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# Required for Mesoderm Induction, but BMP Activity Is Necessary for Dorsal/Ventral Patterning

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**The activity of bone morphogenetic protein (BMP) heterodimers has been shown to be more potent than that of homodimers in a number of contexts, including mesoderm induction. Although BMP-2/7 and -4/7 heterodimers are potent inducers of ventral mesoderm in ectodermal explants, we show that they are not a necessary component of the primary mesoderm-inducing signal in intact *Xenopus* embryos. The secreted BMP antagonists noggin and gremlin both efficiently block mesoderm induction by BMP homo- and heterodimers in animal caps. When these antagonists are ectopically expressed in the ventral marginal zone of early embryos the initial formation of mesoderm as indicated by panmesodermal markers remains unaffected. Only the subsequent dorsal/ventral patterning of this mesoderm appears to be altered, with expression of a number of organizer-specific transcripts observed in the marginal zone where BMP signaling has been abolished. Thus, we conclude that BMPs do not contribute an essential signal to mesodermal induction or patterning until gastrulation. The activities of noggin and gremlin are strikingly different from that of the multifunctional antagonist cerberus, which completely abolishes mesoderm induction when misexpressed during early development.** © 1999 Academic Press

***Key Words:*** **bone morphogenetic protein-2; bone morphogenetic protein-4; bone morphogenetic protein-7; cerberus; gremlin; heterodimer; mesoderm induction; noggin; *Xenopus laevis*.**

## INTRODUCTION

In *Xenopus laevis,* prospective mesoderm is induced in the equatorial region of the late blastula embryo by signals originating in the vegetal hemisphere. Most candidates for the initial mesoderm inducing signal are members of either the transforming growth factor-$\beta$ (TGF-$\beta$) or fibroblast growth factor (FGF) superfamilies. These include bFGF and bone morphogenetic proteins (BMPs), which induce primarily ventral mesoderm (Kimelman and Kirschner, 1987; Slack *et al.*, 1987), and activin (Thomsen *et al.*, 1990; Green *et al.*, 1992), Vg1 (Thomsen and Melton, 1993), and Xnr-1, -2, and -4 (Jones *et al.*, 1995; Joseph and Melton, 1997), which can all induce dorsal mesoderm. The necessity for an activin-like signal in mesoderm induction has been well established by experiments using dominant negative activin receptors. Embryos expressing a truncated activin type II receptor or a truncated ALK-4 fail to form mesoderm (Hemmati-Brivanlou and Melton, 1992; Chang *et al.*, 1997). However, the dominant negative type II receptor blocks signaling by Vg1 (Schulte-Merker *et al.*, 1994) and BMPs (Hemmati-Brivanlou and Thomsen, 1995) in addition to activin. While the truncated ALK-4 is more specific and suggests that an activin-like signal is required, the nature of that signal remains unclear. Cerberus injection prevents mesoderm formation and blocks the activity of several TGF-$\beta$ family members including the Xnrs (Bouwmeester *et al.*, 1996; Hsu *et al.*, 1998; Piccolo *et al.*, 1999). However, a cleavage mutant Xnr-2, which blocks signaling by Xnr-1, -2, and -4, does not prevent mesoderm formation (Osada and Wright, 1999), suggesting that other TGF-$\beta$ family members may cooperate in the induction of mesoderm.

While BMPs are clearly important in the dorsal–ventral patterning of the mesoderm (reviewed in Harland, 1994; Harland and Gerhart, 1997), their role in mesoderm induction remains less certain. BMP-2, -4, and -7 are present as maternal transcripts in the early *Xenopus* embryo along with their receptors and downstream signaling components (Nishimatsu *et al.*, 1992; Graff *et al.*, 1994, 1996), indicating that all elements of the BMP signal transduction pathway are present at the time of mesoderm induction. Animal cap experiments have shown that BMP-2, -4, and -7 can all

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induce ventral types of mesoderm from naive ectoderm when injected as mRNAs (Dale *et al.*, 1992; Jones *et al.*, 1992; Clement *et al.*, 1995; Suzuki *et al.*, 1997). In addition, analysis of whole embryos injected early with BMP-4 RNA has shown an expansion of mesoderm and mesoderm-specific markers into the animal hemisphere (Dale *et al.*, 1992; Schmidt *et al.*, 1995b).

Studies using transgenic mice have demonstrated that BMP signaling plays a crucial role in mesoderm formation. Mouse embryos lacking the BMP-4 gene all die between 6.5 and 9.5 days postcoitus (p.c.) and exhibit a variable phenotype. Most arrest at the egg cylinder stage and generate little or no primitive streak mesoderm as indicated by *T* (Brachyury) expression. Homozygous mutant embryos that survive to the neural fold stage are developmentally retarded but do generate some mesodermal derivatives such as the notochord (Winnier *et al.*, 1995). Similar defects are observed in BMP-2/4 receptor (ALK-3)-mutant mice. ALK-3 encodes a type I TGF-$\beta$ family receptor that binds both BMP-2 and -4 and is expressed ubiquitously in the early mouse embryo. Homozygous mutant embryos all die by 9.5 days p.c. and fail to form mesoderm as judged by histological criteria and *in situ* hybridization analysis of mesodermal marker genes (Mishina *et al.*, 1995).

Loss of function assays using dominant negative BMP receptors have provided the strongest evidence to date that BMPs are not responsible for initial mesoderm induction in *Xenopus* embryos. Transmembrane serine/threonine kinase receptors that bind to BMP-2 and -4 with high affinity have been cloned from both mouse (Suzuki *et al.*, 1994) and *Xenopus* (Graff *et al.*, 1994). Truncated forms of these receptors lacking the intracellular kinase domain have been shown to act in a dominant negative manner. These truncated receptors specifically block the activities of injected BMP-2 and -4 mRNA and can be neutralized by overexpression of the wild-type receptor (Graff *et al.*, 1994; Suzuki *et al.*, 1994). When truncated BMP receptor mRNA is injected into the marginal zone of early *Xenopus* embryos, the expression of the panmesodermal marker Xbra is not altered during gastrulation (Suzuki *et al.*, 1994; Schmidt *et al.*, 1995b).

