On the function of *BMP-4* in patterning the marginal zone of the *Xenopus* embryo

Abraham Fainsod^{1,2,3},
Herbert Steinbeisser^{1,3} and
Eddy M.De Robertis^{1,4,5}

¹Molecular Biology Institute and Department of Biological Chemistry, University of California, Los Angeles, CA 90024-1737, USA and

²Department of Cellular Biochemistry, Hebrew University–Hadassah Medical School, Jerusalem 91010, Israel

³Present address: Howard Hughes Medical Institute, UCLA, USA

⁴Equal first authors

⁵Corresponding author

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Bone morphogenetic protein 4 (*BMP-4*) is expressed in the ventral marginal zone of the gastrulating embryo. At late gastrula stage this gene is expressed in the ventral-most part of the slit blastopore and in tissues that derive from it. At tailbud stages *BMP-4* is expressed in the spinal cord roof plate, neural crest, eye and auditory vesicle. The interactions of *BMP-4* with dorsal genes such as *gooseoid* (*gsc*) and *Xnot-2* were studied *in vivo*. In embryos ventralized by UV irradiation and suramin treatment, *BMP-4* zygotic transcripts accumulate prematurely and the entire marginal zone expresses this gene. The patterning effect of *BMP-4* on ventro-posterior development can be revealed by a sensitive assay involving the injection of *BMP-4* mRNA in the ventral marginal zone of embryos partially dorsalized with LiCl, which leads to the complete rescue of trunk and tail structures. The experiments presented here argue that *BMP-4* may act *in vivo* as a ventral signal for the proper patterning of the marginal zone, actively interacting with dorsal genes such as *gsc* and *Xnot-2*. A model is proposed in which the timing of expression of various marginal zone-specific genes plays a central role in patterning the mesoderm.

Key words: *BMP-4*/gooseoid/LiCl/suramin/UV/*Xenopus*

Introduction

The formation of mesoderm during gastrulation has been studied extensively in *Xenopus laevis*. In this system, dorso-ventral patterning gives rise to notochord (dorsal tissue), muscle and kidney (dorso-lateral tissues), and mesenchyme and blood (ventral tissues). This process of creating dorso-ventral polarity in a radially symmetrical egg requires multiple inductive steps and a regulatory network that is not yet fully understood (for reviews see Kimelman *et al.*, 1992; Sive, 1993). It has been proposed that at the early blastula stage, a dorsalizing signal emanating from the dorsal vegetal blastomeres (Nieuwkoop center) induces Spemann's organizer which leads to the graded patterning of dorsal tissues in the

marginal zone (for reviews see Gerhart *et al.*, 1991; Gilbert and Saxen, 1993). The marginal zone is the equatorial region of the *Xenopus* embryo, which during gastrulation and neurulation gives rise to all mesodermal tissue types.

Growth factors play a key role in mesoderm induction and patterning. Secreted molecules like FGF, Wnt-8 and bone morphogenetic protein 4 (*BMP-4*) have been shown to be inducers or modifiers of mesodermal induction, leading towards a ventral fate. Activin and noggin, in contrast, have a dorsalizing effect on the forming mesoderm (for reviews see Slack, 1993; Smith, 1993). Many studies have focused on the induction of dorsal mesoderm, while ventral mesoderm has been assumed to be the default state (Gerhart *et al.*, 1989). Recently, it has been shown that genes expressed just prior to gastrulation play a role in the dorso-ventral patterning of the marginal zone. The *Xenopus* *gooseoid* (*gsc*) homeobox gene is expressed in Spemann's organizer and can pattern the mesoderm in a concentration-dependent manner on the dorsal side of the embryo (Niehrs *et al.*, 1994). In contrast, a ventro-laterally expressed secretory gene product, *Xwnt-8*, has a partial ventralizing effect limiting head and notochord, but not affecting muscle development (Christian and Moon, 1993).

BMP-4, a growth factor of the transforming growth factor- β superfamily, has been identified as a very potent ventralizing factor when used to treat *Xenopus* embryos (Dale *et al.*, 1992; Jones *et al.*, 1992; Köster *et al.*, 1992). The *Xenopus* *BMP-4* gene has been cloned and studied by a number of groups (Dale *et al.*, 1992; Köster *et al.*, 1992; Nishimatsu *et al.*, 1992). The effects of injected mRNA or of added *BMP-4* protein of *Xenopus* (Dale *et al.*, 1992; Köster *et al.*, 1992) or human (Jones *et al.*, 1992) origin were studied in some detail in frog embryos. This gene has strong ventralizing effects in whole embryos, animal cap assays and Einsteck grafts. In some instances a weak mesoderm-inducing activity has been noted, but the most striking effect of *BMP-4* is its capacity to cause the strong ventralization of mesoderm induced by high concentrations of activin which should have a dorsal identity (Dale *et al.*, 1992; Jones *et al.*, 1992; Köster *et al.*, 1992). *BMP-4* transcripts are present in the egg, but the main burst of zygotic expression initiates after the onset of gastrulation. Although the ventralizing effect of *BMP-4* is well documented, the relationship between this activity and its function *in vivo* is not yet understood, principally because no correlation could be found between its transcript distribution in the embryo and its ventralizing effect. The improvement in sensitivity in the whole mount *in situ* hybridization technique (Harland, 1991) led us to re-examine the *in vivo* distribution of *BMP-4* transcripts in *Xenopus*.

