnt-8 in cap explants reversed this pattern; when *Xfrzb* was coexpressed with Xwnt-8, these effects were blocked.

(E) Frzb prevents induction of Xwnt-8 response genes. Animal cap assays were as in (D), but no activin was used. Coinjection of either Xenopus or bovine *frzb* mRNA (100 pg) blocked induction of *Siamois* and *Xnr3* by *Xwnt-8* (10 pg mRNA).

Xwnt-8 is felt to exert its primary effects on pattern formation during gastrulation (Christian and Moon, 1993). It was therefore appropriate to determine whether Frzb could antagonize the action of Xwnt-8 expressed following the midblastula transition (MBT). An *Xwnt-8* expression plasmid under the control of the cytoskeletal actin (CSKA) promotor has been shown to induce the ventrolateral marker *Xpo* (Sato and Sargent, 1991) and suppress induction of the dorsal marker *goosecoid* in activin-treated animal cap explants (Hoppler et al., 1996); this effect was blocked completely in caps overexpressing a dominant-negative Xwnt-8. Our results confirmed that *Xpo* expression could be increased by *Xwnt-8* plasmid in activin-treated animal cap explants and that *Xfrzb* could block this effect (Figure 3D). *ADMP* is a Spemann organizer–specific marker that is induced by activin in animal cap explants (Moos et al., 1995). Induction of *ADMP* by activin was suppressed in explants injected with *Xwnt-8* plasmid; this suppression was rescued by Xfrzb (Figure 3D). *frzb* overexpression did not affect the expression level of *Xwnt-8* (not shown). These observations are consisent with the interpretation that Frzb exerts its dorsalizing effects by inhibiting the action of Xwnt-8. In a related experiment, we injected the CSKA-*Xwnt-8* plasmid into dorsal blastomeres with or without *Xfrzb* mRNA. In this assay, CSKA-*Xwnt-8* plasmid produced head defects (64/80 embryos, three independent experiments), as described previously (Christian and Moon, 1993). If *Xfrzb* mRNA was coinjected with the CSKA-*Xwnt-8* plasmid, these defects were not observed (0/81 embryos).

Induction of *Siamois* (Lemaire et al., 1995) and *Xnr3* (Smith et al., 1995) in animal cap explants injected with *Xwnt-8* mRNA has been used to assay Xwnt-8 signaling (Carnac et al., 1996; Yang-Snyder et al., 1996). We therefore tested the ability of Frzb to block Xwnt-8-mediated induction of these response genes. Either *Xfrzb* or *Bfrzb* blocked the induction of both *Siamois* and *Xnr3* by Xwnt-8 (Figure 3E).

Frzb Is a Soluble, Secreted Protein That Can Act across Cell Boundaries

Since initial experiments with mammalian Frzb expressed in cell culture suggested that it may be membrane-associated (Hoang et al., 1996), we evaluated the subcellular distribution of the protein expressed in vivo. Endogenous Xfrzb protein was found in 105,000 $\times$ g supernatants isolated from Xenopus embryos (Figure 4A, lane 2) but could not be detected in the pellets (Figure 4A, lane 3). Further, Frzb protein was secreted by oocytes injected with *frzb* mRNA (Figure 4A, lane 5). The apparent molecular weight of 33 kDa is consistent with removal of the signal sequence at the cleavage site predicted (Nielsen et al., 1997) between amino acids 28 and 29; proteolytic processing likely accounts for the difference in molecular weight between secreted Frzb (Figure 4A, lane 5) and Frzb contained in oocyte lysates (Figure 4A, lane 6). Further experiments identified mammalian cell lines and culture conditions in which Frzb was secreted (not shown).