Interpretation of dominant negative experiments is complicated by the fact that BMPs recognize several different type I and type II receptor kinases. There is evidence that the responses generated by BMP signaling depend on the composition of the receptor/ligand complex and are particularly influenced by the specificity of the type I receptor. BMP-2 and -4 have both been shown to bind the type II BMP receptor (BMPR-II) while mammalian OP-1 (BMP-7) shows significant affinity for activin receptors type II and type IIB in addition to BMPR-II (Liu *et al.*, 1995; Rosenzweig *et al.*, 1995; Yamashita *et al.*, 1995). Three type I BMP receptors—ALK-2 (ActR-I), ALK-3 (BMPR-IA), and ALK-6 (BMPR-IB)—have been described which are capable of interacting with BMP-2, -4, and -7 (Koenig *et al.*, 1994; ten Dijke *et al.*, 1994a,b; Macias-Silva *et al.*, 1998). The presence of multiple type I and type II BMP receptors raises the possibility that any single dominant negative construct is only capable of blocking a subset of the total possible BMP signal transduction pathways. A further complication in interpreting experiments with dominant negative receptors is that these constructs act cell autonomously and their effect is restricted by the limited diffusion of injected RNA. Therefore, the domain of gene expression that is affected in such experiments may be extremely localized.

A second common criticism of BMPs as endogenous mesoderm inducers has been that their capacity for induction appears quite limited in a variety of assays. Exposing animal caps directly to purified BMP homodimer proteins either fails to induce mesoderm (Clement *et al.*, 1995) or can only do so at concentrations far higher than what might be considered physiologically relevant (Koster *et al.*, 1991; Jones *et al.*, 1992; Wilson and Hemmati-Brivanlou, 1995). While as little as 1 ng $ml^{-1}$ of activin can induce mesoderm-specific transcripts in animal caps (Green and Smith, 1990), a concentration of 1000 ng $ml^{-1}$ of BMP-4 is required in a similar assay for mesoderm induction (Wilson and Hemmati-Brivanlou, 1995). As with purified proteins, the mesoderm-inducing activity of BMP mRNAs appears to be fairly weak and typically requires injection of 0.5–2 ng. In contrast, activin mRNA can induce mesoderm in animal caps at doses as low as 1 pg.

Recent evidence suggests that although individual BMP homodimers fail to induce mesoderm strongly, BMP-2/7 and -4/7 heterodimers may convey a much more potent activity. Hazama *et al.* (1995) have produced *Xenopus* BMP-2/7 and -4/7 heterodimers using an insect cell/baculovirus expression system and suggest that heterodimers form preferentially when these BMPs are coexpressed. In assays for osteogenic differentiation, both heterodimers exhibit significantly increased activities relative to homodimers (Aono *et al.*, 1995; Hazama *et al.*, 1995). In animal cap experiments, coinjection of BMP-2 and -7 or BMP-4 and -7 mRNAs has a synergistic effect on mesoderm induction (Suzuki *et al.*, 1997; Nishimatsu and Thomsen, 1998). In addition, when applied to animal caps as purified protein the BMP-4/7—and perhaps BMP-2/7—heterodimer is a potent inducer of ventral mesoderm at low and perhaps physiological concentrations (Suzuki *et al.*, 1997; Nishimatsu and Thomsen, 1998). The strength of this activity is in sharp contrast to that of BMP homodimers and suggests that heterodimers have unique activities. Nishimatsu and Thomsen (1998) speculate that the mesoderm induction seen when single BMP mRNAs are injected into animal caps is actually the result of heterodimeric proteins formed from synthetic and endogenous BMP transcripts. The potency of BMP heterodimers, combined with the fact that dominant negative BMP receptors have not been shown to block their activity, raises the possibility that BMPs may play an essential role in mesoderm induction.

The identification and characterization of proteins that act as TGF-$\beta$ antagonists has provided us with an effective tool for specifically inhibiting the BMP pathway. In gastrula stage *Xenopus* embryos, BMP signaling is attenuated in the

dorsal mesoderm by Spemann's organizer. This population of cells secretes a number of proteins, including noggin, Xnr-3, chordin, follistatin, and cerberus, that act as BMP antagonists (Smith and Harland, 1992; Hemmati-Brivanlou *et al.*, 1994; Sasai *et al.*, 1994; Smith *et al.*, 1995; Bouwmeester et al., 1996; Piccolo *et al.*, 1996, 1999; Zimmerman *et al.*, 1996; Fainsod *et al.*, 1997; Hsu *et al.*, 1998). By inhibiting BMP signaling, the organizer creates a BMP-free zone where dorsal and neural fates are expressed. Additional BMP antagonists which function later in development, including gremlin and Dan, have also been described (Hsu *et al.*, 1998). Although most of these molecules share no sequence similarity, many have been shown to act through a common mechanism: they block BMP signaling by binding the ligand extracellularly and preventing it from interacting with its cell surface receptors.

To determine whether BMPs play an essential role in mesoderm induction, we have blocked the BMP signaling pathway in early *Xenopus* embryos by ectopically expressing noggin, gremlin, and cerberus. Using *Xenopus* explant assays, we demonstrate that these three molecules are all effective antagonists of mesoderm induction by BMP homo- and heterodimers. By examining mesoderm-specific transcripts in whole embryos, we show that induction occurs normally even in regions where BMP signaling has been blocked by expression of antagonists. These results provide the first direct evidence that the potent mesoderm-inducing activity reported for BMP heterodimers does not constitute an essential component of the initial mesoderm inducing signal in *Xenopus*. In addition, we have examined the expression of dorsal-specific transcripts and find that inhibition of BMP signaling in the mesoderm leads to the delayed expression of a wide variety of organizer-specific transcripts including goosecoid (gsc) and the BMP antagonists chordin and cerberus.