In this study we determine the localization of the *BMP-4* zygotic transcripts and investigate their relationship with

Fig. 1. *BMP-4* spatial pattern of expression. *BMP-4* expression was determined by whole mount *in situ* hybridization of albino embryos. (A) Vegetal view of a stage 11.5 embryo showing the ventral marginal zone expression of *BMP-4*. The arrowhead marks the position of the dorsal lip of the blastopore. (B) Side view of a stage 11 embryo where the expression of *BMP-4* in the animal cap and the ventral marginal zone is demonstrated. Dorsal is to the left and the position of the dorsal lip is marked by an arrowhead. (C) Lateral view of a stage 14 (slit blastopore) embryo where *BMP-4* expression is evident throughout the ventral region. Strong expression can be observed at the anterior and posterior ends and in the lateral plate mesoderm. The dorsal region of the embryo is devoid of *BMP-4* transcripts. Anterior is to the left and dorsal to the top. (D) Stage 23 embryo, as seen from the side, continues to exhibit strong *BMP-4* expression in the anterior (left) and posterior ends in the ventral region. Expression can still be observed in the lateral region of the embryo and in the dorsal midline neural crest and the eye. (E) Dorsal view of a stage 24 embryo to show the dorsal neural crest cells expressing *BMP-4*. Anterior is to the left. (F–I) Lateral views of embryos at stages 26, 28, 33 and 40, respectively. All embryos show the strong *BMP-4* expression at the anterior (left) and posterior ends of the embryo. At the anterior end of the embryo the expression localizes just ventral to the cement gland. At the posterior end, *BMP-4* transcripts can be seen at the base of the tail. Other regions expressing *BMP-4* include the eye, neural crest cells in the dorsal midline and the otic vesicle.

other genes involved in mesodermal patterning. We show that *BMP-4* is expressed in the ventral marginal zone during gastrulation. The cross-regulatory interactions of *BMP-4* with other genes expressed in the dorsal marginal zone, such as *gsc* and *Xnot-2*, were examined. The results demonstrate that *BMP-4* can repress the expression of genes active in Spemann's organizer, and that *BMP-4*

RNA can pattern the mesoderm and rescue trunk and tail formation in embryos partially dorsalized by treatment with LiCl. In addition, we show that ventralizing treatments prematurely activate *BMP-4* expression during gastrulation. A model is proposed in which the timing of *BMP-4* expression plays a central role in patterning the marginal zone at the gastrula stage.

Results

BMP-4 spatial pattern of expression

An *in situ* hybridization study was performed with digoxigenin-labeled *BMP-4* antisense RNA probes to study the localization of the transcripts during embryonic development. Prior to gastrulation, no localized *BMP-4* transcripts were observed. At the onset of gastrulation (stage 10; Nieuwkoop and Faber, 1967), *BMP-4* expression can be detected in the animal cap. At stage 11, *BMP-4* expression in the ventral marginal zone becomes detectable (Figure 1A and B). The marginal zone expression covers the ventral and lateral regions, and by stage 11.5 the only region in the embryo not expressing *BMP-4* is the dorsal marginal zone. At the end of gastrulation, high *BMP-4* expression is localized to the ventral-most region of the closing slit blastopore (Figure 2A). At this stage *Xwnt-8*, which starts ventro-laterally (see Christian and Moon, 1993), becomes restricted to more lateral regions of the blastopore (Figure 2B), so that *BMP-4* appears to be the ventral-most marker available at present. In the same embryos, a second site of strong *BMP-4* expression is localized to the ventral region at the anterior end of the embryo (Figure 1C). Staining can also be detected in the entire ventral region of the lateral plate mesoderm (Figure 1C). It is of importance to note that at this stage the dorsal midline and the neural plate are completely devoid of *BMP-4* transcripts, as detected by *in situ* hybridization.

As the embryo continues to develop, the main sites of *BMP-4* expression remain the ventro-anterior region surrounding the cement gland and the tissue spanning the region between the tailbud and the anus in the posterior end (Figure 1D–I). Outside these regions, *BMP-4* expression in ventral mesoderm becomes weaker, while in dorsal mesoderm no transcripts can be detected at any stage. This pattern of expression is consistent with the ventralizing activity reported for *BMP-4*. Additional new sites of *BMP-4* expression that do not belong to the ventral mesoderm domain appear in later development: the eye and surrounding cells (Figure 1D), the otic vesicle (Figure 1F), the neural crest migrating to the dorsal fin, and the roof plate of the spinal cord (Figure 1D–H).

These observations were confirmed by histological sections. At neurula and early tailbud stages *BMP-4* transcripts are found in the ventral half of the lateral plate (Figure 3A), including the forming blood islands. The strong staining observed in the ventral head region corresponds to head mesenchyme cells that surround the cement gland (which is negative), as well as the heart anlage. The signal found in the ventro-posterior region corresponds to the thick collar of cells that surrounds the proctodeum (Figure 3B). At later stages, signals are also found in head mesenchyme, eyes and surrounding mesenchyme, auditory vesicles, developing kidney, post-anal gut and neural crest. One site of expression that may be particularly important in view of the signaling properties of *BMP-4* is the roof plate of the central nervous system which starting at stage 28 expresses this gene in a highly localized fashion (Figure 3C) in the hindbrain and spinal cord.

BMP-4 expression in LiCl- and UV-treated embryos

UV irradiation of the vegetal hemisphere at the 1-cell stage has a ventralizing effect (Scharf and Gerhart, 1983),

while LiCl treatment at the 32-cell stage dorsalizes the embryo (Kao *et al.*, 1986). It is of interest to study the localization of the *BMP-4* transcripts in embryos following both treatments to better correlate the ventralizing effect of this factor and its pattern of expression. In LiCl-treated embryos, *BMP-4* expression in the animal cap persists during gastrulation, as it does in untreated embryos, but expression in the marginal zone is missing (Figure 4A). In gastrulating UV-treated embryos, *BMP-4* transcripts are present in the entire marginal zone and animal cap (Figure 4B). Thus, *BMP-4* expression in the marginal zone closely correlates with the formation of ventral mesoderm. The animal cap seems to be regulated by a different mechanism (Figure 4A).