To demonstrate that Frzb can act across cell boundaries, we adapted an experimental design used to study the dominant-negative Xwnt-8 (Hoppler et al., 1996). As shown in Figure 4B, *Xfrzb* reduced the percentage of

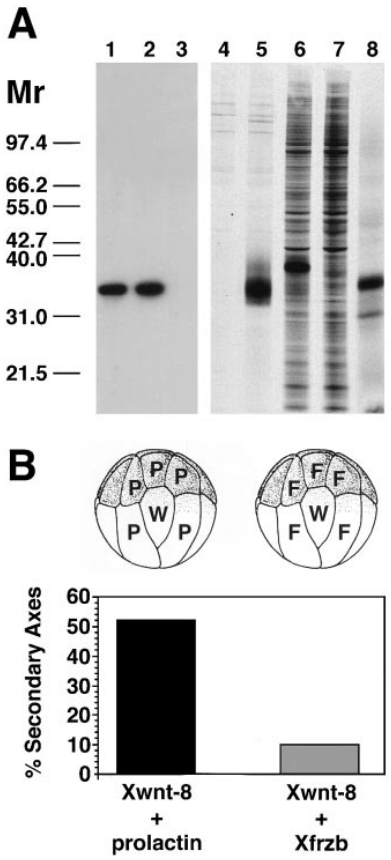

Figure 4. Frzb Is a Soluble, Secreted Protein That Can Act across Cell Boundaries

(A) Immunoblot analysis of normal stage 30 embryos (lanes 1–3) and metabolic labeling pattern of oocytes injected with *Xfrzb* mRNA (lanes 4–8). Lane 1, 20,000 × g supernatant; lane 2, 105,000 × g supernatant; lane 3, 105,000 × g pellet. Frzb was recovered in the soluble fraction. Lane 4, culture supernatant from uninjected oocytes; lane 5, supernatant from oocytes injected with *Xfrzb* mRNA; lane 6, lysate from oocytes injected with *Xfrzb* mRNA; lane 7, lysate from uninjected oocytes; lane 8, bovine Frzb expressed in E. coli. The band in lane 5 corresponds to secreted protein that has undergone proteolytic processing. The somewhat larger band in lane 6 corresponds to unprocessed protein.

(B) Frzb can block Xwnt-8 signaling across cell boundaries. Ventral blastomeres were injected with either *prolactin* (P) or *Xfrzb* (F) mRNA (50–100 pg per blastomere), as shown at the early 16 cell stage. At the late 16 cell stage, single blastomeres surrounded by those injected previously were injected with *Xwnt-8* (W) mRNA (10 pg). They were then scored for secondary axes. This experiment was performed three times with similar results; the data were pooled for the graph presented.

secondary axes induced by *Xwnt-8* from 52% (46/88) to 10% (5/49) when the two mRNAs were injected into different cells.

Frzb and Wnt Proteins Interact Directly

Direct interaction between Frzb and Wnt proteins was demonstrated in two systems: rabbit reticulocyte lysate containing canine microsomal membranes and transfected COS7 cells. *Xwnt-8*^{myc}, *Bfrzb*, *Xfrzb*, and the β-*lactamase* control mRNA were all translated and processed in vitro (Figure 5A), either alone or in the combinations indicated. As expected, the anti-myc antibody precipitated Xwnt-8^{myc} but not β-lactamase, Xfrzb, or Bfrzb (Bfrzb is not shown). Conversely, the 374-PEP antiserum, which recognized both mammalian and amphibian Frzb in immunoblots, precipitated both Xfrzb and Bfrzb (the latter is not shown), but neither Xwnt-8^{myc} nor β-lactamase. However, when Xwnt-8^{myc} and Frzb were cotranslated, both proteins were precipitated by either the myc-specific 9E10 monoclonal antibody or the 374-PEP antiserum (Figure 5B; identical results obtained with Bfrzb are not shown). Neither reagent precipitated β-lactamase cotranslated with Frzb or Xwnt-8^{myc}.

These results were further supported by experiments in which COS7 cells were cotransfected with expression plasmids encoding Bfrzb and an HA-tagged murine Wnt-1, which belongs to the same functional class as Xwnt-8 (Figure 5C) (Nusse and Varmus, 1992). Cell lysates were immunoprecipitated with an anti-HA antibody, immunoblotted, and probed with the Frzb-specific 374-PEP serum. Frzb protein was detected only in lysates from cells transfected with both *frzb* and *Wnt-1* cDNAs.

