Spatial and temporal properties of ventral blood island induction in *Xenopus* *laevis*

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SUMMARY

Questions of dorsoventral axis determination and patterning in *Xenopus* seek to uncover the mechanisms by which particular mesodermal fates, for example somite, are specified in the dorsal pole of the axis while other mesoderm fates, for example, ventral blood island (VBI), are specified at the ventral pole. We report here that the genes *Xvent-1*, *Xvent-2*, and *Xwnt-8* do not appear to be in the pathway of VBI induction, contrary to previous reports. Results from the selective inhibition of bone morphogenetic protein (BMP) activity, a key regulator of VBI induction, by ectopic Noggin, Chordin, or dominant negative BMP ligands and receptors suggest an alternative route of VBI induction. Injection of noggin or chordin RNA into animal pole blastomeres effectively inhibited VBI development, while marginal zone injection had no effect.

Cell autonomous inhibition of BMP activity in epidermis with dominant negative ligand dramatically reduced the amount of αT3 globin expression. These results indicate that signaling activity from the Spemann Organizer alone may not be sufficient for dorsoventral patterning in the marginal zone and that an inductive interaction between presumptive VBIs and ectoderm late in gastrulation may be crucial. In agreement with these observations, other results show that in explanted blastula-stage marginal zones a distinct pattern develops with a restricted VBI-forming region at the vegetal pole that is independent of the patterning activity of the Spemann Organizer.

Key words: *Xenopus*, Blood, Xvent, Mesoderm, Prepattern, Morphogenesis, Gastrulation

INTRODUCTION

Models of mesoderm development and patterning in *Xenopus* have proposed that cell autonomous and inductive events pattern the marginal zone such that dorsal mesoderm is specified near the early forming blastopore lip (the Spemann Organizer), and ventral mesoderm is specified at the opposite side of the marginal zone, where the blastopore lip forms last (Dale, 1997; Heasman, 1997; Kessler and Melton, 1994). This latter region has been called the ‘ventral marginal zone’. Recent reports have drawn into question this model of dorsoventral patterning in the marginal zone (Lane and Smith, 1999; Mills et al., 1999; Tracey et al., 1998). Specifically, the distribution of the presumptive dorsal and ventral mesoderm in the late blastula and early gastrula *Xenopus* embryo differs from what was proposed in these earlier models. It has been shown that the ventral blood islands (VBIs), which constitute the ventralmost mesoderm of the tadpole, arise not only from the ‘ventral marginal zone’, but also from mesoderm originally situated near the Spemann Organizer. Furthermore, blastomeres in the ‘ventral marginal zone’ contribute to both dorsal (somite) and ventral (VBI) posterior mesoderm (Lane and Smith, 1999). Likewise, dorsal and ventral anterior mesoderm arise from cells near the Spemann Organizer. Fate mapping at 16- and 32-cell stages has shown that the VBIs arise primarily from vegetal portions of the marginal zone (Lane and Smith, 1999; Mills et al., 1999). Mesoderm originating at the vegetal limit of the marginal zone, and presumably containing the VBI progenitors, constitutes the leading edge mesoderm. Maps of morphogenetic movements show that this tissue converges towards the ventral midline during gastrulation (Keller, 1991). In contrast to the VBIs, somite progenitors map to more animal blastomeres of the marginal zone (Lane and Smith, 1999). From these results, a new fate map has been proposed in which it is the anteroposterior, not the dorsoventral axis, that is established around the circumference of the marginal zone (Fig. 1). The disposition of the notochord axes in the marginal zone is more complicated and may not exactly parallel those of the non-axial mesoderm (Keller, 1991). These axes in the late blastula and gastrula embryo are approximate and only after the morphogenic movements of gastrulation and tailbud extension do the various mesoderm derivatives come to lie in their final dorsoventral positions. Based on the revised fate map, we will use the term ‘posterior marginal zone’ in place of ‘ventral marginal zone’, as has been suggested previously (Lane and Smith, 1999).