Rescue of LiCl-treated embryos

In the gastrulating LiCl-treated embryo *BMP-4* transcripts are missing from the marginal zone (Figure 4A), raising the possibility that the embryo is dorsalized in part by the lack of a ventralizing signal in the mesoderm. To address this possibility, an experiment was designed to rescue LiCl-treated embryos by providing them with a localized ventral signal. In previous experiments in which *BMP-4* RNA was injected into the entire embryo and a full LiCl treatment applied, completely ventralized embryos lacking an axis were obtained (Dale *et al.*, 1992). For this reason, a new biological assay was devised. It involves a partial LiCl treatment (partial-Li) which results in embryos that lack trunk and tail structures but are not entirely radial [dorso-anterior index (DAI) of 6.5–7.5; Figure 5B; Kao and Elinson, 1988].

To restore localized expression of *BMP-4* in the ventral marginal zone, embryos were injected at the 32-cell stage with 200 pg of *BMP-4* mRNA into one of the C4 blastomeres (Dale and Slack, 1987; Moody, 1987) followed by a partial-Li treatment. The C4 blastomere was chosen because it is ventral and because injections of *BMP-4* mRNA into blastomeres of the dorsal side at the 32-cell stage result in completely ventralized embryos missing axes. In a first set of injections, 39 out of 42 injected embryos grew entirely normal posterior axes (trunks and tails), while in the control uninjected group only one embryo out of 43 had a tail (Figure 5). In a second set of injections, 29 out of 35 *BMP-4*-injected partial-Li embryos developed normal trunks and tails, while control injections of *LacZ* RNA resulted in 26 embryos with the partial-Li phenotype and none of these embryos had a tail. We conclude from these results that to attain ventro-posterior pattern the embryo requires a ventral component. In the partial-Li embryo this ventral component can be restored by *BMP-4* mRNA injection into one of 32 blastomeres.

Regulation of BMP-4 by gsc

To better understand the changes that *BMP-4* expression undergoes in UV- and LiCl-treated embryos, the interactions of *BMP-4* and genes expressed in the dorsal marginal zone were studied. The expression pattern of *BMP-4* during gastrulation revealed a transcript distribution which is complementary to that of genes transcribed in Spemann's organizer, such as *gsc* which in LiCl-treated embryos are expressed radially in the entire marginal zone (Cho *et al.*, 1991). This suggested that dorsal marginal

Fig. 2. Patterning of the slit blastopore marginal zone. (A) Dorso-posterior view of a stage 13 embryo showing *BMP-4* expression in the slit blastopore. *BMP-4* expression encompasses the region surrounding the ventral third of the blastopore. Anterior is to the top and the arrow marks the slit blastopore. (B) Detection of *Xwnt-8* expression in the circumblastoporal region of a stage 13 embryo (dorso-posterior view). *Xwnt-8* transcripts can be detected on both sides of the slit blastopore in the middle third of the marginal zone. The arrow marks the position of the blastopore and anterior is to the top. (C) Diagrammatic representation of the main sites of expression of genes expressed around the slit blastopore. The different localization of these gene transcripts raises the possibility of their role in patterning the mesoderm at this stage. The *Xnot-2* localization is from Gont *et al.* (1993) and the *Xwnt-11* localization is from Ku and Melton (1993).

Fig. 3. Histological analysis of *BMP-4* expression in *Xenopus* embryos. Embryos hybridized for *BMP-4* mRNA were embedded in paraffin, 10 μ m sections were cut and specimens were analyzed using Nomarski optics. (A) Cross-section through the anterior third of a stage 23 embryo showing *BMP-4* mRNA in the ventral mesoderm. No *BMP-4* transcripts can be observed in more lateral regions. (B) The proctodeum of a stage 26 embryo is shown in cross-section. *BMP-4* is expressed in a mesodermal collar around the anus. (C) Cross-section through a stage 38 embryo in which *BMP-4* is expressed in neural crest cells populating the dorsal fin and in cells of the roof plate of the spinal cord. Abbreviations: nt, notochord; sc, spinal cord; pd, proctodeum; nc, neural crest; rp, roof plate. The bar indicates 100 μ m.

zone genes may regulate *BMP-4* negatively to prevent the expression of this ventralizing gene in the dorsal region of the embryo. To test this hypothesis, the effect of *gsc* expression on the *BMP-4* pattern was studied.

Injections of *gsc* mRNA were performed in normal and UV-treated embryos in an attempt to down-regulate *BMP-*

4 transcription. UV-treated embryos were injected diagonally in two non-adjacent blastomeres of the 4-cell embryo. In all uninjected UV-treated embryos ($n = 14$), no gaps in the *BMP-4* pattern of expression were observed. In UV-treated embryos injected in the equatorial region with 100 pg of *gsc* mRNA, 11 out of 13 exhibited gaps in the

Fig. 4. Regulation of *BMP-4* by LiCl and UV treatments, and *gsc* mRNA injection. (A) Lateral view of a LiCl-treated embryo *in situ* hybridized to detect *BMP-4* transcripts. This treatment leads to the down-regulation of *BMP-4* transcription in the marginal zone, while animal cap expression remains constant. (B) *BMP-4* expression in a UV-treated embryo (lateral view). In this case, *BMP-4* transcription becomes spatially deregulated and covers the whole animal cap and marginal zone; the only region without signal is the yolk plug. (C) Injection of *gsc* RNA and its effect on *BMP-4* expression. UV-treated embryo injected with *gsc* RNA in two non-adjacent blastomeres at the 4-cell stage and processed for *BMP-4* whole mount *in situ* hybridization. A gap in the *BMP-4*-expressing region can be seen (arrowhead); the same embryo had a second gap on the opposite side of the marginal zone. The gap caused by *gsc* exhibits a sharp boundary at the transition between the animal cap and the marginal zone, suggesting that *gsc* cannot repress *BMP-4* in the animal cap of UV-treated embryos. (D) Injection of *gsc* RNA in wild-type embryos and its effect on *BMP-4* expression. Normal embryos were injected with *gsc* RNA in all four blastomeres at the 4-cell stage. This view from the dorsal side shows two regions of expansion of the *BMP-4*-free region extending into the animal cap (arrowheads). In all panels the embryos are placed so that the animal pole is to the top; the brackets on the side delineate the marginal zone (MZ). The whole mount *in situ* hybridization method used (Harland, 1991) is unable to detect transcripts in the large yolky vegetal cells of the *Xenopus* embryo (O'Keefe *et al.*, 1991).