## MATERIALS AND METHODS

### *Synthesis and Microinjection of Synthetic mRNA*

Capped synthetic mRNAs were transcribed using the mMessage mMachine kit (Ambion). *Xenopus* noggin (a gift of David Hsu, University of California, Berkeley, CA) and cerberus (Bouwmeester *et al.*, 1996), mRNAs were made from CS2(+) vectors linearized with *Not*I and transcribed by SP6 RNA polymerase. *Xenopus* gremlin mRNA was made from pCS105 (Hsu *et al.*, 1998) linearized with *Not*I and transcribed by SP6. Anti-myc immunoglobulin light chain mRNA was made from p9E10LC (a gift from J. de Jesús) linearized with *Asc*I and transcribed by SP6. Activin–BMP (AB)-2, -4, and -7 chimera mRNAs (gifts from P. Wilson and A. Hemmati-Brivanlou) were made from pSP64T (Krieg and Melton, 1984) linearized with *Eco*RI and transcribed by SP6. Activin–BMP chimeras were used because we consistently found them to be more active than the wild-type counterparts. Nuclear $\beta$-galactosidase mRNA was made from pSP6nuc$\beta$Gal (Smith and Harland, 1992) linearized with *Xho*I and transcribed by SP6. All mRNAs were injected as indicated.

### *Animal Cap Assays and RT-PCR*

*Xenopus* embryos were generated as described previously (Condie and Harland, 1987). Embryos were staged according to the normal table of Nieuwkoop and Faber (1967). For animal cap assays, one-cell embryos were injected in the animal pole. Animal cap ectoderm was dissected at stages 8–9 and cultured in 75% NAM solution (Peng, 1991). RNA was harvested at stages 10.5–11 as described (Condie and Harland, 1987). RT-PCR was performed as described by Wilson and Melton (1994). Primer sets for EF1$\alpha$, Xbra, and goosecoid are described by Wilson and Melton (1994), those for chordin and cerberus are described by Mariani and Harland (1998), and those for Xhox3 are described by Hemmati-Brivanlou and Thomsen (1995). Primers used for Siamois were (listed here 5′ to 3±) **U,** TGA GGC TGA AAT GGA GCA (nucleotide positions 22–39); and **D,** CTG TTG ACT GCA GAC TGT (nucleotide positions 347–330).

### *Whole-Mount in Situ Hybridization and Lineage Tracing*

Synthetic mRNA for noggin, gremlin, cerberus, or immunoglobulin light chain was injected into the marginal zone of both ventral blastomeres of a four-cell embryo. In addition, 250 pg of nuclear $\beta$-galactosidase RNA was coinjected per blastomere as a lineage tracer. All injections were in volumes of about 10 nl. Embryos were allowed to develop to stage 10.25 and fixed for 1 h. $\beta$-Galactosidase protein was then visualized as described by Smith and Harland (1991) with the modification that 6-chloro-3-indolyl-$\beta$-D-galactoside (red-gal; Research Organics, Inc.) was used in place of X-gal. Following staining, embryos were refixed and whole mount *in situ* hybridizations were carried out according to the method of Harland (1991) with the modifications described by Knecht *et al.* (1995). Boehringer Mannheim purple AP substrate was used for all staining. We have observed that activity staining for $\beta$-galactosidase often partially obscures the *in situ* hybridization signal. For this reason some embryos were analyzed without red-gal staining.

## RESULTS

### *Mesoderm Induction by BMP Homo- or Heterodimers in Animal Caps Is Blocked by BMP Antagonists*

Previous studies have shown that BMPs have the ability to induce mesoderm directly in animal caps when injected as mRNA or supplied exogenously as proteins (Koster *et al.*, 1991; Dale *et al.*, 1992; Jones *et al.*, 1992). For both mRNA and protein treatments, BMP-2/7 and -4/7 heterodimers are found to be significantly more potent than homodimers. It has been established that heterodimeric BMP molecules are efficiently produced in cell lines expressing RNAs for both subunits (Hazama *et al.*, 1995). We have therefore used coinjection of mRNAs encoding BMP-2 and -7 or BMP-4 and -7 to assess mesoderm induction by BMP heterodimers in *Xenopus* animal cap explants. In all cases, mesoderm induction was observed in explants expressing either individual BMPs or BMP-2/7 and -4/7 in combination (Fig. 1, lanes 1–5). In addition, treated caps expressed the early

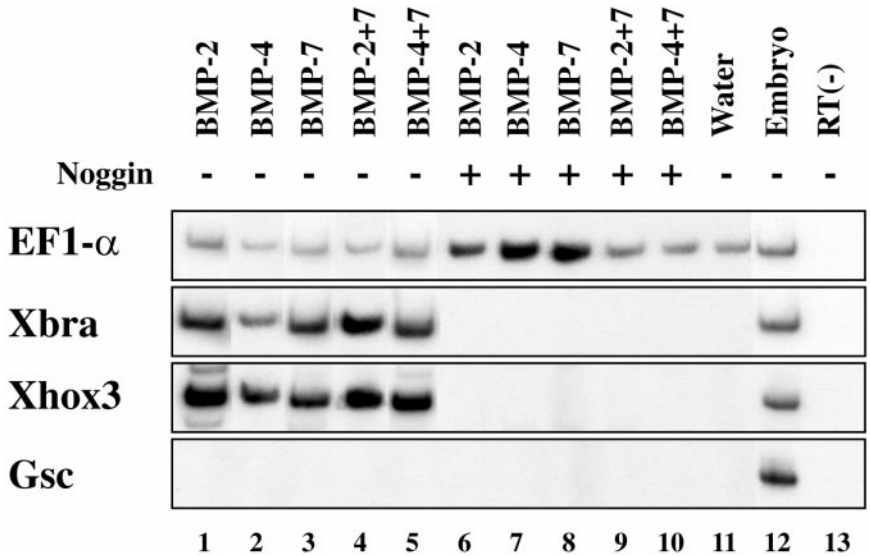


**FIG. 1.** Noggin blocks the mesoderm-inducing activity of BMP- homo and heterodimers in animal caps. 500 pg of BMP-2, -4, or -7 mRNA was injected into the animal poles of single cell embryos (lanes 1–3 and 6–8). To generate BMP-2/7 and -4/7 heterodimers, embryos were coinjected with 250 pg of mRNA for each BMP for a total of 500 pg (lanes 4 and 5 and 9 and 10). Where indicated, embryos were also injected with 100 pg of noggin mRNA. In all cases, animal caps were isolated at stage 8 and RNA was harvested at stage 11. RT-PCR analysis shows that ectopic expression of BMPs either alone or in combination induces Xbra. In all cases, this induction is completely blocked by coinjection of as little as 100 pg of noggin mRNA. EF1-$\alpha$ was used as a control for loading.

ventral mesodermal marker Xhox3 (Ruiz i Altaba and Melton, 1989) but not the dorsal-specific marker gsc (Fig. 1). These data are consistent with previous findings that mesoderm induced by BMPs in ectodermal explants is ventral in character.