The finding that both dorsally and ventrally fated mesoderm
arise from all meridians of the marginal zone requires that we reconsider models of how the marginal zone is patterned. Studies into the molecular nature of mesoderm induction and patterning have largely interpreted results in the context of the three-signal model (Dale and Slack, 1987). According to this model, mesoderm is initially induced in a binary state. One sector centered around the early forming blastopore lip is specified as the Spemann Organizer, while the mesoderm around the remaining circumference of the marginal zone is specified as ventral (blood, mesenchyme and mesothelium). Soluble inducing factors secreted from the Spemann Organizer are proposed to result in the specification of dorsolateral fates, such as somites, pronephros and heart (Harland and Gerhart, 1997; Heasman, 1997; Kessler and Melton, 1994). Many models depict induction from the Spemann Organizer as a gradient of ‘dorsalizing factors’ that diffuse across the marginal zone. The distance of a marginal zone cell from the Spemann Organizer at gastrulation would then determine its dorsoventral identity. Thus the VBIs, which were proposed to arise from tissue furthest away from the Spemann Organizer, were thought to be specified by the absence of Organizer signaling. In molecular terms, dorsoventral patterning of the mesoderm is thought to result from antagonistic interactions between ventral mesoderm inducers bone morphogenetic protein 4 (BMP-4) and BMP-2 and their inhibitory binding proteins, including Chordin, Noggin and Follistatin, that are produced by the Spemann Organizer (Graff, 1997; Thomsen, 1997). Studies that have indicated a central role for BMP-4 in VBI induction are consistent with this model. Ectopic expression of BMP-4 in *Xenopus* embryos or animal caps stimulates blood cell formation and/or globin expression (Dale et al., 1992; Jones et al., 1992), while injection of an RNA encoding dominant negative BMP-4 receptor results in decreased blood formation (Graff et al., 1994; Maeno et al., 1996). These results appear to be consistent with the three-signal model in which the highest levels of BMP activity would be found in the blastula- and gastrula-stage marginal zone furthest from the Spemann Organizer, and would thus lead to VBI induction. However, as stated above, the VBIs arise from all meridians of the blastula embryo, not just those furthest from the Spemann Organizer at the blastula and early gastrula stages (Lane and Smith, 1999; Mills et al., 1999). During the period when these inductions are taking place, the embryo is undergoing extensive morphogenesis. The result is that, by the end of gastrulation, somites, including those in the posterior marginal zone, end up adjacent to the descendants of the Spemann Organizer, the notochord and head mesoderm. The VBIs, on the contrary, migrate as the leading edge mesoderm toward the ventral midline. Thus, the anterior VBIs, which started near the Spemann Organizer, end up on the ventral side of the embryo at the end of gastrulation. A complete model of dorsoventral mesoderm patterning must take into account these morphogenic movements that occur during gastrula-stage inductions and which continuously change the spatial relationship between induced tissue and inducer.

In this report, we have examined spatial and temporal aspects of VBI induction. First, we examined the activity of factors that are expressed in the blastula and gastrula ‘ventral marginal zone’, and found that the proposed blood inducers, Xvent-1 (Gawantka et al., 1995), Xvent-2 (Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996) and Xwnt-8 (Christian and Moon, 1993) all lacked VBI-inducing activity. While other results presented here are consistent with a role for BMP signaling in VBI induction, experiments with injected noggin, chordin or dominant negative BMP-7 (Cm-XBMP-7) RNA point to VBI-inducing factors coming from outside of the marginal zone, and perhaps not acting until late in gastrulation. In addition, experiments with explanted posterior marginal zones suggest that the marginal zone may be patterned along the animal-vegetal axis into regions that are fated to make the VBIs and those fated to make somites independent of the activity of the Spemann Organizer.

**MATERIALS AND METHODS**

**Xenopus embryos**

Ovulation was induced by injecting female *Xenopus* with human chorionic gonadotropin (Sigma) and eggs were fertilized as described previously (Condie and Harland, 1987). Embryos were dejellied 10 minutes after fertilization in 2% cysteine-HCl (pH 8.0), and staged according to Nieuwoop and Faber (1994).

