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The role of *gsc* and *BMP-4* in dorsal–ventral patterning of the marginal zone in *Xenopus*: a loss-of-function study using antisense RNA

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The dorsal-specific homeobox gene *goosecoid* (*gsc*) and the bone morphogenetic protein 4 gene (*BMP-4*) are expressed in complementary regions of the *Xenopus* gastrula. Injection of *gsc* mRNA dorsalizes ventral mesodermal tissue and can induce axis formation in normal and UV-ventralized embryos. On the other hand, *BMP-4* mRNA injection, which has a strong ventralizing effect on whole embryos, has been implicated in ventralization by UV, and can rescue tail structures in embryos dorsalized by LiCl. The above-mentioned putative roles for *BMP-4* and *gsc* are based on gain-of-function experiments. In order to determine the *in vivo* role of these two genes in the patterning of the *Xenopus* mesoderm during gastrulation, partial loss-of-function experiments were performed using antisense RNA injections. Using marker genes that are expressed early in gastrulation, we show that antisense *gsc* RNA has a ventralizing effect on embryos, whereas antisense *BMP-4* RNA dorsalizes mesodermal tissue. These loss-of-function studies also show a requirement for *gsc* and *BMP-4* in the dorsalization induced by LiCl and in the ventralization generated by UV irradiation, respectively. Thus, both gain- and loss-of-function results for *gsc* and *BMP-4* support the view that these two genes are necessary components of the dorsal and ventral patterning pathways in *Xenopus* embryos.

Keywords: antisense/*BMP-4*/*gsc*/mesoderm patterning/*Xenopus*

Introduction

The formation of the mesoderm in *Xenopus* embryos can be divided into two distinct sets of events. Initially, the cortical rotation, which occurs within the first hour after fertilization, defines the dorsal side of the future embryo. Subsequently, the Nieuwkoop center signal that emanates from the dorsal vegetal blastomeres, induces the formation of the dorsal organizing center (Spemann's organizer) in the overlaying dorsal marginal zone (Nieuwkoop, 1969; Gerhart *et al.*, 1989). The molecular basis of the Nieuwkoop center activity remains unknown, but peptide growth factors, especially Vg1 as well as secreted molecules of the Wnt family, are likely key players in this induction event (Kessler and Melton, 1994). These inductive cues trigger the expression of zygotic genes in the developing mesoderm which function in the establishment of its dorsal–ventral patterning. As a result, dorsal tissue types such as notochord and muscle, as well as ventral tissues such as blood and mesenchyme, are formed. Significant progress has been made in understanding mesoderm patterning by studying the roles of genes expressed in the marginal zone of the *Xenopus* embryo, the region from which the mesoderm arises.

Genes expressed in Spemann's organizer with dorsalizing activities, such as the homeobox genes *goosecoid* (*gsc;* Cho *et al.*, 1991); *Xlim* (Taira *et al.*, 1994) and *siamois* (Lemaire *et al.*, 1995), as well as the secreted factors noggin (Smith and Harland, 1992), follistatin (Hemmati Brivanlou *et al.*, 1994; Sasai *et al.*, 1995) and chordin (Sasai *et al.*, 1994) have been described. The predominant view in the past has been that dorsal genes promote the formation of dorsal structures and that ventral mesoderm develops as a default state due to the absence of dorsal factors. More recently, however, experimental evidence suggesting that ventral mesoderm formation requires active induction by genes with ventralizing activity has been steadily accumulating (Köster *et al.*, 1991; Dale *et al.*, 1992; Jones *et al.*, 1992; Fainsod *et al.*, 1994; Graff *et al.*, 1994: Suzuki *et al.*, 1994). Two genes with such ventralizing activities, *Xwnt-8* and bone morphogenetic protein 4 (*BMP-4*), are indeed expressed in the ventral marginal zone (VMZ) of the gastrula embryo (Christian and Moon, 1993; Fainsod *et al.*, 1994; Schmidt *et al.*, 1995). Furthermore, blocking *BMP-4* activity by introducing a dominant negative receptor, which binds *BMP-2* and *BMP-4* and possibly blocks the activity of other members of the *BMP* family as well, results in the development of dorsal structures on the ventral side of the embryo (Graff *et al.*, 1994; Harland, 1994; Suzuki *et al.*, 1994).

Recently we presented gain-of-function studies on the patterning of the marginal zone by *gsc* and *BMP-4* (Fainsod *et al.*, 1994; Niehrs *et al.*, 1994). The *gsc* homeodomain protein is able to dorsalize mesodermal tissue in a dose-dependent manner (Niehrs *et al.*, 1994) and induces secondary axial structures on the ventral side of the embryo (Cho *et al.*, 1991; Steinbeisser *et al.*, 1993). Microinjection of *gsc* mRNA represses the expression of ventral genes such as *Xwnt-8* and *BMP-4* (Christian and Moon, 1993; Fainsod *et al.*, 1994). In LiCl-treated embryos, *gsc* expression becomes expanded throughout

the marginal zone in accordance with their dorsalized phenotype. In embryos ventralized by UV irradiation, *gsc* expression is repressed.

BMP-4 has for several years been known to have a strong ventralizing effect when its mRNA is injected into *Xenopus* embryos, capable of abolishing all dorsalizing signals emanating from the organizer (Köster *et al.*, 1991; Dale *et al.*, 1992; Jones *et al.*, 1992). This effect comes about in part by repressing the expression of organizer-specific genes such as *gsc* and *Xnot/Xnot-2* during gastrulation (Fainsod *et al.*, 1994; Schmidt *et al.*, 1995). In an inverse manner to that described for *gsc*, *BMP-4* expression is absent from the entire marginal zone in embryos dorsalized by LiCl treatment, and its transcripts accumulate precociously in embryos ventralized either by UV irradiation or by suramin treatment (Fainsod *et al.*, 1994).

In *Xenopus* embryos, studies of the genes and activities responsible for the patterning of the mesoderm during gastrulation are based mainly on gain-of-function experiments by injection of synthetic mRNAs in either whole embryos or explants. Such studies can show that a gene is sufficient to carry out a certain function, but not whether it is required *in vivo*. The approach taken here was to generate partial loss-of-function for specific genes by injecting antisense RNA into *Xenopus* embryos. We present evidence that antisense *gsc* RNA has a ventralizing effect in *Xenopus* embryos, judging by the analysis of early marker genes. In addition, decreasing the *gsc* product by injected antisense RNA or DNA expression constructs counteracts the dorsalization caused by LiCl in the *Xenopus* embryo. This indicates that *gsc* is a necessary component of the dorsalizing pathway activated by LiCl. In contrast, antisense *BMP-4* RNA causes dorsalization of mesodermal tissues in normal and UV-treated embryos. This provides further support for the view that ventral mesoderm induction should be considered an active process rather than a default state (Fainsod *et al.*, 1994; Graff *et al.*, 1994; Grunz, 1994; Harland, 1994) and points to a fundamental role for *BMP-4*, rather than a related factor, in establishing the ventral state.

Results

Inhibition of ectopic β-gal activity in Xenopus embryos by injecting antisense RNA

To investigate whether it was possible to inhibit zygotic gene expression in *Xenopus* embryos using an antisense approach, we first devised a system to test the effectiveness of injected antisense RNA. Synthetic β-*gal* antisense RNA was microinjected into the four animal blastomeres of an 8-cell *Xenopus* embryo (400 pg/embryo). Ten minutes later, in a second injection, β-*gal* sense RNA was delivered to two animal blastomeres (10 pg/embryo). At stage 11 (gastrula), embryos were tested for β-*gal* enzyme activity (Figure 1). Injected antisense β-*gal* RNA caused a significant reduction of β-*gal* activity in all the samples (n = 11) compared with the embryos injected with β-*gal* sense RNA alone (n = 10; Figure 1A and B) A quantitative analysis by measuring the enzyme activity in embryo homogenates from two independent experiments showed a 77–85% reduction of β-*gal* activity in embryos that received both sense and antisense β-*gal* RNA compared with those injected with sense RNA alone. For each experiment, two duplicate sets of three pooled embryos were measured

To test whether the antisense RNA is still able to inhibit synthesis of the protein product when the target sense RNA is synthesized at a later stage in development, we injected a β-*gal* DNA construct which produces β-*gal* sense RNA under the control of a cytomegalovirus (CMV) promoter, alone or in combination with β-*gal* antisense RNA. In this experimental design, β-*gal* mRNA is transcribed only after mid-blastula transition, reflecting the onset of zygotic gene expression in the embryo (Niehrs *et al.*, 1993). Similar to the mRNA injection, β-*gal* activity was reduced (52–70%) in all the embryos (n = 23) preloaded with antisense RNA and subsequently injected with the CMV–β-gal plasmid, compared with those that received the DNA alone (Figure 1C and D). These data indicate that antisense RNA has the potential to inhibit zygotic gene expression in *Xenopus* embryos, leading to partial loss-of-function. These results with the β-*gal* system suggested that loss-of-function studies by antisense RNA injection might be possible for genes expressed at the blastula and gastrula stages. A prerequisite for loss-of-function in this experimental design is that reductions of 50–80% in the activity of a gene product must be enough to detect a loss-of-function phenotype. Both *gsc* and *BMP-4* have been shown in gain-of-function experiments to exhibit responses that are highly dose dependent (Fainsod *et al.*, 1994; Niehrs *et al.*, 1994). In addition, zygotic expression of *gsc* and *BMP-4* occurs at the onset of gastrulation, when the activity of the unwinding enzyme that makes antisense experiments in *Xenopus* eggs difficult has decreased greatly (Bass and Weintraub, 1987; Rebagliati and Melton, 1987). Therefore, these two genes were attractive candidates for a loss-of-function study in *Xenopus* embryos.

To test the stability of the injected antisense RNA, we used quantitative reverse transcription (RT)-PCR specific for the injected RNA. Injected antisense *BMP-4* RNA (600 pg/embryo) was present until the mid- to late-gastrula (stage 12.5; 13 h) in amounts comparable with those of the endogenous elongation factor-1α (EF-1α mRNA), and in excess of the endogenous *BMP-4* sense mRNA, using the same number of PCR cycles (Figure 2A). To test whether the transcripts produced by the CMV promoter were abundant and uniformly distributed, embryos were injected with 50 pg of CMV-antis-gsc plasmid in one out of four blastomeres and hybridized with a sense probe (which detects specifically antisense transcripts) as shown in Figure 2C. The CMV vector produces transcripts in considerable excess over those produced by the endogenous *gsc* gene, which can be detected in similarly injected embryos using an antisense probe as shown in Figure 2D.