We next assayed the BMP antagonists noggin, gremlin, and cerberus for their ability to blocking mesoderm induction by BMP homo- and heterodimers in animal cap explants. This was done by coinjecting embryos at the one-cell stage with 500 pg of BMP mRNA and either 100 or 500 pg of mRNA encoding the BMP antagonist being assessed. As before, caps were analyzed by RT-PCR for expression of Xbra. We found that as little as 100 pg of noggin mRNA was able to completely block mesoderm induction by 500 pg of mRNA encoding individual BMPs or combinations of BMP-2/7 and -4/7 (Fig. 1, lanes 6–10). Similar results were obtained for gremlin and cerberus, although in both of these cases injection of 500 pg of mRNA was required to block mesoderm induction completely (Fig. 2, lanes 3–6; data not shown for antagonism of mesoderm induction by BMP-2, -4, and -7 alone or the BMP-2/7 combination). In contrast, mRNA encoding an immunoglobulin light chain, a neutral secreted protein, had no effect on the mesoderm-inducing activities of BMPs. These results show that noggin, gremlin, and cerberus are effective at blocking signaling by BMP heterodimers as well as homodimers.

We were concerned that the loss of Xbra expression in animal caps treated with BMP antagonists could reflect a conversion to extreme dorsoanterior fate rather than a true

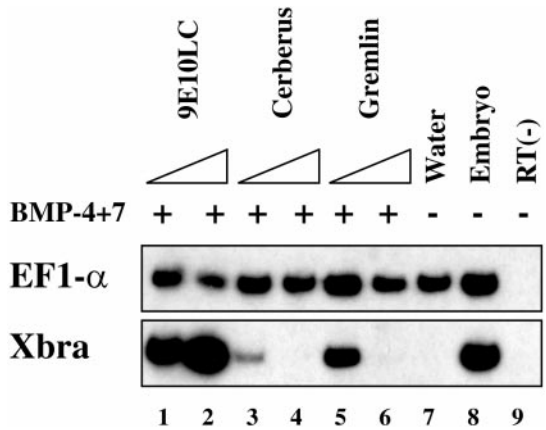


**FIG. 2.** Cerberus and gremlin also block BMP-mediated mesoderm induction in animal caps. Embryos were coinjected with 250 pg of BMP-4 mRNA and 250 pg of BMP-7 mRNA as indicated. In addition, embryos were also injected with 100 or 500 pg of light chain mRNA (lanes 1 and 2), 100 or 500 pg of cerberus mRNA (lanes 3 and 4), or 100 or 500 pg of gremlin mRNA (lanes 5 and 6). All injections were into the animal pole of single-cell embryos. Animal caps were isolated at stage 8 and RNA was harvested at stage 11. RT-PCR analysis indicates that the induction of Xbra by BMP-4/7 is blocked in a dose dependent manner by both cerberus and gremlin, but not by immunoglobulin light chain.

inhibition of mesoderm induction. This can be seen in mesoderm induced by a high dose of activin, which is extremely dorsal in character and expresses the mesodermal marker gsc even in the absence of the panmesodermal marker Xbra (Green *et al.*, 1992). BMP antagonists also possess a strong dorsalizing activity and both noggin and cerberus are thought to be partially responsible for mediating the activities of Spemann's organizer during gastrulation. In such a situation, high levels of gsc transcription might reduce or even abolish Xbra expression (Artinger *et al.*, 1997; Latinkic and Smith, 1999). We therefore analyzed all animal caps coinjected with BMP antagonists for gsc expression. RT-PCR failed to detect any gsc expression in explants injected with noggin (Fig. 1), gremlin, or cerberus (data not shown), suggesting that the mesodermalizing activity of BMPs is truly inhibited.

### *General Mesoderm Induction in Whole Embryos Is Not Blocked by BMP Antagonists*

Having established that noggin, gremlin, and cerberus are effective inhibitors of BMP-mediated mesoderm induction in ectodermal explants, we investigated their effects on mesoderm formation in whole embryos. We reasoned that if signaling by either BMP homo- or BMP heterodimers is required for the initial induction of ventral mesoderm in *Xenopus*, then ectopic expression of BMP antagonists in the ventral marginal zone should block expression of general mesodermal markers at the site of injection. To test this, we injected 200 pg of synthetic noggin mRNA into the marginal zone of both ventral blastomeres of four-cell embryos. This dose was twice that required to completely repress mesoderm induction by 500 pg of BMP mRNA in animal caps and should therefore completely abolish endogenous BMP signaling at the site of injection in intact embryos. Embryos were examined by whole mount *in situ* hybridization to assess general mesoderm induction as indicated by Xbra expression. Figure 3A shows that Xbra continues to be transcribed normally even in regions of the embryo that are also expressing noggin. To confirm that BMP signaling is not required in the formation of general mesoderm, we also looked at transcription of Eomesodermin (Eomes) and VegT. Both of these transcription factors are expressed throughout the marginal zone during gastrulation. Their expression can be induced by a variety of secreted mesoderm inducers and they can activate transcription of mesoderm-specific genes in animal caps (Lustig *et al.*, 1996; Ryan *et al.*, 1996; Stennard *et al.*, 1996; Zhang and King, 1996). Like Xbra, the expression of Eomes and VegT remains unchanged in noggin-injected embryos (Figs. 3E and 3I). These data indicate that the general mesoderm-inducing signal is functioning in regions of the embryo completely lacking BMP activity.

We carried out similar analyses on embryos that were ectopically expressing gremlin or cerberus mRNAs in the ventral marginal zone. Both of these mRNAs were injected at a dose of 500 pg per blastomere, as this higher concentration was shown to be necessary to completely block mesoderm induction by 500 pg of BMP mRNA in animal cap explants. Gremlin-treated embryos were found to behave in a manner that was indistinguishable from those injected with noggin. Xbra, Eomes, and VegT expression persisted at the site of injection, providing additional evidence that BMP homo- or heterodimers are not an essential part of the general mesoderm inducing signal in *Xenopus* (Figs. 3B, 3F, and 3J). In striking contrast, expression of all three mesodermal markers was found to be completely abolished by cerberus. This is apparent in Figs. 3C, 3G, and 3K where mesoderm-specific transcripts have been lost even at some distance from cells expressing injected mRNAs (as indicated by expression of the nonsecreted nuclear $\beta$-galactosidase lineage tracer). The ability of cerberus to completely block mesoderm induction in whole embryos presumably results from its antagonism of growth factors such as nodals and Wnts in addition to BMPs (Hsu *et al.*, 1998; Piccolo *et al.*, 1999). Neither noggin nor gremlin have this capability, as both appear to be specific for members of the BMP subfamily.