Fig. 5. Rescue of LiCl-treated embryos by *BMP-4* and suramin. (A) Control untreated embryos for (B) and (C). (B) Embryos treated to obtain the partial-Li phenotype exhibit head and partial trunk development characteristic of this treatment. Note that posterior trunk and tail are absent. (C) *BMP-4* RNA rescue of embryos treated with partial-Li. Injection of *BMP-4* RNA into one C4 blastomere at the 32-cell stage restored normal trunk and tail development in partial-Li-treated embryos. (D) Control-untreated embryos for (E)–(G). (E) Suramin treatment of embryos. Embryos treated with 400 μ M suramin for 7.5 h starting at the 64-cell stage. These embryos exhibit the ventralization characteristic of the suramin treatment (Grunz, 1992). (F) Partial-Li-treated embryos exhibiting normal head development and reduced trunk development. In these embryos no tail development is noticeable. (G) Suramin rescue of partial-Li-treated embryos. Embryos first treated with LiCl and subsequently with suramin are shown. These embryos show rescue of posterior development so that the trunk and the tail develop as a result of the suramin treatment of the partial-Li embryos; some head defects caused by the suramin treatment remain. The rescue of partial-Li embryos reinforces the view that suramin may ventralize the embryo by inducing the expression of *BMP-4*. (see Figure 7).

BMP-4 expression and almost 50% of them ($n = 5$) had two gaps on opposite sides of the marginal zone (Figure 4C). The gaps in the *BMP-4* pattern of expression were

all localized to the marginal zone, and many of them had sharp boundaries at the junction between the marginal zone and the animal cap (Figure 4C). Injection of the *gsc*

RNA in the animal pole region of UV-treated embryos resulted in a reduced percentage of embryos with gaps in the *BMP-4* expression in the marginal zone (11 out of 21 embryos), but expression in the animal cap itself was unaffected. Thus, *gsc* can inhibit the expression of *BMP-4*. This repression is restricted to the marginal zone and cannot take place in the animal cap region of UV-treated embryos which seems to be regulated by a separate mechanism.

In normal embryos, injections of *gsc* RNA were performed radially in all four blastomeres of 4-cell embryos in the equatorial region. Embryos with one gap were scored as unaffected by the injection and representing the normal *BMP-4* pattern of expression (seven out of 25). Two gaps were observed in 14 of the embryos and the gaps were localized adjacent to each other on the dorsal side of the embryo. These gaps extended the dorsal *BMP-4*-free region into the animal cap (Figure 4D, arrows). Four of the embryos had three gaps, one of them localized to the ventral side of the embryo. None of the embryos exhibited four gaps in the *BMP-4* pattern of expression.

We conclude from these experiments that *gsc* is able to repress *BMP-4* RNA expression in the marginal zone of wild-type and UV-treated embryos. Taken together, these results support the notion that the lack of *BMP-4* expression on the dorsal side of the embryo could in part be attributed to repression by *gsc*.

Regulation of *gsc* by *BMP-4*

Injection of *BMP-4* mRNA results in the strong ventralization of the entire embryo, even when injected at the 32-cell stage in a blastomere destined to form part of Spemann's organizer (Dale *et al.*, 1992; Jones *et al.*, 1992; our unpublished observations). These observations raise the possibility that *BMP-4* may cause the down-regulation of organizer-specific genes like *gsc*. To test this hypothesis, the effect of *BMP-4* on the *gsc* pattern of expression was tested. Normal embryos injected radially with control prolactin mRNA (400 pg) resulted in 100% ($n = 16$) exhibiting the normal *gsc* pattern of expression (Figure 6A). A parallel set of radial injections with *BMP-4* RNA (400 pg) resulted in the elimination of *gsc* transcripts in 14 out of 16 embryos by stage 11 (Figure 6B).

Further support for the observation that *gsc* is down-regulated by *BMP-4* was obtained using LiCl-treated embryos. After LiCl treatment, the *gsc* pattern of expression is expanded into a ring around the forming blastopore (Cho *et al.*, 1991). Diagonal injection of *BMP-4* mRNA in the 4-cell LiCl-treated embryo resulted in the elimination of the *gsc* expression in six out of 14 embryos (Figure 6D). In the remaining eight LiCl-treated embryos, *gsc* transcripts remained in a small region of the marginal zone reminiscent of the normal pattern of expression (Figure 6D) instead of forming a ring. This incomplete repression of *gsc* transcription might be due to the unequal distribution of the injected *BMP-4* mRNA. Similar injections with control prolactin mRNA did not change the *gsc* pattern of expression ($n = 16$; Figure 6C).

We conclude from these results that *BMP-4* mRNA injection is sufficient to initiate the events that will lead to repression of *gsc* expression. Thus, *BMP-4* differs from another ventralizing gene, *Xwnt-8*, which is unable to down-regulate *gsc* expression (Christian and Moon, 1993).