We next tested whether *gsc* expression can be inhibited by antisense *gsc* RNA. The presence of the antisense RNA caused no reduction of the amounts of endogenous *gsc* mRNA (assayed by quantitative RT-PCR and *in situ* hybridization, Figure 2D). Thus, it appears that the antisense RNA does not cause the degradation of transcripts. However, antisense RNA has an inhibitory effect on *gsc* mRNA translation (Figure 2B). As no gsc antibodies are available, we resorted to an indirect method to measure an expected decrease in gsc protein. To test whether protein

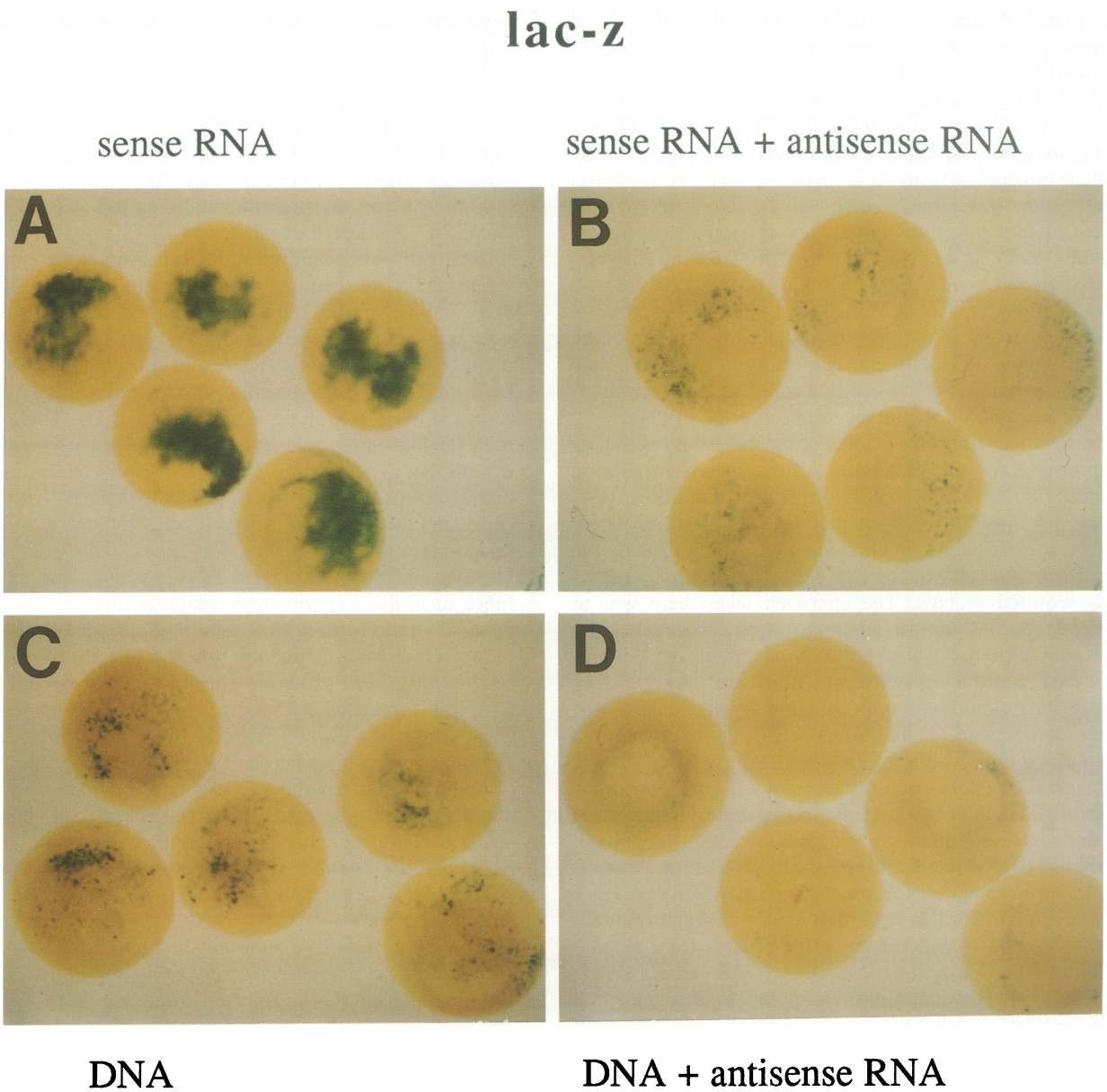

Fig. 1. β-Galactosidase activity is inhibited by injected antisense β-*gal* RNA. (**A**) *Xenopus* embryos injected at the 8-cell stage into two animal blastomeres with sense β-*gal* RNA (5 pg/blastomere). (**B**) 8-cell embryos injected with antisense β-*gal* RNA into four animal blastomeres (100 pg/blastomere) and in a second injection into two blastomeres with sense β-*gal* RNA (5 pg/blastomere). (**C**) Two animal blastomeres of 8-cell embryos were injected with the β-*gal* DNA construct pCH 101 (10 pg/blastomere). (**D**) 8-cell embryos were injected with antisense β-*gal* RNA into four animal blastomeres (100 pg/blastomere) and in a second injection into two blastomeres with the β-*gal* DNA construct pCH 101 (10 pg/blastomere). The embryos were fixed in MEMFA at stage 11 and stained for β-*gal* for 1–3 h, refixed and made transparent in Murray's solution. The samples which had received the antisense as well as the sense β-*gal* RNA or the sense β-*gal* DNA construct showed reduced β-*gal* activity compared with those injected with the sense β-*gal* RNA or pCH101 alone. Similar results were obtained in two independent experiments.

synthesis is decreased, a *gsc* vector which contained a *myc*-epitope tag at the 5′ end was constructed. Synthetic mRNA for *gsc–myc* was injected in combination with either wild-type antisense *gsc* RNA or control prolactin RNA and the presence of the epitope-tagged gsc protein was assayed by Western blot (Figure 2B). Injection of *gsc* antisense RNA decreased the amount of epitope-tagged gsc protein significantly when compared with the control injected with prolactin mRNA, and did not affect the general protein profile (Figure 2B).

Phenotypic effects of injected antisense RNA for gsc and BMP-4

gsc mRNA has a dorsalizing effect on mesodermal tissue whereas *BMP-4* mRNA promotes the formation of ventral mesoderm. Because *BMP-4* and *gsc* act antagonistically in the developing mesoderm, one should expect opposite effects in partial loss-of-function experiments. The *Xenopus* embryo can be dorsalized by LiCl, which increases the expression of dorsal genes such as *gsc* and represses that of ventral genes such as *BMP-4* and causes