To ensure that all BMP antagonists were being functionally expressed by the time of gastrulation, we examined expression of Xvent-2 by whole mount *in situ* hybridization. Xvent-2 is a homeobox gene expressed in the marginal zone and animal cap during gastrulation but excluded from the organizer region. Previous studies using a dominant negative BMP-4 receptor have shown that Xvent-2 expression is abolished when BMP signaling is blocked (Onichtchouk *et al.*, 1996). We found that, as expected, embryos injected with noggin, gremlin, or cerberus mRNA exhibited a significant reduction of Xvent-2 expression at the site of injection (Figs. 3M–3O) while Xvent-2 levels were unaffected by ectopic expression of immunoglobulin light chain mRNA (Fig. 3P). This clearly indicates that BMP signaling is being blocked even at early gastrula stages by expression of antagonists. Several differences were apparent in the way that Xvent-2 expression responded to cerberus as compared to noggin and gremlin. The domain of inhibition was consistently more widespread in noggin- and gremlin-injected embryos. In contrast, Xvent-2 transcription appeared to be most completely blocked at the sites of cerberus expression (compare Fig. 3M to Figs. 3N and 3O). This may indicate that signals in addition to BMPs are involved in activating Xvent-2 transcription or it may simply reflect differences in the diffusibility of the antagonists.

### *BMP Antagonists Induce the Early Dorsal Mesodermal Marker Goosecoid*

Our observation that Xbra expression is not disrupted in the presence of noggin and gremlin indicates that BMP signaling through homo- or heterodimers is not a necessary component of general mesoderm induction. Instead, BMPs are likely to act as short range signals that allow cells already specified as general mesoderm to remain on