Repression of *Xnot-2* by *BMP-4*

The cross-regulation between *gsc* and *BMP-4* raised the possibility that genes expressed in different parts of the marginal zone are part of a cross-regulatory network that patterns the mesoderm. To test further the possibility that a ventral gene can regulate dorsal genes, we studied the effect of *BMP-4* mRNA injection on the expression of the dorsal-specific homeobox gene *Xnot-2* (Gont *et al.*, 1993). *Xnot-2* is expressed in the organizer region and in a thin band of the marginal zone at early stages. Normal and LiCl-treated embryos were injected with *BMP-4* mRNA and *Xnot-2* expression was analyzed. Normal embryos injected radially with control prolactin mRNA exhibited the wild-type pattern of *Xnot-2* expression ($n = 16$; Figure 6E). Radial injection of *BMP-4* mRNA eliminated *Xnot-2* transcripts ($n = 15$; Figure 6F). Corroboration of this result was obtained using LiCl-treated embryos in which *Xnot-2* is expressed as a uniform ring in the marginal zone. Diagonal injection of *BMP-4* mRNA caused the partial repression of the *Xnot-2* in 14 out of 17 embryos (Figure 6H). Diagonal injection of control prolactin mRNA resulted in the expected *Xnot-2* ring of expression ($n = 15$; Figure 6G). These results support and expand observations on *Xnot* regulation by von Dassow *et al.* (1993). We conclude that the ventral signal provided by *BMP-4* can repress dorsal-specific genes such as *gsc* and *Xnot-2*.

BMP-4 is prematurely expressed in ventralized embryos

The UV-treated embryo becomes ventralized, forming tissues such as blood, mesenchyme and mesothelium, and lacking dorsal tissues such as muscle and notochord (Gerhart *et al.*, 1989). These same embryos express *BMP-4* transcripts in the animal cap and, more importantly, the entire marginal zone (Figure 4B). Furthermore, previous work has shown that *BMP-4* mRNA and protein have a strong ventralizing effect when injected or added prior to gastrulation (Dale *et al.*, 1992; Jones *et al.*, 1992; Köster *et al.*, 1992). These observations raise the possibility that ventralization of the UV-treated embryo might at least in part result from premature zygotic expression of the *BMP-4* gene, which in turn would down-regulate dorsal marginal zone-specific genes such as *gsc*.

To test this hypothesis, the kinetics of zygotic *BMP-4* transcript accumulation were analyzed in normal and UV-treated embryos. RNA was prepared every hour from 8 to 11 h post-fertilization. All the samples were reverse transcribed and quantitative PCR (RT-PCR) performed for *BMP-4*; histone H4 was used as a loading control in the same reaction (Figure 7A). In each experiment the number of cycles necessary to obtain results in the quantitative range of the PCR was determined. As shown by others (Dale *et al.*, 1992; Jones *et al.*, 1992; Köster *et al.*, 1992), *BMP-4* transcripts peak at about stage 11–11.5 and then remain high and almost constant until the beginning of neurulation. Comparison of the 9 and 10 h time points in normal and UV-treated embryos shows a premature increase in *BMP-4* levels in the UV sample. The same conclusion is reached when the transcripts are quantitated (Figure 7B). These results show that in the UV-treated embryo *BMP-4* transcripts reach a high level 1 h before normal embryos. In our experiment, the 9 h sample was

Fig. 6. Regulation of marginal zone genes by *BMP-4*. Injections of *BMP-4* RNA were used to study the interactions between this gene and other marginal zone-expressed genes. (A) Control wild-type embryos injected radially with control prolactin mRNA and probed for *gsc*. These embryos exhibit the normal dorsal marginal zone expression of *gsc*. (B) The effect of radial *BMP-4* RNA injection on *gsc* expression in wild-type embryos. Ectopic expression of *BMP-4* RNA causes a strong down-regulation of *gsc* expression. (C) *gsc* expression in LiCl-treated embryos injected diagonally with prolactin RNA. The expression of *gsc* in LiCl-treated embryos becomes radial as a result of this treatment and is unchanged by prolactin mRNA injection. (D) Down-regulation of *gsc* expression in LiCl-treated embryos by *BMP-4*. Diagonal injection of *BMP-4* mRNA causes a strong down-regulation of the *gsc* expression, leaving only a small arch region of expression. (E) Expression of *Xnot-2* in control embryos radially injected with prolactin mRNA. These embryos exhibit the normal *Xnot-2* pattern of expression. (F) Effect of *BMP-4* on *Xnot-2* expression. Radial injection of *BMP-4* RNA into normal embryos at the 4-cell stage resulted in the complete disappearance of the *Xnot-2* transcripts. (G) *Xnot-2* pattern of expression in LiCl-treated embryos injected radially with prolactin RNA. LiCl treatment results in the expression of *Xnot-2* as a ring in the marginal zone which is unchanged by the prolactin mRNA injection. (H) *BMP-4* RNA injection into all four blastomeres at the 4-cell stage results in the down-regulation of *Xnot-2* expression. In all cases a group of embryos is shown, while the insert shows an embryo at higher magnification.

Fig. 7. Premature expression of *BMP-4* transcripts in ventralized embryos. (A) RNA samples from untreated and UV- and suramin-treated embryos were processed for RT-PCR to detect the levels of *BMP-4* and histone H4 transcripts. The time points for 8 (stage 9), 9 (stage 10), 10 (stage 10.5) and 11 h (stage 11–11.5) are shown. In normal embryos the increase in *BMP-4* transcripts takes place between 9 and 10 h. In UV-treated embryos this increase in *BMP-4* transcription occurred between 8 and 9 h, while in suramin-treated embryos the *BMP-4* transcript levels were already high at 8 h. (B) Quantitation of the *BMP-4* transcript levels at the different time points using a PhosphorImager. Suramin- and UV-treated embryos exhibit at 8 and 9 h of incubation, respectively, almost double the level of *BMP-4* transcripts in normal embryos prior to the onset of gastrulation.

composed mostly of stage 10 embryos, while at 10 h most embryos were at stage 10.5–11. This 1 h difference would result in high *BMP-4* expression at the onset of gastrulation. As shown above, this premature expression, in combination with the expansion of *BMP-4* transcription throughout the marginal zone (Figure 4B), could result in the repression of dorsal-specific genes. The *BMP-4* signaling pathway could play an important role in the chain of events that lead to the ventralization of UV-treated embryos.