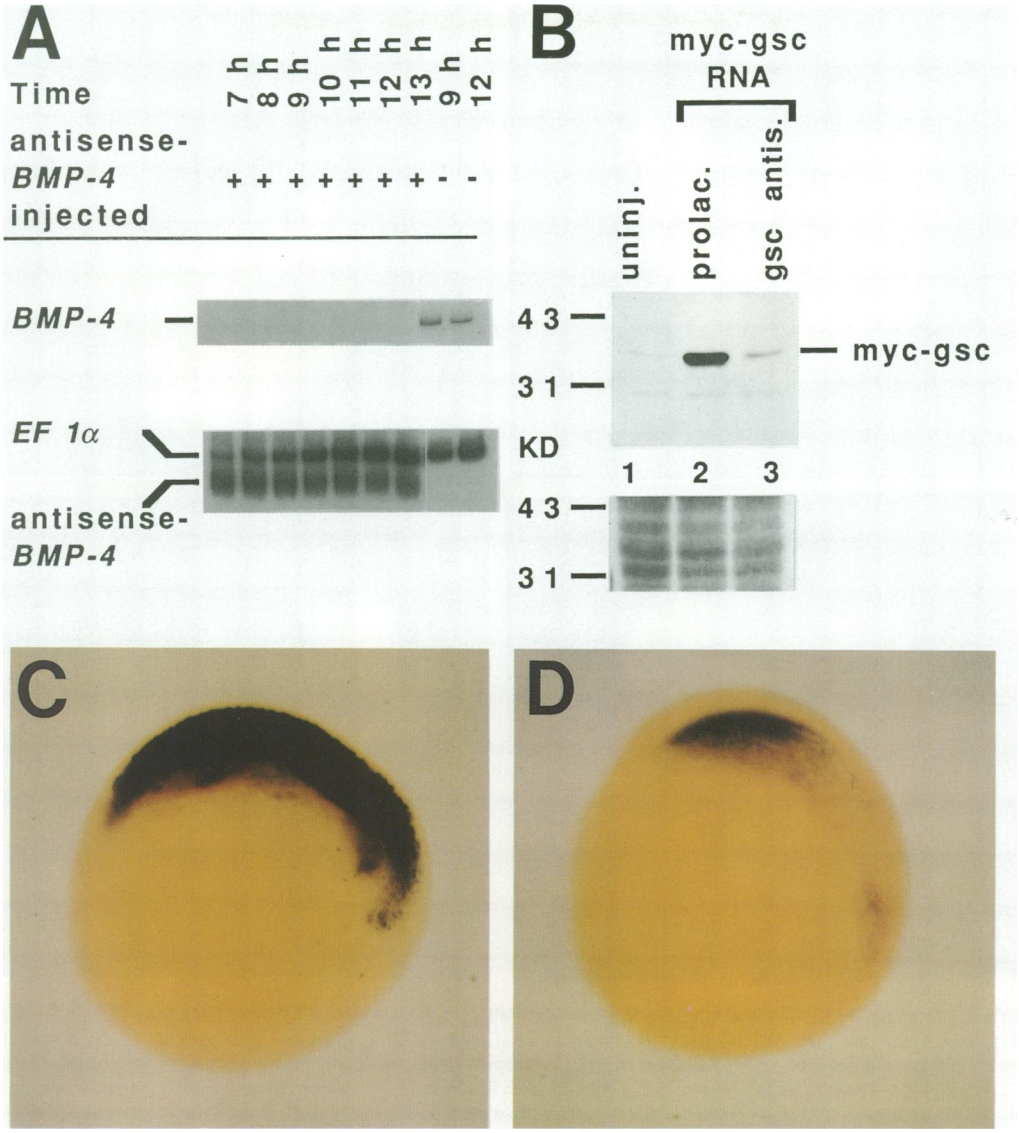

Fig. 2. Stability and distribution of microinjected antisense RNA or DNA until late gastrulation and inhibition translation of mRNA translation. (**A**) Injected *BMP-4* antisense RNA is still present by the late gastrula stage. Embryos injected radially with antisense *BMP-4* RNA were harvested at different times after fertilization and processed for RT-PCR with antisense *BMP-4*-specific primers. The antisense *BMP-4* injected RNA is still present after 13 h post-fertilization (late gastrula, stage 12.5). The elongation factor-1α (EF-1α) endogenous transcripts were used as internal controls. The last two lanes show RT-PCRs using control uninjected embryos to show the endogenous levels of *BMP-4* transcripts under the same PCR conditions. (**B**) Antisense *gsc* RNA interferes with the translation of myc–gsc protein. Embryos were injected into the animal pole region at the 4-cell stage with 6 pg *myc–gsc* mRNA in combination with 300 pg of prolactin or antisense *gsc* RNA. When these embryos reached the gastrula stage, the proteins were extracted, separated by SDS–PAGE, blotted on nitrocellulose and the myc–gsc protein was visualized using the anti-myc antibody 9E10 and the ECL detection system. Myc–gsc protein is absent in uninjected control embryos (lane 1) and reduced in samples injected with antisense *gsc* and sense *myc–gsc* RNA (lane 3) compared with embryos which had received prolactin (control) as well as *myc–gsc* RNA (lane 2). The nitrocellulose filter was subsequently stained with India ink to visualize the proteins; the region including the myc-tagged gsc protein is shown under the Western blot. Note that similar amounts of protein were loaded for all three samples. (**C**) Expression of the antisense *gsc* RNA produced by the injected CMV-antis-gsc plasmid. Embryo injected in one out of four blastomeres with the CMV-antis-gsc and analyzed by *in situ* hybridization with a *gsc* sense probe specific for the antisense RNA. Note the high level and uniform expression generated by the injected plasmid. (**D**) Endogenous *gsc* transcripts in CMV-antis-gsc-injected embryos. An embryo identically injected and treated as in (C) but probed with an antisense *gsc* probe to detect the endogenous *gsc* transcripts. Note that the endogenous *gsc* pattern of expression appears normal.

a reduction or loss of tail and trunk. To test whether *gsc* is required for this effect, we asked whether injection of antisense *gsc* RNA could rescue trunk and tail formation (Figure 3). *gsc* antisense RNA was delivered to two neighboring ventral blastomeres of 4-cell embryos (200 pg/blastomere) which were then treated with 120 mM LiCl at the 32-cell stage for 30 min. Control embryos treated with LiCl showed the typical dorsalized phenotype lacking tail structures, whereas embryos injected with *gsc* antisense RNA developed tail structures in 85% of the embryos (n = 33) in two independent experiments (Figure 3A and B). Tail formation was not observed in control embryos injected with β-*gal* or prolactin RNAs. This suggests that *gsc* is required for the dorsalizing effect of LiCl on *Xenopus* embryos.

To analyze the effects of antisense *BMP-4* RNA in axis formation, we used embryos irradiated with UV light, a treatment which abolishes the formation of axial structures

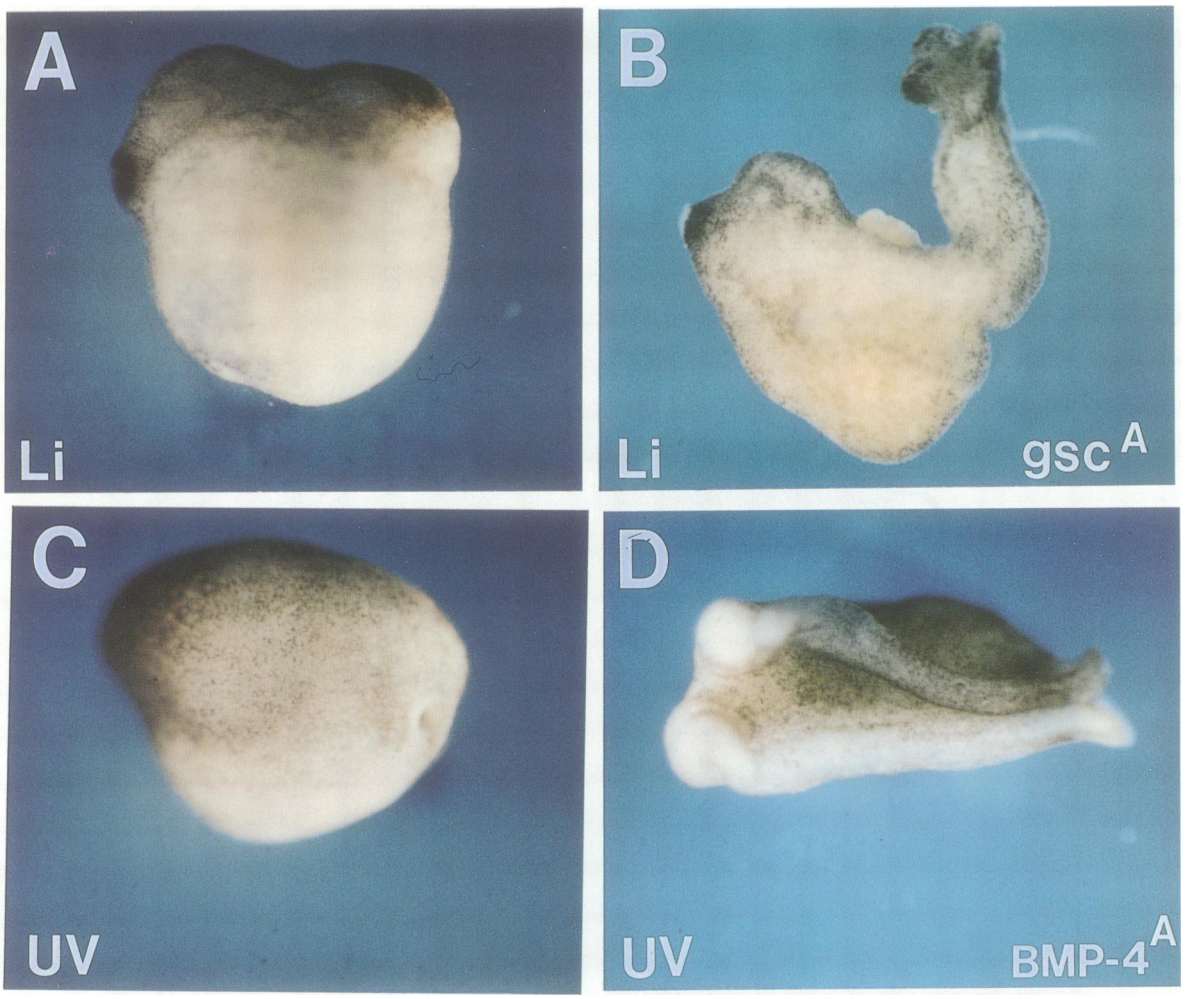

Fig. 3. Rescue of axial structures in LiCl- and UV-treated *Xenopus* embryos by antisense *gsc* and *BMP-4* RNA. (**A**) Embryo incubated in LiCl at the 32- to 64-cell stage for 25 min and cultured for 2 days. (**B**) Embryo dorsalized by LiCl as in (A) that was injected into two ventral blastomeres at the 4-cell stage with 200 pg of antisense *gsc* RNA/blastomere. These embryos developed partial tails which were not observed in uninjected samples treated with LiCl. (**C**) Embryo ventralized by UV irradiation 30 min after fertilization. (**D**) UV-irradiated embryos injected into two non-adjacent blastomeres at the 4-cell stage with 200 pg of antisense *BMP-4* RNA/blastomere and cultured for 2 days. Twinned axis structures, which extended anteriorly to the hindbrain level, seen in embryos injected with antisense *BMP-4* RNA but not in uninjected UV-irradiated controls or those injected with control prolactin RNA.

concomitantly with the repression of dorsalizing components and the premature expression of *BMP-4* (Fainsod *et al.*, 1994). We asked whether antisense *BMP-4* RNA was able to rescue axis formation in embryos ventralized by UV light (Figure 3C and D). *Xenopus* eggs were irradiated with UV for 60 s at 30 min after fertilization and two non-adjacent blastomeres were injected with antisense *BMP-4* RNA (400 pg/blastomere) at the 4-cell stage. These embryos developed two partial axes, lacking complete heads, with high frequency (42%, $n = 95$, three independent experiments), whereas uninjected or prolactin-injected UV-treated controls were strongly ventralized [dorso-anterior index (DAI) <1; Kao and Elinson, 1988]. The induction of twinned axes in the embryos injected with antisense *BMP-4* RNA excludes the possibility that these embryos escaped the UV treatment, in which case only one axis would develop. In addition, in the antisense *BMP-4*-injected embryos, 95% ($n = 95$) had at least one partial axis. This suggests that antisense *BMP-4* can partially rescue the UV phenotype with high efficiency and that *BMP-4* is required for the ventralization caused by UV in *Xenopus* embryos.