Frzb Blocks MyoD Expression

Xwnt-8 was recently implicated in somite development (Hoppler et al., 1996) through the use of a carboxyl-terminal deletion construct that acted in a dominant negative fashion. Since our data suggested that Frzb could also act as a Wnt inhibitor, we evaluated its effects on somite formation and *MyoD* expression, both of which are suppressed by the dominant-negative Xwnt-8. When *Xfrzb* mRNA was injected radially into all blastomeres at the four cell stage, trunk development was grossly abnormal (Figure 6B), resembling that seen in embryos overexpressing the dominant-negative Xwnt-8. Furthermore, *Xfrzb* blocked *MyoD* expression both in gastrulating embryos (Figure 6D) and in activin-treated animal cap explants (Figure 6E).

Discussion

Frzb May Act in Both Axial and Limb Patterning

Mammalian Frzb was first identified in a highly purified protein fraction isolated from bovine articular cartilage. This fraction contained potent osteoinductive activity (Hoang et al., 1996). Its similarity to Drosophila *frizzled*, a gene implicated in polarity determination, and its graded expression pattern in developing mammalian limbs suggested a potential role for *frzb* in embryonic pattern formation. We therefore evaluated the activity of bovine *frzb* in several assays of axial patterning. The results presented in Figure 1 indicated that mammalian *frzb* could indeed influence axial patterning in Xenopus embryos. When overexpressed ventrally, *Bfrzb* was able to induce secondary axes containing dorsal structures in normal embryos and rescue partial dorsal axes in UV-irradiated embryos, which are incapable of axis formation. These findings prompted us to identify and characterize an orthologous gene in Xenopus and examine its expression pattern and biological activities in greater detail.

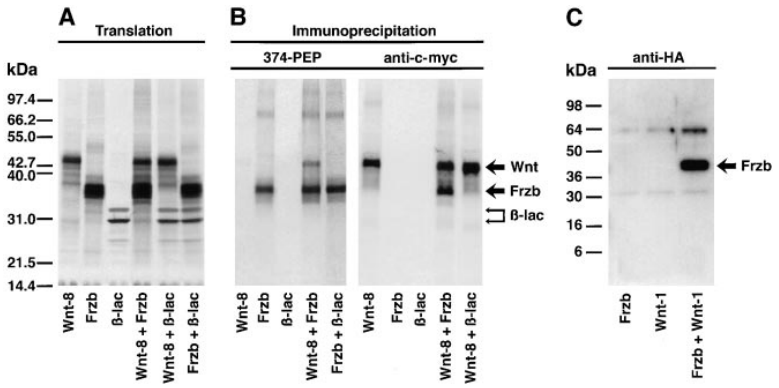

Figure 5. Frzb Binds to Wnt Proteins Directly

(A) In vitro translation of *Xwnt-8*^{myc}, *Xfrzb*, β-*lactamase*, or the indicated combinations of these mRNAs. Each of these proteins contains a signal sequence; the observed patterns are consistent with a mixture of processed and unprocessed translation products.

(B) Immunoprecipitation of in vitro translation products. The antiserum 374-PEP was used to immunoprecipitate Frzb; a commercial monoclonal was used to precipitate myc-tagged Xwnt-8. β-lactamase was used as a control for nonspecific interactions. When Frzb and Xwnt-8 were cotranslated, either antibody precipitated both proteins. These analyses were done at least twice with both Xenopus and bovine Frzb.

(C) Coimmunoprecipitation of Frzb and Wnt-1 from COS7 cells. Cells were transfected with expression plasmids encoding Frzb, an HA-tagged Wnt-1, or both together. They were then lysed, immunoprecipitated with an anti-HA antibody, and immunoblotted with the 374-PEP antiserum to detect Frzb. The specific band is indicated by the arrow. Frzb was detected in the immunoprecipitate only if cotransfected with Wnt-1. This experiment was performed at least four times with identical results.