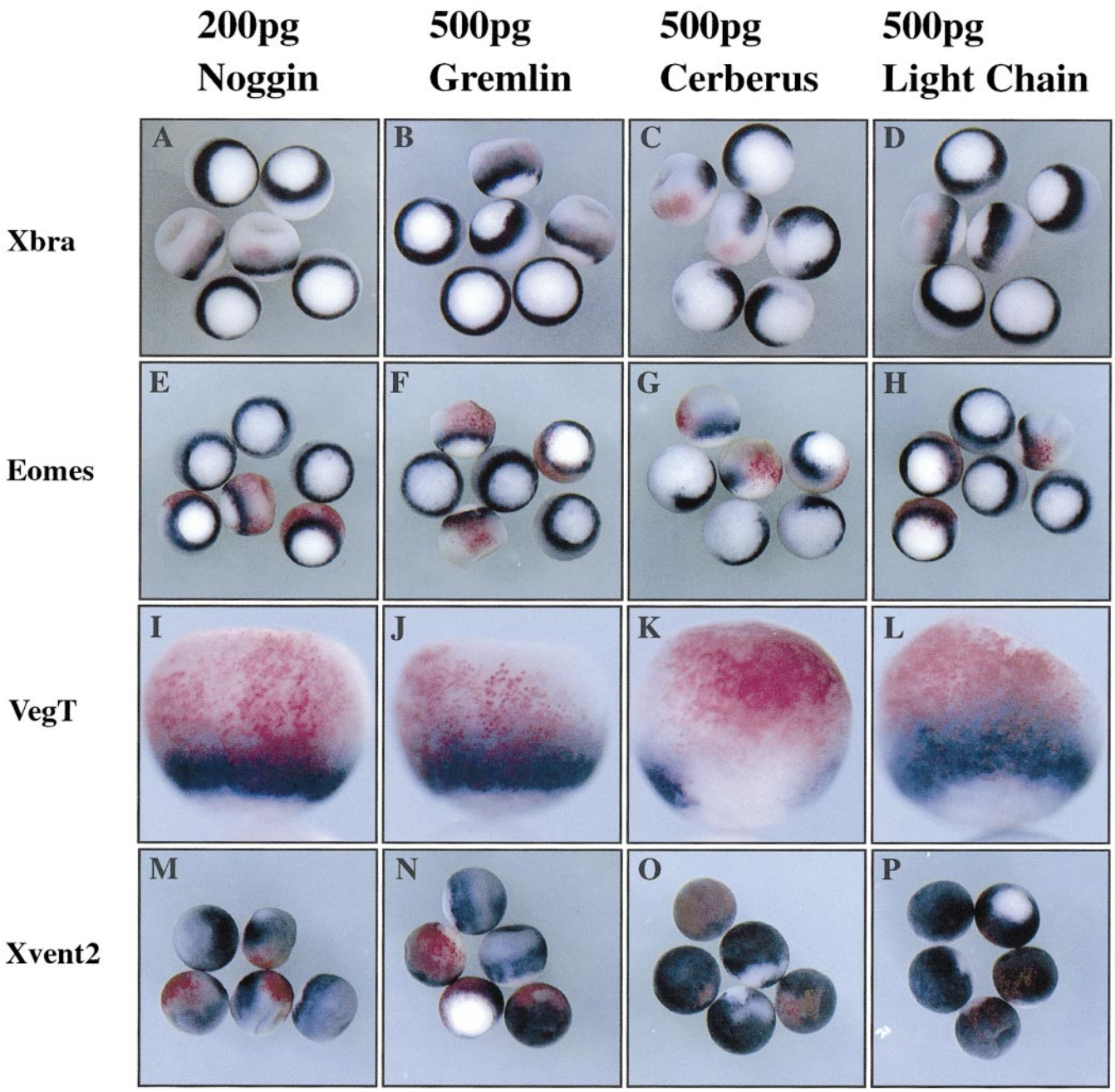


**FIG. 3.** Effects of BMP antagonists on expression of the panmesodermal markers Xbra, VegT, and Eomesodermin and the ventral marker Xvent-2 in stage 10.5 *Xenopus* embryos. Whole mount *in situ* hybridization analysis was carried out on embryos injected ventrally with 200 pg of noggin mRNA or 500 pg of gremlin, cerberus, or immunoglobulin light chain mRNA. All embryos were coinjected with a $\beta$-galactosidase lineage tracer and a subset of these were stained with red-gal to mark the site of injection and confirm targeting of mRNA to the ventral marginal zone. Ectopic expression of noggin (A, E, and I) and gremlin (B, F, and J) in the ventral marginal zone does not inhibit normal expression of the panmesodermal markers Xbra, Eomesodermin, and VegT. In contrast, cerberus—which blocks signaling by nodal-related molecules in addition to BMPs—completely abolishes expression of all three transcripts at the site of injection (C, G, and K). Note that embryos in M–P are shown at higher magnification to clearly illustrate the overlapping domains of VegT expression and the red-gal activity stain at the site of noggin and gremlin injections. All three antagonists are active as shown by their inhibition of Xvent-2 (M–O), a marker of ventral and lateral mesoderm that requires BMP signaling for transcription. RNA-encoding immunoglobulin light chain, a neutral secreted protein, has no effect on the expression pattern of any marker (D, H, L, and P).

paths leading to ventral posterior development. To more carefully evaluate the effects of inhibiting BMP signaling in the ventral marginal zone, we examined embryos injected with BMP antagonist RNAs for gsc expression. Gsc is an organizer-specific homeobox gene that can mimic some activities of Spemann's organizer when ectopically expressed (Cho *et al.*, 1991; Niehrs *et al.*, 1993; Steinbeisser and De Robertis, 1993). High-level transcription of gsc is thought to be activated in the dorsal mesoderm by a combination of Wnt-like and Activin-like signals (Steinbeisser *et al.*, 1993; Watabe *et al.*, 1995). We were interested to see whether gsc and other organizer-specific transcripts would be expressed in regions of the ventral marginal zone where BMP signaling had been blocked by antagonists.

As in previous experiments, embryos were injected ventrally with noggin, gremlin, or cerberus mRNA. The embryos in Figs. 4A and 4B clearly show weak patches of ectopic gsc expression at the sites of noggin and gremlin injection. This expression appears to be limited to the marginal zone, an observation consistent with previous findings that gsc can only be expressed in cells that have received a general mesoderm-inducing signal (Steinbeisser *et al.*, 1993). Expression of gsc in response to noggin and gremlin is delayed relative to endogenous expression in the dorsal marginal zone. Ectopic transcripts are not detected in stage 9 embryos treated with noggin although organizer expression is clearly visible at this time (Fig. 4F). By stage 10.5 noggin- and gremlin-induced gsc transcription is apparent by *in situ* hybridization, but levels are considerably weaker than endogenous expression. This difference is confirmed by comparison to embryos injected ventrally with 10 pg of Xwnt-8 mRNA (Fig. 4D). Xwnt-8 mRNA is a potent mesoderm-dorsalizing agent and is capable of inducing complete secondary axes in *Xenopus* embryos, although it cannot induce mesoderm on its own (Christian *et al.*, 1992). In Xwnt-8-injected embryos, gsc mRNA is strongly induced throughout most of the marginal zone at levels that are indistinguishable from endogenous transcripts. Unlike noggin and gremlin, ventral injection of cerberus mRNA was not observed to induce gsc expression (Fig. 4C). Given the ability of cerberus to block general mesoderm induction in the marginal zone completely, this finding is not surprising.

To more thoroughly characterize the effects of blocking BMP signaling in the ventral mesoderm, we performed RT-PCR analysis on ventral marginal zone (VMZ) explants from embryos injected with noggin, gremlin, or Xwnt-8 mRNA. Consistent with results from whole mount *in situ* hybridization analyses, both BMP antagonists and Xwnt-8-induced goosecoid expression in the VMZ (Fig. 5, lanes 1 and 2). Xwnt-8, noggin, and gremlin were also found to induce expression of chordin and cerberus, additional transcripts which are restricted to the organizer during gastrulation (Fig. 5, lanes 1 and 2). Siamois, another organizer-specific homeobox gene, appears to be induced by Xwnt-8 but not noggin or gremlin. As expected, the ventral and lateral mesoderm marker Xhox-3 is downregulated in VMZs expressing BMP antagonists.