Having demonstrated clear phenotypic effects of antisense RNA for *gsc* and *BMP-4* in experimentally manipulated embryos (dorsalized or ventralized), we analyzed the phenotypic effects of loss of *gsc* or *BMP-4* function in the wild-type *Xenopus* embryo. Four-cell embryos were injected radially in the equatorial region with capped, full-length antisense *gsc* or antisense *BMP-4* RNA and cultured until stage 30. Various amounts of antisense RNA, ranging from 50 to 800 pg/embryo, were tested. Phenotypic effects were observed in embryos injected with >300 pg of antisense RNA/embryo. Eleven out of 15 embryos injected with 800 pg of antisense *gsc* RNA displayed moderate but consistent defects in the anterior head region and, in the most severe cases (two embryos), anterior head structures were missing (Figure 4B). Staining of the notochord with the monoclonal antibody MZ 15 showed that the notochord extended more anteriorly in 11 out of 15 embryos injected with *gsc* antisense RNA, perhaps as a result of a reduced amount of prechordal plate tissue (Figure 4E). In the two headless embryos, no notochord could be detected. The same amount of injected control prolactin mRNA (800 pg/embryo) caused neither the head

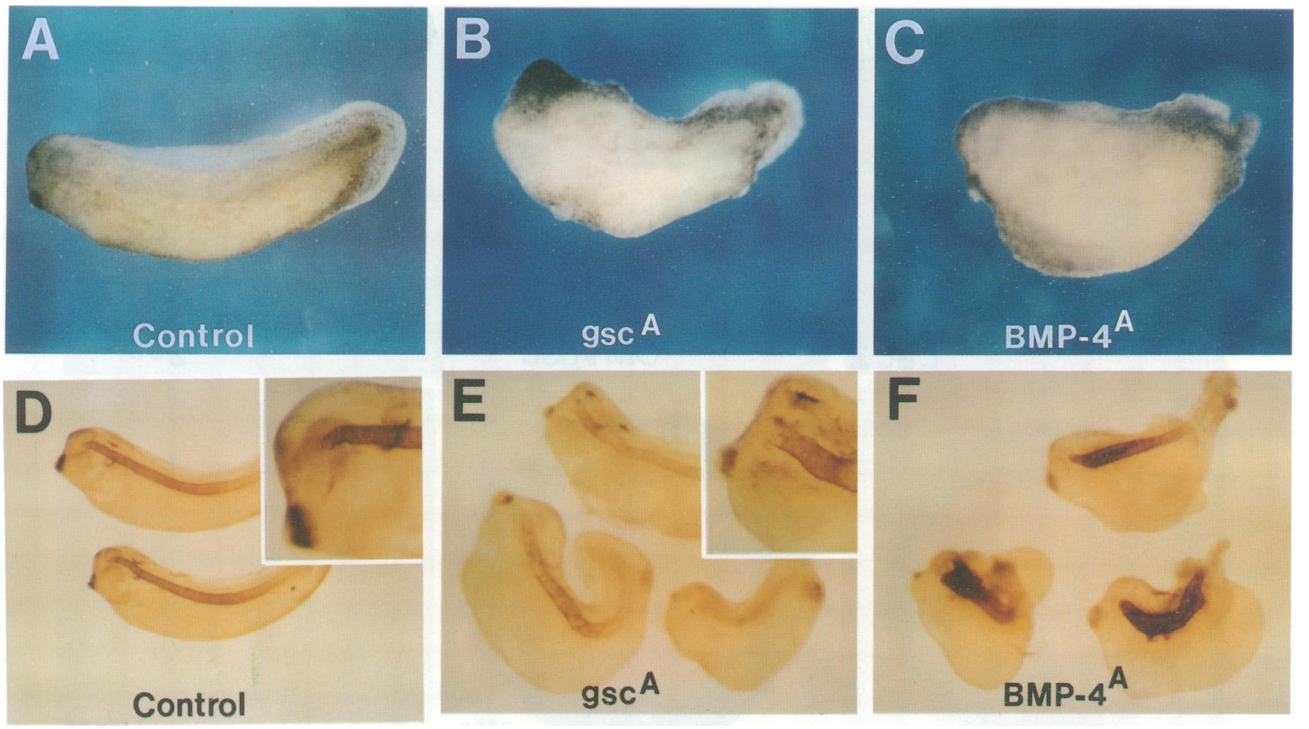

Fig. 4. Phenotypic effects of injected antisense *gsc* and *BMP-4* RNA in wild-type *Xenopus* embryos. (**A**) Uninjected control embryos at stage 30. Four-cell embryos were injected radially into the equatorial region with 800 pg of antisense *gsc* (**B** and **E**) or antisense *BMP-4* RNA (**C** and **F**) and cultured for 2 days. Embryos injected with antisense *gsc* RNA consistently showed defects in the anterior body region (B). When stained to detect notochord with the MZ 15 antibody, an abnormal notochord morphology was observed and the notochord extended more anteriorly (E). Antisense *BMP-4* RNA affected the trunk and tail portion which was shortened (C) compared with the uninjected controls, and the notochords were short and massive (F). (**D**) Notochord staining of uninjected control embryos. The percentages of embryos displaying these effects are listed in the main text.

nor the notochord phenotype (n = 12). The antisense *gsc* phenotype was also seen when the antisense RNA was delivered to the dorsal side of the embryo, but not in the case of ventral injections (not shown).

Injections of wild-type embryos with antisense *BMP-4*, either radially or diagonally, resulted in embryos with a shortened body axis (Figure 4C and F) resembling LiCl-treated embryos with a DAI between 7 and 8 (Kao and Elinson, 1988). In the case of radial injections, out of 26 surviving embryos, 20 (77%) exhibited the shortened axis phenotype. Diagonal injections into two blastomeres, as expected, resulted in a smaller percentage of affected embryos with the same phenotype (33%; n = 30). The injection of antisense *BMP-4* RNA did not cause the formation of secondary dorsal axes in the wild-type embryo.

The injection of antisense *gsc* affected anterior development of the wild-type embryo (Figure 4B and E) and the phenotype caused by antisense *BMP-4* showed dorsalization and impaired posterior development (Figure 4C and F). Therefore, the phenotypes of antisense *gsc* and *BMP-4* in wild-type embryos are consistent with those observed in the LiCl- and UV-treated embryos (Figure 3). However, in wild-type embryos, the phenotypic effects are not as strong as those observed in the sensitized assays provided by LiCl and UV treatment, in which the formation of new structures (tails and secondary axes) can be used to score the requirement for *gsc* and *BMP-4* in the dorsalizing and ventralizing pathways.

Repression of Xwnt-8 expression in the dorsal marginal zone requires gsc activity

We next asked whether the phenotypic defects observed after antisense *gsc* injection were reflected in an altered expression pattern of early marker genes at the gastrula stage. We chose as a marker *Xwnt-8*, a gene expressed in the ventral and lateral marginal zone of the *Xenopus* gastrula (Figure 5A), because it is known that its expression can be inhibited by injected *gsc* mRNA (Christian and Moon, 1993 and Figure 5F). Antisense *gsc* RNA (150 pg/blastomere) or a CMV plasmid (50 pg/blastomere) which transcribes antisense *gsc* RNA after the mid-blastula transition, were injected radially into wild-type embryos and the expression pattern of *Xwnt-8* was detected by *in situ* hybridization at the early gastrula stage. The *Xwnt-8* region of expression was expanded on the dorsal side in 21 out of 32 injected embryos (65%). In an additional four cases, *Xwnt-8* had an almost ring-shaped expression pattern in the marginal zone (12.5%; Figure 5B and C). The expansion of *Xwnt-8* expression was also seen when a truncated antisense *gsc* RNA lacking the homeobox (Cho *et al.*, 1991) was injected (800 pg/embryo).

Dorsalization of embryos with LiCl abolishes the expression of *Xwnt-8* in the marginal zone (Smith and Harland, 1992; Figure 5D). *Xwnt-8* expression can be restored in LiCl-treated embryos by injection of antisense *gsc* RNA (in 14 out of 19 injected embryos) (Figure 5E). This result was confirmed by quantitative RT-PCR (Figure 6A). Importantly, the induction of *Xwnt-8* expression by antisense *gsc* RNA can be reversed in a dose-dependent

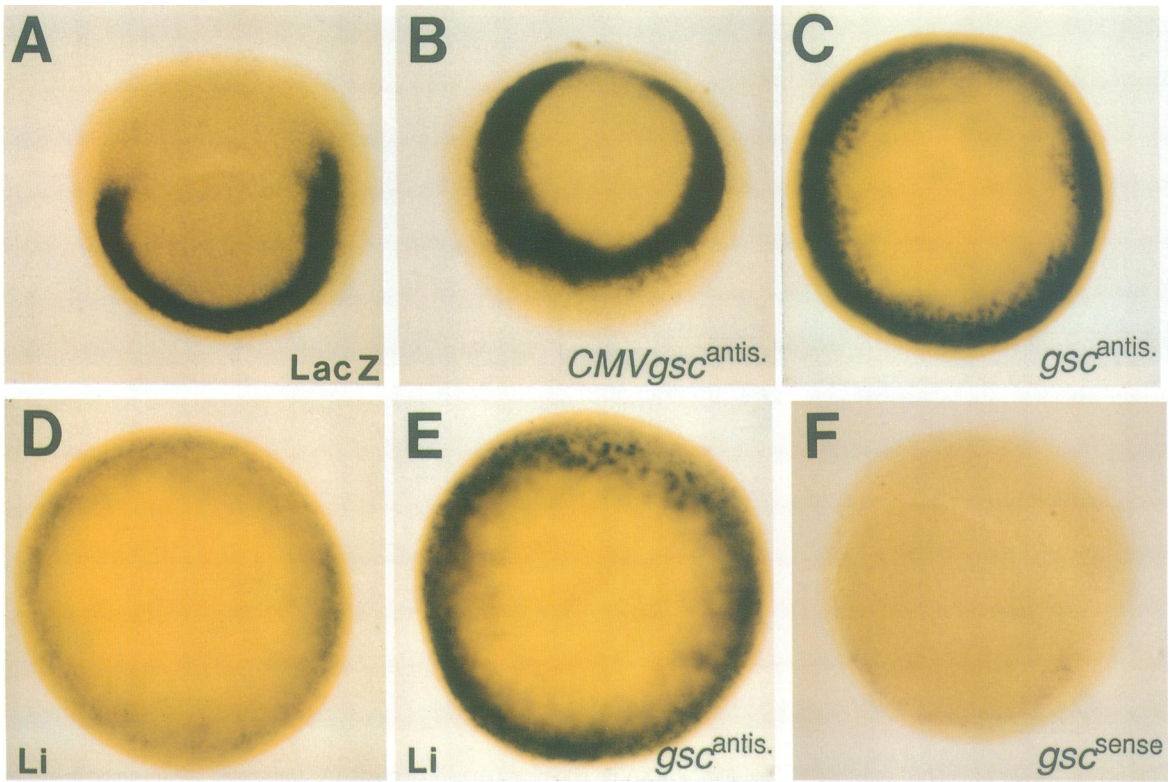

Fig. 5. Antisense *gsc* RNA changes the expression pattern of *Xwnt-8*. Wild-type embryos were injected radially at the 4-cell stage with (**A**) 600 pg of β-*gal* RNA, (**B**) 200 pg of CMV-antis-gsc plasmid, (**C**) 600 pg of antisense *gsc* RNA or (**F**) 160 pg of sense *gsc* RNA. Embryos dorsalized with LiCl were injected radially at the 4-cell stage with (**D**) 600 pg of β-*gal* RNA, or (**E**) 600 pg of antisense *gsc* RNA. At stage 11, the embryos were fixed and a whole mount *in situ* hybridization was performed to detect *Xwnt-8* transcripts. β-*gal* RNA did not change the expression pattern of *Xwnt-8*, which is restricted to the lateral and ventral marginal zone. CMV-antis-gsc plasmid and antisense *gsc* RNA expand *Xwnt-8* expression to the dorsal marginal zone. Sense *gsc* RNA represses *Xwnt-8* expression as does treatment with LiCl. Note that in LiCl-treated embryos, *Xwnt-8* expression is restored after injection of antisense *gsc* RNA.

manner by injection of *gsc* sense RNA (Figure 6A, lanes 4 and 5). We conclude from these results that the homeobox gene *gsc* is required for the dorsalizing effect of LiCl in *Xenopus* embryos and that this effect is apparent by early gastrula stages.