The Expression Pattern of *Xfrzb* Is Consistent with a Role in Axial Patterning

Xfrzb is expressed in the Spemann organizer, a region of the developing amphibian embryo associated with a variety of signals that act in concert to specify dorsoventral patterning. By stage 10, endogenous Xfrzb protein could be detected by immunoblot analysis. Later in gastrulation, *Xfrzb* is expressed in the involuted mesoderm, which is thought to convey to the overlying neuroectoderm signals that participate in specification of the nervous system. Expression then becomes progressively restricted to prechordal mesoderm and finally to the putative pituitary. A region of expression remains near the proctodeum, which may be associated with residual organizer activity (Gont et al., 1993). Thus, *Xfrzb* is expressed at the appropriate time and place to participate in specification of the body axis.

Frzb Can Be Secreted

The finding that Frzb was secreted both by mRNA-injected oocytes (Figure 4A) and transfected COS7 cells suggested that it could act extracellularly. In earlier experiments, solubilization of mammalian Frzb required guanidine or sodium dodecyl sulfate (Hoang et al., 1996). Immunoblot analyses suggested that the protein examined in these experiments contained signal peptide. In contrast, immunoblot analysis of supernatants from normal embryos indicated a molecular weight consistent with removal of the signal peptide. Release of soluble protein may thus be dependent on proteolytic processing, which may vary with the cell or tissue type. When *Xfrzb* and *Xwnt-8* were overexpressed in different cells (Figure 4B), Xwnt-8 signaling was inhibited. This finding indicates that effects of Frzb on Xwnt-8 occur following secretion.

The Effects of Frzb Overexpression Depend on Cellular Context

Overexpression of Frzb can induce partial dorsal axes containing muscle (Figure 1C) or suppress *MyoD* expression (Figure 6), effects which may appear incompatible. These observations can be reconciled by consideration of the cellular context in which overexpression of Frzb occurs. Ectopic gene expression may generate a secondary axis directly by an inductive effect or indirectly by inhibition of a ventralizing signal. Frzb blocks the actions of Xwnt-8 but does not induce mesoderm, muscle, or neural tissue when overexpressed in animal cap explants, which do not express Xwnt-8. The dorsalizing actions of Frzb are thus likely to be indirect, resulting from inhibition of the ventralizing effects of Xwnt-8.

Local overexpression of a molecule acting in such an indirect manner will produce effects different from generalized overexpression. Injection of *frzb* mRNA into a single blastomere within the expression domain of *Xwnt-8* would be expected to block its ventralizing activity locally. Generation of a partial dorsal axis by *frzb* (Figure 1C) is consistent with this prediction. On the other hand, generalized overexpression should block all actions of Xwnt-8 throughout the embryo, including both its ventralizing activity and its effects on somite formation.

Recently, a dominant-negative Xwnt-8 was reported to suppress development of the trunk and somites (Hoppler et al., 1996). When Frzb was overexpressed using an identical protocol (all blastomeres at the four cell stage), the same phenotype was produced (Figure 6B). Thus, the induction of muscle tissue by local overexpression of Frzb in one type of experiment and suppression of somite development by generalized overexpression in another are compatible findings consistent with the interpretation that Frzb acts through inhibition of Xwnt-8 signaling.

Frzb Is a Functional Antagonist of Xwnt-8 In Vivo

The similarity in primary structure between Frzb and the putative ligand binding domain of the Frizzled proteins and the recent identification of these proteins as potential receptors for the Wnt family of growth factors (Bhanot et al., 1996; Wang et al., 1996; Yang-Snyder et al., 1996) provided strong impetus to examine the possibility of functional interactions between Frzb and Wnts. When overexpressed ventrally prior to the midblastula transition (MBT), Xwnt-8 is a powerful inducer of secondary

