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

Abraham Fainsod1,2,3, Herbert Steinbeisser1,3 and Eddy M.De Robertis1,4,5

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

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 goosecoid (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/goosecoid/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 goosecoid (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 Einstick 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
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 mesenchymal 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 μm 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 μm.

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 transcript 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 yolk 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 μM 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.

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.
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 ventralization 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 μM) 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 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 DA1 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.
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-I, 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-1I 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 cDNA 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 by 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/lacZ 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'-GCGATGAAATAAGTGCCATC and 5'-GACTCGACCTCAAGGCAC, resulting in a 478 bp fragment after 26 cycles. The histone H4 primers were 5'-CGGATAAACTGGGATACCT and 5'-ATCCATGCCGAAAACTGGTCT, resulting in a 188 bp product after 19 cycles. Quantitation was performed on a Phosphorlmager (Molecular Dynamics) and the results were normalized to the level of histone H4 transcripts.

Acknowledgements

We wish to thank Yoshiki Sasai and Sarah Cranston for critically reading the manuscript. We are indebted to Ken Cho and Bruce Blumberg for the BMP-4 cDNA, to Linda Gont for the Xnot-2 probe, to Bin Lu for the histological sections and to Anatalia Cuellar for technical assistance. H.S. was supported by a DFG postdoctoral fellowship. A.F. was supported by an American Cancer Society International Cancer Research Fellowship. This work was supported by grants from The Council for Tobacco Research (3332) to A.F. and the NIH (HD 21502-09) and the HFSPO to E.D.R.

References


Received on July 12, 1994; revised on August 9, 1994

Mesoderm patterning