Antisense gsc RNA ventralizes mesodermal tissue

gsc has a dorsalizing effect on mesoderm which is reflected by an enhanced expression of this gene in embryos dorsalized by LiCl (Cho *et al.*, 1991; Fainsod *et al.*, 1994). Since antisense *gsc* RNA is able to partially rescue tail formation in dorsalized embryos (Figure 3B) and can induce the ventro-lateral marker *Xwnt-8* (Figure 5E), we attempted to characterize this ventralizing activity of antisense *gsc* RNA at a molecular level using marker genes for dorsal and ventral tissue types. Embryos dorsalized with LiCl and radially injected with antisense *gsc* RNA were harvested, and the expression pattern of α-actin, α-globin and UVS2 was analyzed by RT-PCR at stage 22 (Figure 6B). Expression of α-actin, a marker for embryonic muscle, is reduced in dorsalized embryos due to the lack of trunks and tails, as depicted diagramatically in Figure 6C. The amount of α-actin mRNA expressed in LiCl-dorsalized embryos injected with antisense *gsc* RNA is increased to wild-type levels (Figure 6B, lanes 4 and 5). UVS2, a hatching gland marker, is inhibited by high levels of *gsc* (Niehrs *et al.*, 1994), a result also found in LiCl-treated embryos (Figure 6B, lanes 1 and 2). Injected antisense *gsc* RNA strongly induces the expression of UVS2 (Figure 6B, lanes 4 and 5). Globin, a marker for blood, a tissue derived from ventral mesoderm, is repressed by dorsalizing treatments and is unaffected by injected *gsc* antisense RNA in this assay system, in agreement with only a partial rescue of the LiCl-dorsalizing effect (see diagram in Figure 6C). As a control for RNA loading, the histone H4 (H4) gene was used as an internal standard in this quantitative RT-PCR assay (Niehrs *et al.*, 1994). As controls for non-specific ventralization by RNA injection, embryos were injected with either β-*gal* or antisense *XlHbox-1* RNA, neither of which had any effect on the markers analyzed (Figure 6B, lanes 2 and 3). From this analysis of marker genes, we conclude that antisense *gsc* shifts tissue differentiation in dorsalized embryos towards a more ventral fate.

Antisense BMP-4 RNA induces the expression of gsc

We next asked whether the injection of antisense *BMP-4* RNA would induce the expression of *gsc*, a marker of dorsal mesoderm. Figure 7 shows an experiment in which wild-type and UV-irradiated embryos were injected with antisense *BMP-4* RNA or DNA expression constructs and analyzed by *in situ* hybridization for the expression of *gsc* at the gastrula stage. Embryos were injected with either antisense *BMP-4* RNA or a DNA construct in which the CMV promoter drives antisense *BMP-4* transcripts after the mid-blastula transition. As negative controls, prolactin RNA and a CMV–β-gal construct were used. Microinjec-

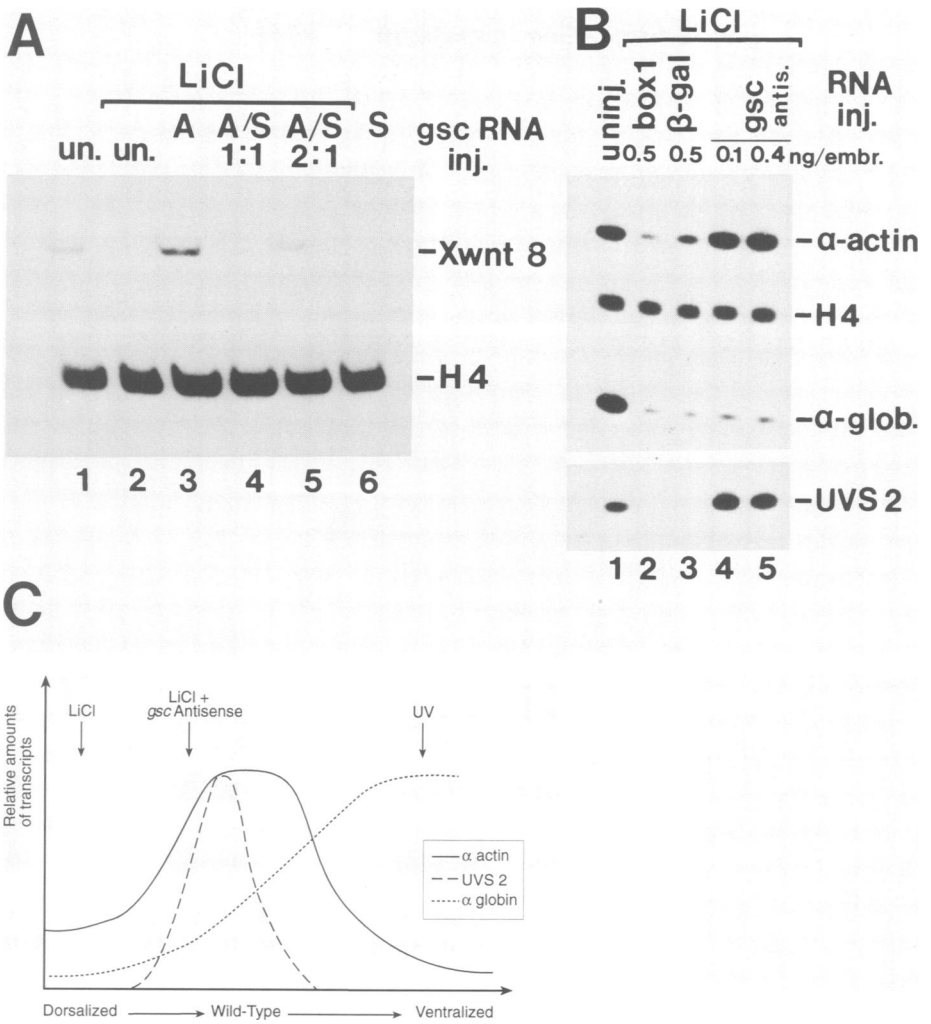

Fig. 6. Quantitative RT-PCR analysis of marker gene expression in LiCl-treated embryos injected with antisense *gsc* RNA. RNA from LiCl-treated embryos which were injected radially with antisense *gsc* RNA was analyzed by RT-PCR at stage 11 for *Xwnt-8* (A) and at stage 22 for α-actin, α-globin, UVS2 and histone H4 (B). (**A**) *Xwnt-8* mRNA is expressed in uninjected stage 11 embryos, (lane 1) and repressed in LiCl-treated embryos (lane 2) as well as in those injected with 200 pg of sense *gsc* RNA (lane 6). *Xwnt-8* expression was restored in LiCl-treated embryos when 400 pg of antisense *gsc* RNA was injected (radially into four blastomeres at the 4-cell stage) (lane 3) but was counteracted by injecting radially 400 pg (lane 4) or 200 pg of sense *gsc* RNA (lane 5). (**B**) In stage 22 embryos treated with LiCl and injected with control RNAs (antisense *XlHbox-1*, lane 2; β-*gal*, lane 3), α-actin, α-globin and UVS2 expression is repressed in comparison with the untreated controls (lane 1). LiCl-treated samples injected with antisense *gsc* RNA show increased levels of α-actin and UVS2 expression, indicating a ventralization of the embryos (lanes 4 and 5). As an internal standard, histone H4 was used. (**C**) Diagram of the expected expression pattern of α-actin, UVS2 and α-globin in wild-type, dorsalized and ventralized embryos. The shift towards a more dorsal condition caused by injecting antisense *gsc* RNA into LiCl-treated embryos is indicated.

tion of sense *BMP-4* RNA radially in the embryos results in an inhibition of *gsc* expression (Figure 7G). Microinjection of either antisense *BMP-4* RNA or the DNA antisense-expressing construct has the opposite effect, leading to an expansion of the area of *gsc* expression, or to ectopic patches of the *gsc* expression in the marginal zone (Figure 7B and C). Despite the radial injections in this experiments, circular expression of *gsc* was observed in only one embryo. In the ventral marginal zone where the concentration of BMP-4 is maximal, no induction of *gsc* expression was generally seen. It appears that the antisense RNA is unable to inhibit BMP-4 synthesis below a level which would permit *gsc* induction in the ventral region. In UV-treated embryos, the expression of endogenous *gsc* transcripts is greatly decreased (Figure 7D), but is restored by injection of *BMP-4* antisense RNA or the DNA construct (Figure 7E and F), consistent with the dorsalizing effect of antisense *BMP-4*. Antisense RNA injection resulted in the activation of *gsc* expression in 66% of the embryos (n = 21). The antisense *BMP-4*-expressing construct restored *gsc* expression in 72% of the injected embryos (n = 18). Once again, expression is maximal on one side, presumably the side corresponding to the original organizer region

Quantitative RT-PCR corroborates the results of the *in situ* hybridization studies. Embryos which were radially injected with antisense *BMP-4* RNA showed enhanced *gsc* expression levels compared with the uninjected controls (Figure 7H and I). In contrast, sense *BMP-4* RNA reduces the amount of *gsc* transcripts dramatically in 9 and 10 h old embryos; however, this inhibition is not detectable in the 8 h samples (Figure 7J). This is in agreement with results of Hogan *et al.* (1994), who reported essentially normal *gsc* mRNA levels in late blastula embryos injected