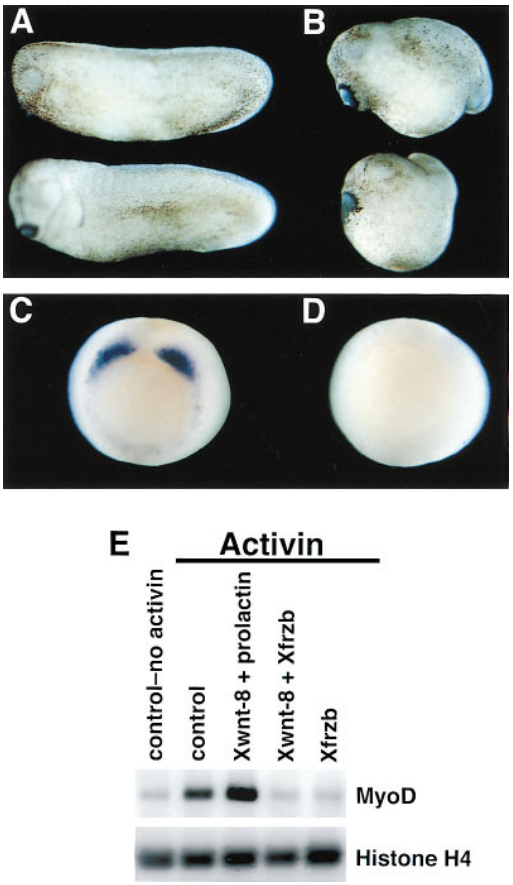

Figure 6. Frzb Blocks *MyoD* Expression

Embryos were injected at the four cell stage with 500 pg *prolactin* (A and C) or *Xfrzb* (B and D) mRNA into the marginal zone of each blastomere (instead of a single ventral blastomere as in Figure 1). Though radial injection of *Xfrzb* mRNA produced severe shortening of the trunk, anterior structures (cement gland, eyes) were present. *MyoD* expression in control (prolactin-injected) stage 11 embryos is shown in (C). Ubiquitous overexpression of *Xfrzb* completely blocked expression of *MyoD* (D). (E) shows RT–PCR analysis of animal cap explants as in Figure 3D. *Xfrzb* blocked induction of *MyoD* by activin.

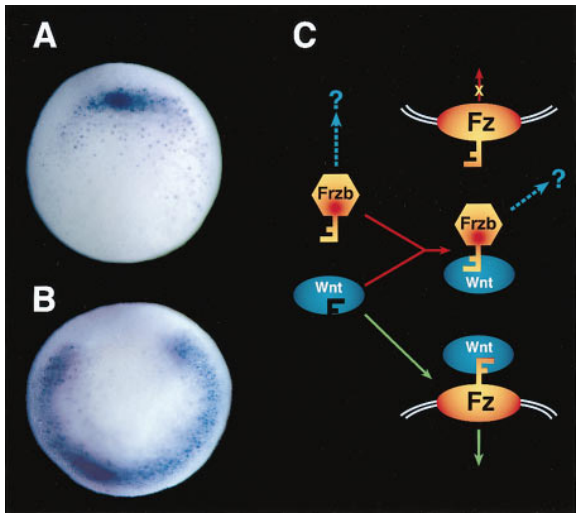

Figure 7. Proposed Interaction of Frzb and Wnt-8 in Dorsoventral Mesoderm Patterning

During gastrulation, Xfrzb (A) is concentrated in the Spemann organizer, which is associated with specification of dorsal structures. Xwnt-8 (B) is excluded from this region, but is expressed in lateral and ventral mesoderm. In (C), the stylized (F) denotes the Frizzled-like amino-terminal sequence conserved between Frzb and Frizzled proteins (indicated by [Fz]), which are proposed as receptors for Wnt proteins. In the dorsal marginal zone, Xfrzb could compete with a Frizzled protein for Wnt binding and prevent signaling. In the ventral regions of the embryo, where Frzb is not present, Wnt-8 binding to the cognate receptor will not be affected. Question marks denote possible alternative actions of Frzb or Frzb–Wnt complexes.