BMP-4 is also prematurely expressed in embryos ventralized by suramin treatment, another ventralizing treatment available in *Xenopus*. The incubation of animal caps, dorsal marginal zones or whole embryos in the drug suramin results in their ventralization (Grunz, 1992). In agreement with this ventralizing activity, suramin is also able to rescue axis formation in the partial-Li assay, as shown in Figure 5D–G. Quantitative RT-PCR analysis of embryos treated with suramin (400 μ M) revealed that from the earliest time point studied (8 h) *BMP-4* transcript levels were already high (Figure 7A). Quantitation of *BMP-4* mRNA levels in these samples revealed that in suramin-treated embryos the level of *BMP-4* mRNA increases 2 h earlier than in normal embryos (Figure 7B). These observations support the hypothesis that ventralization in suramin-treated, as well as in UV-treated, embryos may be due to the premature expression of *BMP-4*.

Discussion

BMP-4 is expressed in the ventral marginal zone

As shown by a number of groups, one component of the *BMP-4* expression is maternal (Dale *et al.*, 1992; Jones

et al., 1992; Köster *et al.*, 1992). This component is not detectable by the whole mount *in situ* technique. One important gap in our knowledge was whether the zygotic *BMP-4* transcripts are expressed in the embryo in a pattern congruent with the ventralizing properties of this factor. We now report that the expression of zygotic *BMP-4* transcripts is highly localized in *Xenopus* embryos. At about stage 11 an increase in *BMP-4* transcripts takes place and expression begins in the ventral marginal zone. Ultimately, *BMP-4* expression encompasses the animal cap and the marginal zone, respecting only the organizer region. Although it has been reported previously that *BMP-4* transcripts are ubiquitous in the stage 11 embryo, it would be difficult to detect the *BMP-4*-free region with the approach used, which involved dissection of embryo fragments (Dale *et al.*, 1992).

At late gastrula and early neurula stages, *BMP-4* transcripts occupy the ventral third of the slit blastopore (Figure 2A). Lineage tracing of this region has shown that it gives rise to the ventral-most mesoderm (Gont *et al.*, 1993). The ventral half of the lateral plate mesoderm contains *BMP-4* transcripts in *Xenopus* (Figures 1 and 3A). This is in agreement with the lateral plate expression reported in mouse embryo (Jones *et al.*, 1991). Therefore, expression of the *BMP-4* gene in the late blastopore correlates closely with the establishment of the ventral mesodermal lineage. *Xwnt-8* gene expression during gastrulation is restricted to the ventro-lateral marginal zone but is excluded from the organizer. This gene has also been shown to be repressed by the injection of *gsc* mRNA (Christian and Moon, 1993). By the late gastrula stage *Xwnt-8* expression weakens ventrally until ultimately *Xwnt-8* transcripts occupy only the lateral region of the slit blastopore (Figure 2B). At the same time, *BMP-4* expression becomes restricted to the ventral third of the slit blastopore (Figure 2A). Thus, *Xwnt-8* appears to be a marker for the lateral mesoderm while *BMP-4* is a ventral marker in the late gastrula.

A role for *BMP-4* in the formation of ventral mesoderm has been proposed in earlier work based on its ventralizing effect (Dale *et al.*, 1992; Jones *et al.*, 1992; Köster *et al.*, 1992). The expression pattern described here supports a role for *BMP-4* in the patterning of ventral mesoderm *in vivo*. A localized source of *BMP-4* may function in patterning mesoderm in the *Xenopus* gastrula.

The ventral nature of *BMP-4* expression can also be revealed by experimental manipulations in which the normal dorso-ventral patterning of the *Xenopus* embryo is perturbed (Figure 4). In LiCl-treated embryos, no *BMP-4* transcripts can be detected in the marginal zone and little ventral mesoderm is formed (Kao and Elinson, 1988). On the other hand, in UV-treated embryos, which form almost exclusively ventral mesoderm, *BMP-4* expression extends throughout the marginal zone, including the dorsal region. Thus, the expression of *BMP-4* in the marginal zone correlates closely with the formation of ventral mesoderm.

At later stages of development *BMP-4* is expressed in several other regions of the embryo (Figures 1 and 3). It is worth noting that at tailbud stages *BMP-4* is specifically expressed in the dorsal region (roof plate) of the spinal cord. Given the current interest in dorso-ventral patterning of the spinal cord (Basler *et al.*, 1993) and the potent inducing activities of *BMP-4*, it should be straightforward

to test whether *BMP-4* functions in central nervous system patterning as well.

BMP-4 restores marginal zone patterning in the partial-Li embryo

In the LiCl-treated embryo *BMP-4* expression is eliminated from the marginal zone. This raised the possibility of studying the patterning of the marginal zone by manipulating gene expression in this region. The experimental design involved the localized restoration of *BMP-4* expression in a partial-Li-treated embryo by injecting RNA into a blastomere which will form part of the ventral marginal zone. This proved sufficient to restore the dorso-ventral polarity of the marginal zone, resulting in normal trunk-tail development (Figure 5). Partial-Li rescue provides a sensitive and novel phenotypic assay that can be added to others already available in *Xenopus* embryology.

BMP-4 and patterning of the marginal zone

Based on the normal spatial pattern of *BMP-4* expression, it appeared possible that genes normally expressed in the organizer may prevent *BMP-4* expression on the dorsal side. A similar model has been proposed for *Xwnt-8* (Christian and Moon, 1993). The results of the injection experiments indicate that *gsc* can repress *BMP-4* in the marginal zone. When UV-treated embryos were utilized, the effect of *gsc* was restricted entirely to the marginal zone and exhibited a strong boundary at the transition to the animal cap (Figure 4C). These observations confirmed the initial assumption that *gsc* is able to repress *BMP-4* expression, but also suggested the existence of other factors present in the marginal zone, but not in the animal cap, which cooperate with *gsc* in performing this down-regulation.