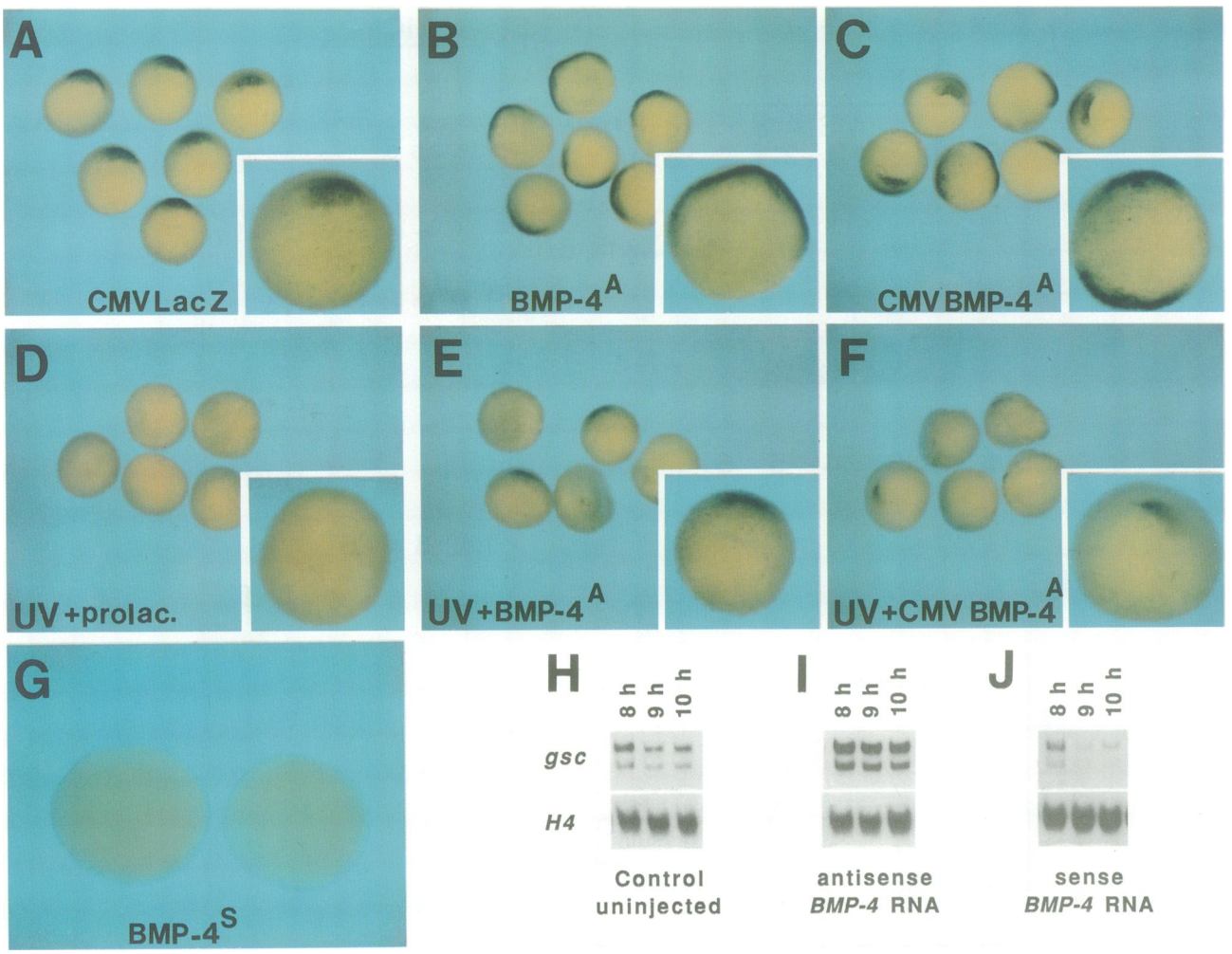

Fig. 7. *gsc* expression is induced by antisense *BMP-4* RNA. Wild-type embryos were injected at the 4-cell stage (four radial injections) with (**A**) 160 pg of pCMV β-gal, (**B**) 600 pg of antisense *BMP-4* RNA, (**C**) 200 pg of pCMV-antis-BMP-4 DNA or (**G**) 200 pg of sense *BMP-4* RNA. Embryos ventralized by UV irradiation were injected at the 4-cell stage (four radial injections) with 600 pg of prolactin RNA (**D**), 600 pg of antisense *BMP-4* RNA (**E**) or 200 pg of pCMV-antis-BMP-4 (**F**). At stage 10.5, embryos were fixed and probed for *gsc* mRNA by the whole mount *in situ* hybridization procedure. UV irradiation and sense *BMP-4* RNA injection abolish *gsc* expression (D and G). Antisense *BMP-4 RNA* (B and E) and pCMV-antis-BMP-4 (C and F) induce ectopic *gsc* mRNA expression in wild-type and UV-treated embryos. (**H**) RT-PCR analysis of RNA for *gsc* and H4 from uninjected embryos, or injected with 600 pg of antisense *BMP-4 RNA* (**I**), or 200 pg of sense *BMP-4 RNA* (**J**) and isolated 8, 9 or 10 h after fertilization. Injected antisense *BMP-4* RNA causes increased *gsc* expression (I) and sense *BMP-4* RNA inhibits *gsc* expression 9 and 10 h after fertilization (J) when compared with the uninjected controls (H). Histone H4 was used as an internal standard.

with sense *BMP-4* RNA, and supports the idea that *BMP-4* executes its ventralizing function after the initiation of gastrulation (Jones *et al.*, 1992; Fainsod *et al.*, 1994). We conclude that antisense *BMP-4* RNA has an opposite effect to that of its sense counterpart, leading to the activation of the dorsal marker *gsc*.

Antisense BMP-4 RNA dorsalizes ventral marginal zone explants

BMP-4 is expressed in the ventral marginal zone of the *Xenopus* gastrula, which will develop into blood and other ventral mesodermal derivatives when explanted. In addition, *BMP-4* is sufficient to induce ventral development (Köster *et al.*, 1991; Dale *et al.*, 1992; Jones *et al.*, 1992). To test whether *BMP-4* is required to maintain ventral differentiation, we analyzed its effect on VMZ explants (Figure 8A). VMZs from embryos injected with antisense *BMP-4* RNA were explanted at stage 10.5 and cultured for 2 days. When the explants were analyzed for muscle development using the monoclonal antibody 12/101, blocks of muscle tissue were detected (Figure 8D). No notochord tissue was detected using the antibody MZ 15 as a marker (data not shown). These VMZ explants were morphologically indistinguishable from uninjected lateral marginal zone explants of the same size which also develop muscle tissue (Figure 8B). In our experiments, the lateral marginal zone explants contain ~90° of the marginal zone and develop muscle with high frequency (smaller lateral fragments from stage 10.5 embryos differentiate into ventral mesoderm). VMZs normally differentiate into ventral mesoderm and, as expected in uninjected VMZ explants or those injected with control prolactin RNA, no muscle was formed (Figure 8C).

The dorsalization of VMZ explants was confirmed at a molecular level by the expression pattern of a panel of marker genes for dorsal and ventral tissue types (Niehrs *et al.*, 1994). As a further control for specificity, a synthetic antisense *BMP-4* RNA lacking the carboxy-terminal

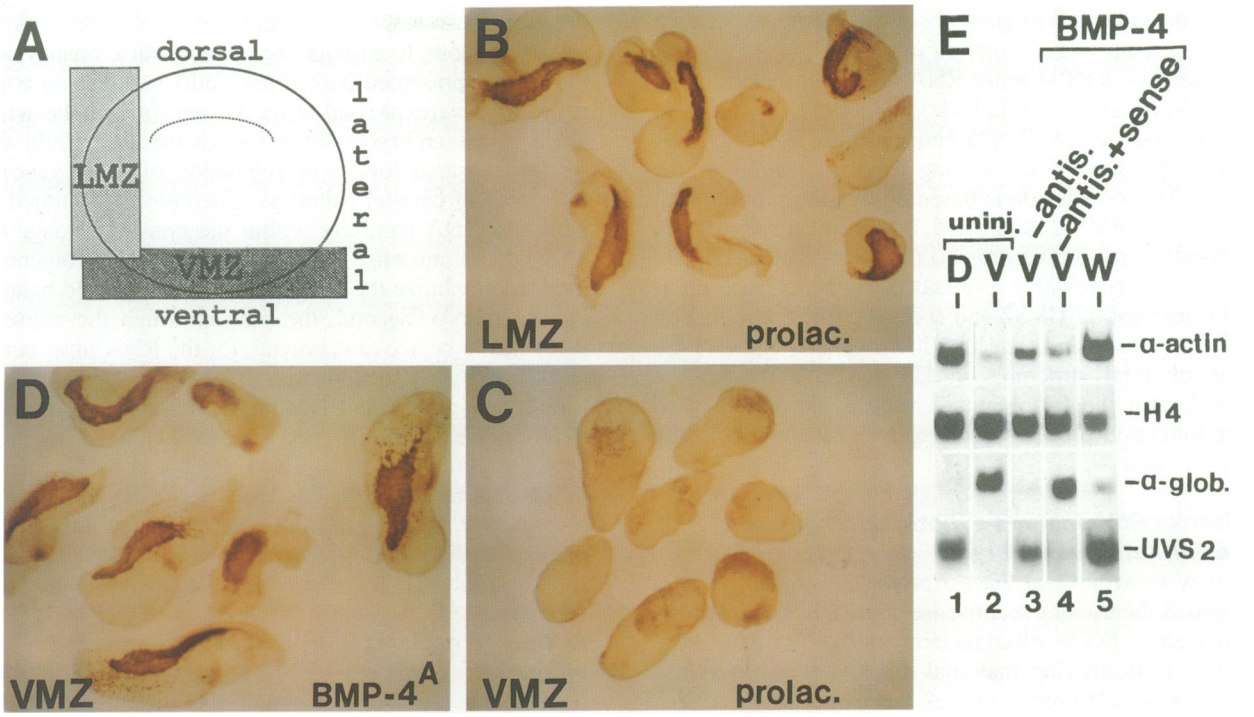

Fig. 8. Antisense *BMP-4* RNA dorsalizes ventral marginal zone tissue. Embryos were injected at the 4-cell stage with 400 pg of prolactin RNA or 400 pg of antisense *BMP-4* RNA in the ventral or lateral region of the embryo. (**A**) At stage 10, ventral (VMZ) and lateral marginal zones (LMZ) were explanted, cultured for 2 days and stained for muscle tissue using the 12/101 antibody. (**B**) LMZ injected with prolactin RNA developed muscle (12, n = 18) as uninjected LMZ explants do (11, n = 15; not shown). (**C**) VMZ explants injected with prolactin RNA developed no muscle tissue (n = 17; in two embryos a few isolated positive cells were seen). (**D**) Large blocks of muscle were found in VMZs that had received antisense *BMP-4* RNA (21, n = 26). (**E**) Quantitative RT-PCR assay of RNA from dorsal or ventral marginal zones at stage 22 for α-actin, α-globin, UVS2 and H4. Dorsal marginal zone explants (lane 1) express strongly the dorso-anterior markers α-actin and UVS2, whereas VMZs (lane 2) preferentially express the blood marker α-globin. In VMZs injected with 400 pg of antisense *BMP-4* RNA, expression of α-actin and UVS2 is elevated and α-globin expression is decreased (lane 3). Induction of α-actin and UVS2 can be reversed by injection of 20 pg of the pCSK-BMP-4 DNA construct in addition to the antisense *BMP-4* RNA (lane 4); α-globin expression in those explants is increased. RNA from whole embryos (lane 5). H4 was used as an internal standard for this quantitative RT-PCR analysis.