body axes (Smith and Harland, 1991; Sokol et al., 1991). We interpreted the ability of Frzb to block this effect (Figures 3A–3C) as preliminary evidence that it may act as a functional Wnt antagonist. However, induction of secondary axes by overexpression of *Xwnt-8* prior to the midblastula transition may not reflect its function during gastrulation (Christian and Moon, 1993). We therefore tested the ability of Frzb to counteract effects of Xwnt-8 expressed under control of the cytoskeletal actin promotor, which is activated after the MBT. Frzb blocked post-MBT ventralization of animal cap explants by Xwnt-8 (Figure 3D) and rescued suppression of anterior structures in whole embryos injected dorsally with the CSKA-*Xwnt-8*. To provide additional evidence that the effects of Frzb can be attributed to inhibition of Wnt signaling directly, we demonstrated that Frzb could inhibit induction of *Siamois* and *Xnr3* by Xwnt-8. Recently, a dominant-negative Xwnt-8 has been shown to suppress somite formation and block expression of *MyoD* (Hoppler et al., 1996). Duplication of these actions by Frzb further confirms its ability to inhibit Wnt signaling (Figure 6). These lines of evidence provide strong support for the conclusion that Frzb can inhibit signaling by Xwnt-8.

The high degree of sequence similarity between the amino-terminal region of Frzb and the putative ligand binding domains of the Frizzled proteins would predict direct protein–protein interactions between Frzb and various Wnt proteins. The immunoprecipitation data presented in Figure 5 demonstrate that Frzb can indeed bind Wnt proteins directly. These results are complemented by experiments demonstrating that Frzb binds to cells transfected with a membrane-tethered Wnt-1 (Leyns et al., 1997). Since coimmunoprecipitation experiments with Frizzled and Wnt proteins have not been described, our results also further corroborate the identification of these proteins as Wnt receptors.

Our findings support the concept that Frzb counteracts Xwnt-8 signaling by direct extracellular binding. Its expression pattern suggests that under normal circumstances, Xfrzb may act to prevent Xwnt-8 from ventralizing mesoderm inappropriately in the Spemann organizer. This model is presented schematically in Figure 7. Precedent for a similar concept has been established by the observation that BMP-4, a key ventralizing factor in vertebrate development, can bind and be inactivated by two secreted factors, noggin (Zimmerman et al., 1996) and chordin (Piccolo et al., 1996). These proteins, like Frzb, are both expressed in the Spemann organizer, a

region of the developing Xenopus embryo destined to form dorsal tissues, and are thought to suppress the ventralizing activity of BMP-4. If chordin or noggin are overexpressed ventrally, within the normal expression domain of BMP-4, secondary dorsal axes are produced (Smith and Harland, 1992 Sasai et al., 1994). A similar effect is also observed with truncated receptors that block BMP signaling (Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994) or with dominant-negative BMP ligands (Hawley et al., 1995; S. W. and M. M., unpublished data).

Since the Wnt proteins have been implicated in a wide variety of developmental processes, including limb patterning (Dealy et al., 1993; Parr et al., 1993; Parr and McMahon, 1995; Tickle, 1995) and certain neoplastic states, Frzb could modulate some of these processes as well. The amino-terminal region of Frzb that exhibits homology to the Frizzled proteins accounts for less than half of the molecule. The carboxyl-terminal region appears to be related to the netrins, which are chemotactic signals that influence axon guidance during development (Wadsworth and Hedgecock, 1996). Alternative actions for Frzb may thus be possible.

Conclusion

Frzb is a secretable protein expressed in the Spemann organizer, a region crucial to control of fundamental patterning events. It counteracts several actions of Xwnt-8. Frzb coimmunoprecipitates with Wnt proteins; this finding provides the first direct biochemical evidence for interaction between Wnts and other proteins. We propose that Frzb is a naturally occurring inhibitor of Wnt signaling that contributes to dorsoventral patterning of the mesoderm during vertebrate development.

Experimental Procedures

Isolation of *Xfrzb* cDNA

The primers 5′-TGGAACATGACTAAGATGCCC-3′ and 5′-CATATAC TGGCAGCTCCTCG-3′ were used for PCR labeling of a region of the bovine *frzb* cDNA sequence (Hoang et al., 1996) found to show a high degree of sequence identity to related genes from human and avian sources. Screening at low stringency (35°C in 20 mM Na_2HPO_4 [pH 7.2], 1 mM EDTA, 1% SDS) by standard procedures (Sambrook et al., 1989) allowed isolation of several clones from a stage 13 cDNA library (Richter et al., 1988); purified plaques were characterized by direct sequencing (Wang et al., 1995). Two closely similar clones were isolated, and one of these was chosen for further study.