The observation that premature expression of *BMP-4* (which can be attained by RNA injection, UV or suramin treatment) prevents dorsalization made us investigate the possible down-regulation of dorsally expressed genes like *gsc* and *Xnot-2* by *BMP-4*. *BMP-4* can down-regulate the expression of *gsc* and *Xnot-2* in normal and LiCl-treated embryos which exhibit expanded expression of these genes. The results suggest that in the embryo the expression of these organizer-specific genes may be restricted to the dorsal marginal zone by the ventral expression of *BMP-4*.

The *XFKH-1* gene (Dirksen and Jamrich, 1992; Knöchel *et al.*, 1992; Ruiz i Altaba and Jessell, 1992), another dorsal-specific gene, may also be down-regulated by *BMP-4*. In a study of the effects of suramin on the expression of a number of genes, it was shown that high suramin concentrations inhibit the expression of *XFKH-1* (Oschwald *et al.*, 1993). As we have shown, suramin causes the de-regulation of the *BMP-4* gene which in turn might down-regulate *XFKH-1* expression as in the case of *gsc* and *Xnot-2*. These observations suggest the existence of important interactions between dorsal- and ventral-specific genes necessary to obtain proper patterning of the marginal zone.

Comparison of the ventralizing genes Xwnt-8 and BMP-4

The relationship between *BMP-4* and *Xwnt-8*, a gene expressed latero-ventrally during gastrulation and also repressed by *gsc* (Christian and Moon, 1993), is of interest.

From the analysis of *in situ* hybridizations it became clear that *BMP-4* and *Xwnt-8* expression patterns overlap along the marginal zone during early gastrulation. During late gastrulation, at the slit blastopore stage, the patterns of expression of these two genes resolve into neighboring regions of expression, with *Xwnt-8* occupying the lateral regions of the slit blastopore and *BMP-4* the ventral-most region (Figure 2). The difference between *Xwnt-8* and *BMP-4* is strengthened further by injection experiments that result in the overexpression of these genes. Overexpression of *Xwnt-8* in the dorsal part of the embryo after midblastula transition diverted the prospective notochord cells to a more ventral fate, muscle (Christian and Moon, 1993). *Xwnt-8* DNA injection in more lateral regions did not further ventralize prospective muscle cells (Christian and Moon, 1993). On the other hand, overexpression of *BMP-4*, even in a very localized manner, results in the severe repression of dorso-anterior development, sometimes giving rise to embryos with a DAI close to zero (Dale *et al.*, 1992; Jones *et al.*, 1992; our unpublished observations). The expression of *BMP-4* in lateral plate mesoderm (Figure 3A) is more ventral than that of *Xwnt-8* (Christian and Moon, 1993). Another difference between these two genes is that *Xwnt-8* is unable to repress *gsc* expression (Christian and Moon, 1993), as *BMP-4* does. Taken together, these observations suggest that while *BMP-4* provides a ventral signal, *Xwnt-8* may play a role in more lateral mesodermal development.

BMP-4 is prematurely expressed in ventralized embryos

One of the most interesting features of *BMP-4* is that it requires another mesoderm inductor to cause strong ventralization (Dale *et al.*, 1992; Jones *et al.*, 1992). Even when high concentrations of activin are utilized to promote dorsal mesodermal induction, *BMP-4* is capable of diverting the fate of the mesoderm formed to a ventral pathway. In other words, *BMP-4* can overcome the dorsalizing effect of activin (Dale *et al.*, 1992; Jones *et al.*, 1992). Similarly, it is accepted that suramin works by inhibiting the interaction between extracellular growth factors and their receptors (Papkoff and Schryver, 1990; Chakrabarti *et al.*, 1992; Grunz, 1992). Even though this type of effect has been shown to take place in a number of systems, the activity of suramin in *Xenopus* animal caps has been shown to be activin-dependent, suggesting that at least one growth factor can interact with its receptor in the presence of this drug (Grunz, 1992).

Temporal regulation of the *BMP-4* gene could play an important role in the proper development of the embryo. The relatively late onset of zygotic *BMP-4* expression may be crucial for normal development. As gastrulation gets underway, dorsal-specific genes such as *gsc* and *Xnot-2* are activated, establishing and maintaining the dorsal state. Dorsal-determining genes must reach levels high enough to prevent expression of *BMP-4* from spreading dorsally. Once zygotic *BMP-4* expression starts in the ventral and lateral marginal zone, further spreading of the organizer region is prevented. When the kinetics of *BMP-4* transcript accumulation were studied in embryos ventralized by UV or suramin treatments, it was found that both ventralizing treatments resulted in prematurely high *BMP-4* transcript levels at the onset of gastrulation.

Fig. 8. Patterning of the marginal zone: a model. Gastrula stage embryo summarizing the interactions between genes expressed in the marginal zone. *BMP-4* can down-regulate the dorsal genes *gsc* and *Xnot* (von Dassow *et al.*, 1993). The dorsally expressed gene *gsc* can repress the expression of the ventro-lateral gene *Xwnt-8* (Christian and Moon, 1993) and the ventral gene *BMP-4*. The genes *gsc* and *BMP-4* have a positive autoregulatory effect on themselves (Jones *et al.*, 1992; Niehrs *et al.*, 1994). These genes and presumably others, such as *Xlim-1*, *XFKH-1* and *noggin*, establish a regulatory network to properly pattern the marginal zone during gastrulation.

This premature expression of *BMP-4* would result in the inhibition of dorsal-specific genes such as *gsc* and *Xnot-2*, preventing dorsal development.