sequence including the portion encoding the mature growth factor (see Materials and methods), was injected. The mature regions of transforming growth factor-βs (TGF-βs) tend to be conserved, in particular BMP-2 is 92% conserved at the amino acid level with BMP-4 (Nishimatsu *et al.*, 1992). The *Xenopus* embryo does contain zygotic *BMP-2* transcripts at the gastrula stage, but these transcripts are located on the dorsal side of the embryo and not in the VMZ (H.S.Bin Lu, Y.S. and E.D.R., unpublished observations).

In molecular analyses, the truncated antisense *BMP-4*, but not *BMP-2*, RNA repressed the expression of the ventral blood tissue marker α-globin while it induced the dorso-anterior marker UVS2 as well as α-actin in VMZ explants (Figure 8E, lanes 2 and 3). Importantly, the dorsalizing effect of antisense *BMP-4* RNA could be rescued by the injection of a sense DNA construct which produces *BMP-4* mRNA under the control of a cytoskeletal actin (*CSKA*) promoter (Harland and Misher, 1988). In VMZ explants that had received both the antisense *BMP-4* RNA and the sense *CSKA–BMP-4* DNA construct, α-actin and UVS2 expression was strongly reduced, whereas α-globin was increased to levels close to those of control VMZs (Figure 8D, lane 4).

We conclude from these experiments that *BMP-4* is required for the formation of ventral mesoderm. Antisense *BMP-4* has a dorsalizing effect that can be counteracted by sense *BMP-4*. These observations extend the results of Graff *et al.* (1994) and Suzuki *et al.* (1994) who reported that a dominant negative receptor mutant that binds BMP-2 and BMP-4, promotes dorsal development in *Xenopus* by supporting the notion that indeed BMP-4, and not a related growth factor, is the ventralizing signal affected by the mutant receptor.

Discussion

Patterning of the marginal zone during gastrulation in the *Xenopus* embryo has been studied extensively by gain-of-function approaches. This effort has led to the identification of a number of genes which can affect this developmental process. Loss-of-function experiments are needed to determine whether a gene is required for a postulated function during embryonic development. In previous studies, we have shown the effect of *gsc* and *BMP-4* on mesodermal patterning. These studies have now been extended by taking advantage of partial loss-of-function by antisense RNA injection. While *gsc* sense RNA is a dorsalizing agent (Niehrs *et al.*, 1994), *gsc* antisense RNA has ventralizing effects on the *Xenopus* marginal zone. This conclusion is based on (i) the induction of *Xwnt-8*, a marker for latero-ventral mesoderm, in the dorsal marginal zone, (ii) the shift in the expression of marker genes in dorsalized embryos to a more ventral tissue pattern and (iii) the phenotypic rescue of tail structures in embryos treated with LiCl. We conclude from these experiments

that the dorsalization process observed in embryos treated with LiCl requires *gsc* function *in vivo*.

Conversely, *BMP-4* sense RNA is a potent ventralizing agent (Köster *et al.*, 1991; Dale *et al.*, 1992; Jones *et al.*, 1992; Fainsod *et al.*, 1994) and antisense *BMP-4* RNA has the opposite effect, leading to dorsalization of the embryo. This conclusion is based on the observations that (i) antisense *BMP-4* RNA and DNA constructs lead to increased expression of *gsc*, (ii) the shift of other dorsal–ventral markers towards a dorsal pattern (decrease in α-globin, increase in UVS2 and α-actin), (iii) the induction of blocks of muscle tissue in VMZ explants and (iv) the rescue of axial structures in UV-treated embryos. We conclude that the formation of ventral mesoderm is an active induction process requiring *BMP-4* function in the *Xenopus* embryo.

Antisense RNA inhibits zygotically expressed genes in Xenopus embryos

One way to achieve at least a partial loss-of-function in *Xenopus* is the use of the antisense approach. The injection of antisense DNA oligonucleotides has proven very effective in destroying maternal mRNAs (via the RNase H pathway) in *Xenopus* oocytes (Heasman *et al.*, 1994). Despite some cases in which the antisense RNA approach has been successful (Giebelhaus *et al.*, 1988; Schmid *et al.*, 1991), this method is complicated by the presence of the double-stranded RNA deaminase in *Xenopus* eggs and early embryos. This enzyme unwinds RNA duplexes and converts adenosine residues into inosine (Bass and Weintraub, 1987; Rebagliati and Melton, 1987; Kim and Nishikura, 1993). This unwinding activity makes the use of antisense RNA to prevent protein synthesis targeted against maternal mRNAs difficult, because it inhibits RNA duplex formation between the mRNA and the antisense RNA in eggs and pre-mid-blastula embryos. On the other hand, the adenosine/inosine substitution changes the coding capacity of the mRNA, and can result in the synthesis of inactive protein which could increase the effect of the antisense technique (Kimelman and Kirschner, 1989; Woolf, 1992).

Our experiments using an injected β-*gal* gene which becomes transcriptionally active after the mid-blastula transition showed that β-galactosidase enzyme activity can be decreased by antisense β-*gal* RNA (Figure 1). This result implies that antisense RNA can be used to partially inhibit zygotic genes in *Xenopus*. The decrease in double-stranded RNA deaminase activity after mid-blastula (Rebagliati and Melton, 1987) makes this method possible. However, these experiments do not reveal the mechanism of the inhibition, which could be due to RNA degradation or to a block of protein synthesis. Experimental evidence that antisense RNA interferes with the translation of mRNAs has been described in other systems and in *Xenopus* as well (Liebhaber *et al.*, 1992). We found that injected antisense *gsc* RNA in *Xenopus* embryos did not lead to *gsc* mRNA degradation, but that the amount of myc-tagged gsc protein was significantly reduced when sense *myc–gsc* RNA and antisense *gsc* RNA were co-injected (Figure 2B). This suggests that the antisense RNA interferes with the translation step.

Previous reports showed no effect for antisense *BMP-4* RNA injection in *Xenopus* (Dale *et al.*, 1992) and indeed used antisense RNA as a negative control. There may be several reasons to explain the apparent discrepancy with the results presented here. First, unlike studies in which genes are overexpressed at the wrong time or the wrong place in the embryo, partial loss-of-function could well be compensated for by up-regulation of other genes or activation of parallel pathways. Therefore, we titrated the antisense RNA carefully within the non-toxic range and injected the antisense RNA into multiple blastomeres in order to maximize the distribution of the injected material in the embryo. Second, the time at which the antisense RNA has to be active is crucial, for the RNA must persist until activity of the unwinding enzyme decreases. The stability of antisense RNA might differ among various RNA species, and we used capped RNA, which might have a stabilizing effect. Third, the assay system has to be chosen carefully. In our case, early markers such as *Xwnt-8, BMP-4* and *gsc* were appropriate and informative markers at the mid-gastrula stage, whereas morphological studies at later stages were less informative.

Antisense gsc and BMP-4 RNA have opposite effects

As the quantitation of endogenous gsc and BMP-4 proteins is not possible due to the lack of specific antibodies, we tested the specificity of the effects caused by *gsc* and *BMP-4* antisense RNA in three ways. First, we over-expressed the myc-tagged gsc protein and showed a quantitative decrease in its expression following antisense *gsc* injection. Second, we asked whether the presence of antisense RNA could have a non-specific effect. By using antisense RNAs of the two antagonizing molecules, *gsc* and *BMP-4*, we were able to show opposite effects in embryos. As discussed below, antisense *gsc* and *BMP-4* RNAs showed ventralizing and dorsalizing effects respectively, which are the opposite of the effects of their respective sense counterparts. This fact strongly supports the view that the effects of antisense *gsc* and *BMP-4* are specific. Third, we tested if the effect of antisense *gsc* and *BMP-4* RNA could be reversed specifically by exogenous *gsc* or *BMP-4* gene products respectively. Microinjection of *gsc* and *BMP-4* mRNA rescued the effects of the antisense RNAs.

Antisense *gsc* and antisense *BMP-4* behave as ventralizing and dorsalizing agents, respectively. They cause specific phenotypic changes in wild-type as well as in LiCl- or UV-treated embryos (Figures 3 and 4). The effects of antisense *gsc* and *BMP-4* RNA become even more apparent when marker genes for the dorsal and ventral marginal zone are analyzed at the gastrula stage. *gsc* sense RNA inhibits the expression of *Xwnt-8*, a marker gene for the lateral and ventral marginal zone (Christian and Moon, 1993). In contrast, antisense *gsc* RNA expands *Xwnt-8* expression to the dorsal side in wild-type embryos and restores *Xwnt-8* expression in LiCl-treated embryos (Figures 5 and 6). In wild-type embryos at the tail bud stage, the phenotypic effects of antisense *gsc* are modest (Figure 4), and presumably the embryo is able to regulate the changes seen at the gastrula stage. This situation is not unlike that seen in the mouse in which knockout of *gsc* has modest phenotypic defects in the head region (Yamada *et al.*, 1995; Rivera-Perez *et al.*, 1995; our unpublished observations).

In the complementary experiment, antisense *BMP-4* RNA induces ectopic expression of *gsc* and other dorsal marker genes and promotes the development of dorsal structures, while sense *BMP-4* RNA represses the expression of dorsal-specific genes (Fainsod *et al.*, 1994; von Dassow *et al.*, 1993). In the mouse, a knockout of *BMP-4* inhibits the formation of posterior and ventral mesoderm (Hogan *et al.*, 1994), a phenotype comparable with that observed in antisense *BMP-4*-injected embryos (Figures 3 and 4).