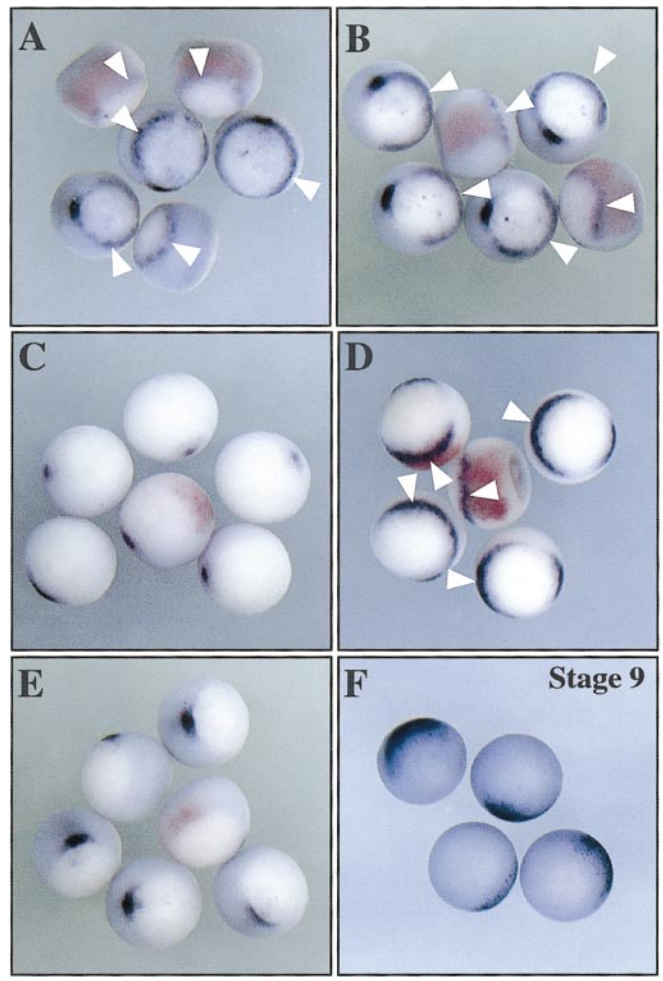


**FIG. 4.** Gsc is weakly induced by blocking BMP signaling in the mesoderm. *In situ* hybridization analysis of gsc expression in embryos injected ventrally with 10 pg of Xwnt-8 mRNA, 200 pg of noggin mRNA, or 500 pg of gremlin, cerberus, or immunoglobulin light chain mRNA. A subset of the injected embryos was lineage traced with $\beta$-galactosidase to demonstrate that ectopic gsc expression coincides with the site of injection. Noggin (A) and gremlin (B) both induce low levels of ventral gsc transcription in stage 10.5 embryos as indicated by arrowheads. Note that ectopic gsc expression is limited to the marginal zone, suggesting that it cannot be induced in nonmesodermal tissues. Cerberus, which blocks mesoderm formation, also fails to induce ectopic gsc expression (C). Xwnt-8 is a potent mesoderm dorsalizer and strongly induces ventral gsc expression (D). Light chain mRNA has no effect on gsc (E). No ventral gsc transcripts are detected in stage 9 embryos injected with 200 pg noggin, indicating that ectopic gsc expression begins subsequent to endogenous expression in the organizer (F). Note that stage 9 embryos have been stained for an extended period of time relative to the other embryos shown to ensure that no ectopic gsc induction is detectable by *in situ* hybridization.

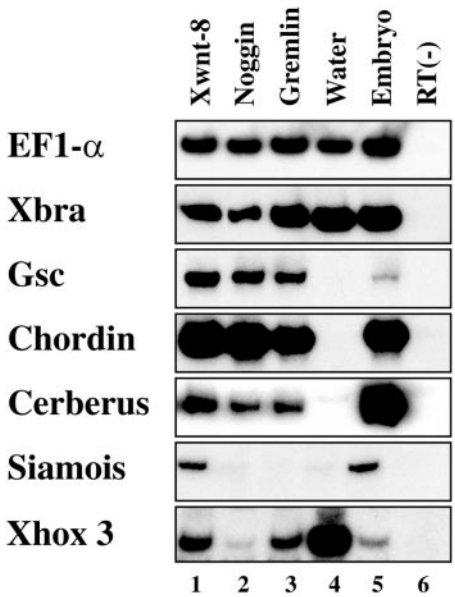


**FIG. 5.** Inhibition of BMP signaling by noggin induces many organizer-specific transcripts and inhibits markers of ventral mesoderm in VMZ assays. Embryos were injected ventrally with 500 pg of noggin mRNA or 100 pg of Xwnt-8 mRNA. VMZs were cut at stages 10.25–10.5 and RNA was harvested at stage 12. RT-PCR analysis shows that noggin, gremlin, and Xwnt-8 can induce expression of gsc, chordin, and cerberus—transcripts which are specific to Spemann's organizer during gastrulation. In addition, they all substantially reduce transcription of the ventral mesoderm marker Xhox-3. Only Xwnt-8 was observed to induce the homeobox gene siamois.

## DISCUSSION

### *BMP Heterodimers Are Not Required for Primary Mesoderm Induction in Xenopus*

In the present study we have employed secreted BMP antagonists to evaluate the necessity for BMP signaling in the initial induction of mesoderm in *Xenopus.* Previous experiments have established that BMP-2, -4, and -7 are all capable of inducing ventral types of mesoderm from naive ectoderm in embryonic explants (Koster *et al.,* 1991; Nishimatsu *et al.,* 1992). Whether or not this activity reflects a need for BMP signaling during mesoderm induction in normal development was unclear. Studies using dominant negative BMP receptors, combined with the observation that BMPs are relatively weak mesoderm inducers when compared to other TGF-βs, has led some to conclude that BMP signaling is not required for the initial induction of mesoderm but only for its subsequent patterning. Recent reports on BMP-2/7 and -4/7 heterodimers, however, show these to be much more potent mesoderm inducing factors than homodimers. There is evidence that BMP homodimers may in fact be incapable of inducing mesoderm when supplied exogenously as proteins (Nishimatsu and Thomsen, 1998). Because of these concerns, we opted to use secreted BMP antagonists rather than truncated receptor constructs when addressing the need for BMP signaling in mesoderm induction.

Using animal cap explants, we have demonstrated that the BMP antagonists noggin, gremlin, and cerberus are all effective at blocking BMP-mediated mesoderm induction. Significantly, all three antagonists were found to inhibit signaling by BMP-2/7 and -4/7 heterodimers as well as by the various BMP homodimers. This activity proved to be very robust, with as little as 100 pg of noggin mRNA and 500 pg of gremlin or cerberus mRNA sufficient to completely block mesoderm induction by 500 pg of BMP-2/7 or -4/7 mRNA. The ability of noggin to inhibit a fivefold excess of BMP mRNA likely reflects differences in the efficiency of protein expression from the injected RNAs. Noggin, gremlin, and cerberus mRNAs were all transcribed from CS2 derived vectors (Turner and Weintraub, 1994) while BMP mRNAs were produced from pSP64T vectors (Krieg and Melton, 1984). By injecting animal caps with 500 pg of BMP mRNA we have presumably greatly exceeded the low levels of endogenous maternal BMP transcripts found in the early *Xenopus* embryo (Dale *et al.,* 1992; Nishimatsu *et al.,* 1992). We are therefore confident that, by injecting antagonist mRNAs at levels capable of inhibiting 500 pg of synthetic BMP mRNA, we can block all endogenous BMP signaling in the embryo at the site of injection. This is confirmed by the inhibition of Xvent-2 in whole embryos at the site of antagonist injection, as Xvent-2 transcription has been shown to require BMP signaling (Onichtchouk *et al.,* 1996).