Plasmids and Probes

The *Xfrzb* open reading frame was subcloned into pCR-Script (Stratagene) to generate probes for hybridization in situ. Both *Bfrzb* and *Xfrzb* were subcloned into pSP64R1 (Sergei Sokol, Harvard University) for mRNA injection experiments. The pSP64T-*Xwnt-8*^{myc} plasmid used for mRNA injections and translation in vitro and the CSKA-X8 expression plasmid (Christian and Moon, 1993) were provided by Dr. R. T. Moon (University of Washington, Seattle). A pGEM-5R-*Xwnt-8* plasmid (Smith and Harland, 1991) was used to generate probe for hybridization in situ. In vitro transcription was done using mMessage mMachine or MEGAscript kits from Ambion (Austin, TX). The plasmid p*LNCWnt1HA*, containing the open reading frame of mouse *Wnt1* and an HA tag near the C terminus, was kindly offered by Dr. Jan Kitajewski (Columbia University). The Xlmf25 plasmid used for hybridization in situ analysis of *MyoDa* (Scales et al., 1990) was the kind gift of Dr. Jon Scales (NICHD). The p*frzb* expression plasmid was described previously (Hoang et al., 1996). The prolactin plasmid (Amaya et al., 1991) was provided by Enrique Amaya.

Oocyte Injections

Enzymatically defolliculated oocytes were injected with 50 ng of mRNA and cultured with oocyte Ringer's solution (Kay, 1991). ^{35}S-methionine (500 μCi/mL) was added to the incubation medium for metabolic labeling studies.

Embryo Manipulations

Frogs and their embryos were maintained and manipulated using standard methods (Gurdon, 1967; Gurdon, 1977). All embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967) and Keller (Keller, 1991). mRNA injection experiments were performed by standard procedures, as described previously (Moos et al., 1995). Dorsal and ventral blastomeres were identified by size and pigment variations (Nieuwkoop and Faber, 1967). Lithium treatment was for 1 hr at 0.1 M (Kao and Elinson, 1988), and UV irradiation was done with a Stratalinker™ (Smith and Harland, 1991). Animal cap explants were cultured in 0.7–1× Marc's Modified Ringer's solution (Kay, 1991). Activin was a gift from Michael Kuehn (National Cancer Institute), and bFGF was from Life Technologies.

Expression of Frzb and Wnt Proteins in COS7 Cells

COS7 cells (1.6×10^6 initial seeding density) were transfected with 5 μg of p*frzb* or p*LNCWnt1HA* or cotransfected with 4 μg of p*frzb* and pLNCWnt1HA in 100 mm dishes using 30 μl LipofectAMINE™ (Life Technologies, Inc., Gaithersburg, MD). Transfections were carried out for 6 hr in serum-free Opti-MEM I® (Life Technologies). Thereafter, cells were incubated for 18 hr in media containing 10% fetal bovine serum. Subsequently, cells were cultured at 37°C for 24 hr in serum-free Opti-MEM I™. The cells were extracted for 30 min on ice with 50 mM Tris, 150 mM NaCl, 1.0% NP-40, 0.5% Deoxycholic acid, and 0.1% SDS and centrifuged at 12,000 × g for 5 min. The supernatants were saved for immunoprecipitation.