In VMZ explants, antisense *BMP-4* RNA led to the formation of muscle, a more dorsal tissue (Figure 8). Similar dorsalizing effects have been reported recently using a dominant negative receptor that binds BMP-2 and BMP-4 (Graff *et al.*, 1994; Harland, 1994; Maéno *et al.*, 1994; Suzuki *et al.*, 1994; Schmidt *et al.*, 1995). There are some differences, however, between these two experiments. First, the dominant negative receptor is able to induce secondary axes in wild-type embryos (Suzuki *et al.*, 1994), while antisense *BMP-4* does not. This could be due to the partial nature of the inhibition by antisense RNA. Such a partial inhibition may be due to uneven distribution of the injected material and could be one reason that Dale *et al.* (1992) did not observe a dorsalizing effect of antisense *BMP-4* RNA in induced animal cap explants. In addition, recently we have been able to apply the antisense method also to the study of the early patterning of *Xenopus* ectoderm, showing an anti-neurogenic activity of BMP-4 (Sasai *et al.* 1995), and that BMP-4 is required in the animal cap to repress neural differentiation. In UV-treated embryos and VMZ explants, the dominant negative BMP-2/4 receptor and antisense *BMP-4* RNA have rather similar effects, rescuing secondary axes that lack notochords (Graff *et al.*, 1994). Second, the antisense approach affects signaling by a single gene product, BMP-4, while the dominant negative receptor blocks a signaling pathway that may involve BMP-4, BMP-2 and perhaps other growth factors of the TGF-β family. The recent finding that a dominant negative activin receptor can also block signaling by the Vg1 growth factor (Schulte-Merker *et al.*, 1994) provides an example of how unexpected interactions might affect dominant negative studies. Thus, our experiments on the dorsalizing activity of antisense *BMP-4* extend the observations of Graff *et al.* (1994) and Suzuki *et al.* (1994), implicating BMP-4 itself as one of the growth factors required for ventral differentiation in the *Xenopus* marginal zone.

Material and methods

In situ hybridization and immunohistochemistry

Digoxigenin-labeled antisense *Xwnt-8* and *gsc* probes were prepared from full-length cDNA clones in Bluescript plasmid linearized with *Xho*I (*Xwnt-8*) and *Eco*RI (*gsc*) and transcribed with T7 RNA polymerase (Blumberg *et al.*, 1991; Christian *et al.*, 1991), using a commercially available nucleotide mix according to the manufacturer's instructions (Boehringer, Mannheim). Whole-mount *in situ* hybridization was performed according to the method of Harland (1991), with the modification that for the detection of *gsc* RNA the alkaline phosphatase staining was performed with the BM purple substrate (Boehringer), which gives very low background in *Xenopus*. Embryos were cleared in benzyl alcohol:benzyl benzoate (1:2). Notochord was detected using the mouse monoclonal antibody MZ 15 at a dilution of 1:1000 (Smith and Watt, 1985). Muscle tissue in marginal zone explants, excised at stage 10.5 and cultured for 2 days, was stained as described by Dent *et al.* (1989) using the monoclonal antibody 12/101 at a dilution of 1:2000 (Kintner and Brockes, 1984) and an anti-mouse–horseradish peroxidase (HRP) secondary antibody.

β-Galactosidase activity was detected in embryos injected with sense β-*gal* RNA from pCDM8-β-gal linearized with *Pst*I using T7 RNA polymerase (Sasai *et al.*, 1992) or with the pCH 101 plasmid (Pharmacia) as described by Sanes *et al.* (1986). To reduce non-specific background, staining was performed at pH 6.8.

Detection of myc-tagged gsc protein

A double-stranded oligonucleotide encoding the *myc* tag was introduced into the *Nco*I site of pSP-gsc (Niehrs *et al.*, 1994) placing the myc peptide (EQKLISEEDL) at the N-terminus of the protein. Capped synthetic *myc–gsc* mRNA was injected into embryos at the 4-cell stage. The embryos were homogenized in 100 mM Tris–HCl pH 7.5 at stage 10.5, and the yolk removed by centrifugation at 10 000 *g*. The proteins were separated on SDS–polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose filters (Schleicher and Schuell) using the semi-dry blotting method (Kyhse-Anderson, 1984) and the MultiphorII apparatus (Pharmacia). Myc-tagged gsc protein was visualized using mouse monoclonal anti-myc antibody (9E10; Santa Cruz Biotechnology) at a dilution of 1:1000 and the enhanced chemiluminescence detection system (ECL, Amersham) according to the manufacturer's instructions. The proteins on the nitrocellulose filter were stained with Indian Black ink.

RT-PCR

RNA from embryos or marginal zone explants was prepared by the phenol–guanidinium thiocyanate method (Chomczynski and Sacchi, 1987) using the RNA STAT-60 reagent (Tel-Test 'B'). Quantitative RT-PCR was performed as described by Niehrs *et al.* (1994). For the detection of *gsc*, *BMP-4*, antisense *BMP-4*, *Xwnt-8*, histone H4, α-actin, α-globin and UVS2 mRNA, the following primers were used. *gsc*: 5′-GGAGAGGACTGCAGTCTGCATG and 5′-TCTAGAGTCTGACTCAACTCTGCAGCTCAGTCCTGGA (590 bp, 33 cycles). *Xwnt-8*: 5′-GCAGGCACTCTCGTCCCTCTGT and 5′-TATCTGGAAGTTGCAGCATACA (270 bp, 26 cycles). *BMP-4*: 5′-GCATGTAAGGATAAGTCGATC and 5′-GATCTCAGACTCAACGGCAC (478 bp, 26 cycles). Antisense *BMP-4*: 5′-GCACATAGACTTGACAAGTGATATG and 5′-GGGCGAATTGGGTACCGGGC (262 bp, 26 cycles). H4: 5′-CGGGATAACATTCAGGGTATCACT and 5′-ATCCATGGCGGTAACTGTCTTCCT (188 bp, 19 cycles). α-Globin: 5′-TTGCTGTCTCACACCATCCAGG and 5′-TCTGTACTTGGAGGTGAGGACG (126bp, 30 cycles). α-Actin: 5′-TCCCTGTACGCTTCTGGTCGTA and 5′-TCTCAAAGTCCAAAGCCACATA (252 bp, 20 cycles). UVS2: 5′-CTCATGAACAGAACAGAAGCGAGCGA and 5′-GAAGCGTAGTCATATTCAATCC (130 bp, 32 cycles).

UV and LiCl treatments of embryos

Embryos were irradiated with UV light for 60 s at 30 min after fertilization using a quartz chamber and a preheated GL25 lamp (UVP). Embryos were not moved for 1 h after the treatment. Embryos were dorsalized by incubating in 120 mM LiCl in 0.1× Barth's solution at the 32-cell stage for 25–30 min. (Fainsod *et al.*, 1994).

Preparation of capped sense and antisense RNA

Capped RNAs were prepared using the commercially available Megascript kit (Ambion) according to the manufacturer's instructions. The cap analog:GTP ratio was 5:1 for both sense and antisense RNAs. Sense *gsc* RNA was transcribed by SP6 RNA polymerase from pSp-gsc linearized with *Eco*RI (Niehrs *et al.*, 1994) and pSP-myc-gsc (see above). Full-length antisense *gsc* RNA was prepared from pSp-gsc linearized with *Eco*RI using T7 RNA polymerase (Cho *et al.*, 1991). Antisense *gsc* RNA lacking the homeobox was transcribed from pΔgsc linearized with *Sma*I using T3 RNA polymerase (Cho *et al.*, 1991). Sense *BMP-4* RNA was transcribed from a Bluescript SK+ plasmid linearized with *Xho*I, which contains the full-length *BMP-4* cDNA (B12) using T3 RNA polymerase (Fainsod *et al.*, 1994). Full-length antisense *BMP-4* RNA was transcribed from the plasmid linearized with *Eco*RI using T7 RNA polymerase. Truncated antisense *BMP-4* RNA lacking the mature peptide region, pΔ3′-BMP-4, was linearized with *Eco*RI and transcribed with T3 RNA polymerase. To create pΔ3′-BMP-4 plasmid, the 5′ region of the *BMP-4* cDNA extending up to two amino acids into the mature growth factor peptide was amplified by PCR and cloned into the *decor*I–*Sal*I sites of Bluescript KS. Sense β-*gal* mRNA was synthesized from pCDM8-β-gal (Sasai *et al.*, 1992) linearized with *Pst*I using T7 RNA

polymerase. Antisense β-*gal* RNA was synthesized from pKS-β-gal linearized with *Sal*I using T7 RNA polymerase. pKS-β-gal was generated by excising the coding region of β-*gal* from pCH 101 with *Hin*dIII and *Pst*I, adding a *Sal*I linker to the *Hin*dIII site and cloning into Bluescript KS which was cut with *Sal*I and *Pst*I. Antisense *XlHbox-1* RNA was transcribed using T7 RNA polymerase from pG10 linearized with *Eco*RI (Cho *et al.*, 1988).

Sense and antisense DNA constructs

For the expression of sense and antisense transcripts in embryos after mid-blastula transition, the following plasmids were used: sense β-*gal* RNA, pCH101 (Pharmacia); sense *BMP-4* RNA, CSKA-BMP-4 (gift of M.Jones and C.V.E.Wright) which contains the full-length *BMP-4* cDNA under the control of a cytoskeletal actin promoter (Harland and Misher, 1988). For antisense *BMP-4* transcripts, CMV-antis-BMP-4 was used. To generate this construct, the full-length cDNA of *BMP-4* was excised with *Eco*RI and *Xho*I and filled in with Klenow DNA polymerase. This fragment was then cloned into pCMV 2 linearized with *Sma*I. For antisense *gsc*, pCMV-antis-gsc was used. This clone was constructed by excising the *gsc* cDNA from pSP-gsc with *Eco*RI and *Hin*dIII, generating blunt ends by filling in, and cloning this fragment into the *Eco*RI site of pCMV which was also made blunt by filling in. For sense *gsc*, pCMV-gsc (Niehrs *et al.*, 1993) was used. A number of DNA concentrations were tested; the most effective range was plasmid DNA when injected at 10–25 μg/ml.

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