Analysis of whole embryos injected ventrally with noggin or gremlin mRNA revealed no disruption of the panmesodermal markers Xbra, Eomes, or VegT (Figs. 3A, 3B, 3E, 3F, 3I, and 3J). These findings provide compelling evidence that BMPs are not a necessary component of the general mesoderm inducing signal in *Xenopus* embryos. In contrast, injection of cerberus completely abolishes expression of all three mesodermal markers (Figs. 3C, 3G, and 3K). The ability of cerberus to inhibit mesoderm induction can likely be attributed to the broad range of growth factors it antagonizes. Previous studies have shown that cerberus can completely inhibit the induction of Xbra in ectodermal explants by Xnr-2 and partially block mesoderm induction by activin. This latter activity may be limited to animal caps exposed to low levels of activin. Under these conditions, expression of Xbra is thought to be mediated in part through a relay, in which Xnr-1 and -2 are induced by activin and then go on to activate Xbra transcription (Hsu *et al.,* 1998; Osada and Wright, 1999). Cerberus has recently been shown to bind Xnr-1 and Xwnt-8 directly in addition to BMPs (Piccolo *et al.,* 1999). The observation that cerberus mRNA injected into the ventral marginal zone of a four-cell embryo efficiently blocks general mesoderm induction provides an important positive control for noggin- and gremlin-treated embryos. It demonstrates that a growth factor

antagonist can be translated from ectopically expressed mRNAs in time to completely inhibit primary mesoderm induction provided the antagonist has the proper activity. We conclude that, although BMP heterodimers are capable of forming ventral types of mesoderm in animal cap assays (Fig. 1), they do not constitute an essential component of the primary mesoderm-inducing signal in *Xenopus.* This does not preclude the possibility that, at least in the case of ventral mesoderm, there are two redundant pathways for induction, one through BMPs and the other through growth factors such as Xnrs which are not inhibited by the BMP antagonists noggin and gremlin.

### *Blocking BMP Signaling Induces Organizer Specific Transcripts in Ventral Mesoderm*

We have shown that both noggin and gremlin induce ectopic expression of the organizer-specific homeobox gene goosecoid (Figs. 4A, 4B, 5). Previous attempts to address the effects of loss of BMP signaling in the early embryo have reached conflicting conclusions regarding gsc induction. In zebrafish BMP-2 (*swirl*) mutants, gsc expression appears normal while more lateral mesodermal markers are dramatically expanded (Mullins *et al.*, 1996; Kishimoto *et al.*, 1997). Suzuki *et al.* (1994) and Schmidt *et al.* (1995b) both find no ectopic induction of gsc in whole embryos injected ventrally with truncated type I BMP receptor RNA. In contrast, Graff *et al.* (1994) detect gsc transcripts in VMZs that have been dorsalized by a similar truncated receptor. In addition, ventral injection of 6 ng of mRNA encoding sog, the *Drosophila* homologue of chordin, weakly induces gsc in *Xenopus* embryos (Schmidt *et al.*, 1995a). We find that gsc is consistently induced by BMP antagonists even in the most ventral regions of the marginal zone. Based on whole mount *in situ* hybridization analysis, ectopic transcription is delayed and appears less robust than endogenous gsc expression in the dorsal marginal zone (Figs. 4A, 4B, and 4F; arrows indicate ectopic gsc expression). We also find that ectopic expression is limited to the marginal zone, although the injected antagonists are expressed in much larger domains as indicated both by lineage tracing and their effect on Xvent-2 transcription. These results strongly suggest that inhibition of BMP signaling leads to gsc expression only in cell populations that are already committed to mesodermal fates. This is supported by the observation that cerberus, the only known BMP antagonist which also abolishes mesoderm induction, fails to induce gsc expression.

BMPs are known to promote ventral mesodermal fates and can override the effects of Spemann's organizer and dorsal mesoderm inducers such as activin when overexpressed (Dale *et al.*, 1992). This has been observed in experiments using dominant negative BMP receptors where ventral mesodermal markers such as T $\alpha$-globin, Xpo, and Xwnt-8 are downregulated while expression of dorsolateral markers such as MyoD and cardiac $\alpha$-actin are increased (Maeno *et al.*, 1994; Suzuki *et al.*, 1994; Schmidt *et al.*, 1995b). In contrast, overexpression of BMP-4 in *Xenopus* embryos leads to ventralization during gastrulation; dorsal transcripts are initially expressed normally and then become downregulated (Jones *et al.*, 1996). It is not entirely surprising, therefore, that blocking the BMP signaling pathway leads to the induction of more dorsal mesodermal markers. What is unexpected is the finding that even the most dorsal transcripts such as gsc, chordin, and cerberus, whose expression patterns are normally limited to the organizer, are induced in the absence of BMP activity. This differs from most studies using dominant negative BMP receptors, which report that genes expressed along the dorsal midline are not ectopically induced in the absence of BMP signaling (Suzuki *et al.*, 1994; Schmidt *et al.*, 1995b). Our findings raise the possibility that BMP signaling in the ventral and lateral marginal zone is necessary for complete repression of organizer-specific transcripts in addition to promoting the differentiation of ventral types of mesoderm instructively.

Our analysis of noggin- and gremlin-injected embryos suggests that BMP-mediated inhibition of gsc (and perhaps other organizer-specific transcripts) is necessary throughout lateral and ventral regions of the mesoderm during gastrulation. The ability of BMPs to suppress gsc and other organizer-specific genes has been well characterized. Transcription of gsc, Xnot, noggin, and pintallavis is downregulated in the organizer of embryos injected dorsally with BMP-4 mRNA (von Dassow *et al.*, 1993; Fainsod *et al.*, 1994; Schmidt *et al.*, 1995b; Jones *et al.*, 1996). It remains unclear what factors are responsible for inducing the low levels of gsc transcription seen in these regions in the absence of BMP signaling. The gsc promoter contains *cis*-acting distal and proximal elements which are required for induction by activin/BVg1 and Wnt induction, respectively (Watabe *et al.*, 1995). It is thought that normal expression of gsc in the organizer requires input from both of these signaling pathways. In the *Xenopus* embryo, there is an early activation of the Wnt signaling pathway on the dorsal side of the embryo during blastula stages and a later expression of Xwnt-8 in the ventrolateral mesoderm during gastrulation. Gsc expression in the organizer is thought to result from an overlap of the early dorsal Wnt signal with the activin like mesoderm inducing signal. It seems possible that one function of BMP signaling in the ventral and lateral mesoderm is to prevent ectopic gsc induction in response to ventral Xwnt-8 expression during gastrulation. This is consistent with the observation that ectopic gsc expression in response to BMP antagonists is delayed relative to endogenous expression of gsc in the organizer (Fig. 4F).

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