Dorso-ventral patterning of the marginal zone: a model

Based on these observations, a model can be proposed to explain the patterning of the marginal zone and ultimately of the mesoderm (Figure 8). It is clear that the interactions identified here can only take place after the midblastula transition, after zygotic transcription of the genes involved begins in the marginal zone (Newport and Kirschner, 1982). It should be noted that it is not known whether any of the interactions shown in Figure 8 are direct or require a number of intermediate steps. In this model a central element is the time sequence in which the different genes are activated. Based on temporal studies it appears that dorsal genes such as *gsc*, *Xlim-1*, *XFKH-1*, *Xnot* and *Xnot-2* (Blumberg *et al.*, 1991; Dirksen and Jamrich, 1992; Knöchel *et al.*, 1992; Ruiz i Altaba and Jessel, 1992; Taira *et al.*, 1992; Gont *et al.*, 1993; von Dassow *et al.*, 1993) are activated first, followed by more lateral genes such as *Xwnt-8* (Smith and Harland, 1991; Christian and Moon, 1993), and finally by the ventral genes represented by *BMP-4* (Dale *et al.*, 1992; Jones *et al.*, 1992; Köster *et al.*, 1992; this study). As shown here, premature expression of *BMP-4* disrupts the normal patterning of the marginal zone and results in ventralization of the embryo. Premature expression can be obtained by microinjection of *BMP-4* mRNA or ventralizing treatments such as UV and suramin. Dorsal-specific genes can establish the dorsal marginal zone before *BMP-4* is activated (Niehrs *et al.*, 1994). Once zygotic *BMP-4* signaling starts, further spreading of the organizer field is prevented.

Patterning of the marginal zone is probably completed by the slit blastopore stage, with the late blastopore lip becoming subdivided into regions that express various markers (Figure 2C). The subsequent cell lineages in which these genes are expressed correspond to lineage tracing experiments (Gont *et al.*, 1993). In this fate map, *BMP-4* is expressed in the ventral region of the blastopore and ultimately will be expressed in the ventral mesoderm (Figures 2A and C, and 3A). *Xwnt-8* is expressed in a lateral intermediate region of the blastopore and then it is expressed in lateral mesoderm (Figure 2B and C; Smith and Harland, 1991; Christian and Moon, 1993). Dorsal to *Xwnt-8*, the main region of expression of *Xwnt-11* can be seen (Figure 2C); later in development this same gene is expressed in the somites (Ku and Melton, 1993). Finally, *Xnot-2* expression can be detected in the dorsal-most position of the slit blastopore; later on, this gene is expressed in the cells giving rise to the notochord and the tip of the tail (Gont *et al.*, 1993).

As shown here, at the late gastrula stage the *BMP-4* signal becomes localized to the ventral mesoderm. *BMP-4* can limit the size of the organizer and restore postero-ventral development in partial-Li embryos. It appears that *BMP-4* plays an active role in determining ventral development *in vivo* and that dorsalizing and ventralizing genes may interact by negative control of their counterparts.

Materials and methods

In situ hybridization

The *BMP-4* probe was a cDNA clone isolated by K.Cho and B.Blumberg in this laboratory. This full-length clone is in the Bluescript SK⁺ plasmid and corresponds to the *BMP-4_i* clone reported by Köster *et al.* (1992). Antisense *BMP-4* RNA probes were generated by linearizing with *EcoRI* and transcribing with T7 RNA polymerase. The *Xwnt-8*, *gsc* and *Xnot-2* probes were prepared from full-length cDNA clones in the Bluescript plasmid (Blumberg *et al.*, 1991; Christian *et al.*, 1991; Gont *et al.*, 1993). Digoxigenin-labeled antisense probes used a commercially available nucleotide mix according to the manufacturer's instructions (Boehringer). Whole mount *in situ* hybridization was performed according to the method of Harland (1991), with the modification that the alkaline phosphatase staining was performed with the BM purple AP substrate (Boehringer), which gives very low background in *Xenopus*. Embryos were cleared in benzyl alcohol:benzyl benzoate (1:2).

UV, LiCl and suramin treatments

UV treatment of embryos was performed 30 min after fertilization by placing the embryos in 1× modified Barth's solution (Gurdon, 1976) on quartz slides. Embryos were irradiated for 60 s using a GL 25 lamp (UVP). The embryos were not moved for 1 h after the UV treatment.

To achieve a partial-Li treatment, embryos at the 32-cell stage were incubated for 20 min in a solution of 120 mM LiCl in 0.1× Barth's solution. After the treatment, the embryos were washed twice in 0.1× Barth's solution and then incubated in the same solution at room temperature. For a full LiCl treatment the incubation time was extended to 35 min.

Embryos at the 64-cell stage were incubated in 0.1× Barth's solution containing 400 μM suramin (Grunz, 1992). The embryos were incubated for 7.5 h and then washed twice before they were cultured further.

Preparation of capped mRNAs

Capped mRNAs were prepared using the commercially available Megascript kit (Ambion) according to the manufacturer's instructions. The cap-analog:GTP ratio used was 5:1. The *LacZ* RNA was prepared from the pCDM8-βgal plasmid (Sasai *et al.*, 1992) and the prolactin RNA was prepared from the pSP35-T plasmid (Amaya *et al.*, 1991).

RT-PCR

RNA from embryos was prepared by the phenol/guanidinium thiocyanate method (Chomczynski and Sacchi, 1987) using the RNA STAT-60

reagent (TEL-TEST 'B'). RT-PCR was performed as described by Niehrs *et al.* (1994). The primers used for *BMP-4* were 5'-GCATGTAAGG-ATAAGTCGATC and 5'-GATCTCAGACTCAACGGCAC, resulting in a 478 bp fragment after 26 cycles. The histone H4 primers were 5'-CGGGATAACATTCAGGGTATCACT and 5'-ATCCATGGCGGTAA-CTGTCTTCTT, resulting in a 188 bp product after 19 cycles. Quantitation was performed on a PhosphorImager (Molecular Dynamics) and the results were normalized to the level of histone H4 transcripts.

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