Immunoblotting and Immunoprecipitation

Embryos and oocytes were lysed by sonication on ice in 40 mM Tris base, 10 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride in a volume of 10 μl/embryo or oocyte. In some experiments, 20,000 × g supernatants were extracted with an equal volume of 1, 1, 2-trichlorotrifluoroethane (Evans and Kay, 1991). In vitro translations were performed in the presence of ^{35}S-methionine with nuclease-treated rabbit reticulocyte lysate and canine pancreatic microsomal membranes (Promega, Madison, WI) according to the instructions of the manufacturer. β-*lactamase* mRNA supplied with the kit was used as a positive control for translation and processing and as a negative control for nonspecific protein–protein interaction. SDS–PAGE was done with Novex 10% Nu-PAGE gels and the MOPS buffer system. Samples from embryos were precipitated with methanol/chloroform (Wessel and Flugge, 1984) prior to analysis. For metabolic labeling studies, gels were dried onto a single sheet of cellophane and imaged with BioMax MR2 film (Kodak) or a phosphor screen (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation was performed according to standard procedures (Harlow and Lane, 1988). Antiserum N374-PEP was prepared as described previously (Hoang et al., 1996); preliminary experiments confirmed that it was reactive against Xenopus Frzb. The clone 9E10 antibody (Boehringer) was used for precipitation or detection of the c-myc epitope, and hybridoma supernatant containing the 12CA5 monoclonal antibody was used for precipitation of the HA epitope. Immunoblot analyses of separated proteins were performed following transfer to nitrocellulose membranes, using 1:20,000 dilutions of primary antisera and 1:100,000 dilutions of peroxidase-conjugated secondary antibody. Bands were detected with the Super Signal Ultra peroxidase substrate (Pierce, Rockford, IL).

RT–PCR

Separate pools of embryos or explants were prepared from at least two different fertilizations for each condition reported. Total RNA was prepared with Trizol™ and treated with Amplification Grade DNase (Life Technologies). Reverse transcription was done with

Superscript II (Life Technologies) as described by the manufacturer, with 1 μg of total RNA per reaction; 2% of the appropriate cDNA pool was used in each PCR. Amplification was performed in 10 μl reactions containing 50 mM TRIS·Cl (pH 8.3), 2 mM $MgCl_2$, 0.25% bovine albumin, 2.5% Ficoll 400, 5 mM tartrazine, 200 μM dNTPs, 1 μM each primer, and 0.2 U Taq polymerase (Boehringer Mannheim, Indianapolis, IN). Each cycle comprised 94°C, 0 seconds; 55°C, 0 seconds; 72°C, 40 seconds. A 1 min denaturation at 94°C preceded cycling, and a 2 min extension at 72°C was done at the end. An Idaho Technologies air thermal cycler was used in all experiments. Optimal cycle numbers were determined for each primer set by pilot experiments (Rupp and Weintraub, 1991; Niehrs et al., 1994). PCR products were separated on 2.5% agarose gels in TAE buffer, stained with SYBR Green 1™ (Molecular Probes, Eugene, OR), and scanned with a Molecular Dynamics Fluorimager. PCR analysis was performed at least twice for each cDNA to confirm that the amplifications were reproducible. The primers for *Histone H4*, *MyoD*, *c-src*, *ADMP*, and *Xbra* were described previously (Niehrs et al., 1994; Hemmati-Brivanlou et al., 1994; Moos et al., 1995). The primers for *Siamois*, *Xnr3*, and *Xpo* have also been reported (Hoppler et al., 1996; Yang-Snyder et al., 1996). The *Xfrzb* primers for RT–PCR were F (5′-AGTAAGCCTACACATACAGGTTGG-3′) and R (5′-GCAGACTC CTCTGTCATATACGG-3′).

Hybridization In Situ

The procedures outlined by Harland (Harland, 1991) were followed, with modifications as described (Moos et al., 1995).

Histology

All embryos were embedded in JB-4 resin (Polysciences, Warrenton, PA). For conventional histological analysis, 1–3 μm sections were cut and stained with hematoxylin and eosin; 10–20 μM sections were taken from embryos stained by hybridization in situ.

Microscopy and Photography

Dark-field images of embryos were photographed with low angle oblique illumination and a Zeiss Stemi-6 dissecting microscope. Embryos cleared with benzyl alcohol/benzyl benzoate and the histological sections in Figure 1 were photographed under diascopic illumination with a Nikon FXA microscope. The sections in Figure 2A were photographed under multiple oblique illumination (Edge Scientific, Santa Monica, CA).

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