**RNA synthesis and microinjection**

Plasmids for in vitro RNA synthesis were first linearized with restriction enzymes: NotI for Xvent-1, Xvent-2 and noggin (Gawantka et al., 1995; Onichtchouk et al., 1996; Smith and Harland, 1992). *Eco*RI for the dominant negative BMP-4 receptor (dnBMP-R), *Xho*I for the chordin, and *Xho*I for the dominant negative BMP-7 (*Cm*-XBMP-7) plasmids (Suzuki et al., 1994; Sasai et al., 1994; Hawley et al., 1995). Capped RNAs were transcribed using mMessage mMACHINE (Ambion). Dejellied fertilized eggs were transferred to 1/3x MMR/2.5% ficoll and injected by air pressure. Injections were made to 1-cell embryos with 10 nl containing either 1.0 ng of Xvent-1, 4.0 ng of Xvent-2, 1.0 ng of Xvent-1 and 4.0 ng of Xvent-2 RNAs or 100 pg of pCSKA-Xvent-8 (Smith et al., 1993). For injections at 32-cell stage, fertilized eggs were cultured in 1/10x MMR until 8-cell stage before being dejellied, and a single B1, C1, C4 or A4 blastomere was injected as above with 1 nl containing either 500 pg each noggin and GFP (or lacZ) RNAs, 50 pg of each pCSKA-noggin (Smith et al., 1995) and 500 pg of GFP RNA, 500 pg of each chordin and GFP (or lacZ) RNAs or 500 pg GFP (or lacZ) RNA alone as a control. Both C1, C4 or A4 blastomeres were also injected with 1 nl containing either 500 pg each *Cm*-XBMP-7 and GFP (or lacZ) RNAs or 500 pg...
each dnBMP-R and GFP (or lacZ) RNAs. The injected embryos were cultured in 1/3× MMR plus 10 μg/ml gentamycin until appropriate stages.

**Posterior marginal zone explants**

Embryos were injected at the 32-cell stage in both B4 blastomeres with 50 pg each GFP and lacZ RNAs. Dissection of posterior marginal zones at stage 9 was performed in 3/4 NAM (82.5 mM NaCl, 1.5 mM KCl, 0.75 mM Ca(NO₃)₂, 0.75 mM MgSO₄, 75 mM EDTA, 1.5 mM sodium phosphate (pH 7.5)) with 25 μg/ml gentamycin and 1 mg/ml BSA. The posterior sides of embryos were identified by GFP fluorescence. The dissected explants contained B4 (GFP-expressing), C4 and probably D4 descendants. The explants were cultured under the pressure of glass coverslips in 3/4 NAM with gentamycin/BSA until intact embryos reached stage 32.

**RNA extraction and northern blotting**

Total RNA was isolated from embryos at stage 24, 30/31 or 37/38 using Trizol Reagent (Gibco). For northern analysis, 10 embryos were homogenized together for each determination. One-tenth of the extracted RNA, about 5 μg, was electrophoresed on formaldehyde-containing agarose gels, with the exception of experiments for the detection of SCL transcript when approximately 20 μg was used. RNA was transferred to Hybond-N nylon membrane (Amersham) by capillary action overnight and hybridized with random primed 32p-labeled probes in QuikHyb (Stratagene). Probes were prepared using the Prime-a-Gene system (Promega) with isolated fragments from Xpo (Sato and Sargent, 1991), myoD (Hopwood et al., 1989), otx2 (Lamb et al., 1993), αT3 globin (Bancille and Williams, 1985), muscle actin (Dworkin-Rastl et al., 1986), SCL (Mead et al., 1998) and EF1α (Krieg et al., 1989) plasmids. The hybridized probes were visualized by autoradiography. For quantitative densitometry, short exposures were used. Densitometry readings were quantified using NIH Image software.

**β-galactosidase staining**

Embryos were fixed in MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO₄, 3.7% formaldehyde) for 20 minutes and rinsed three times in 1× PBS. Staining was performed in 1× PBS containing 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂ and 1 mg/ml X-gal at room temperature. Stained embryos were refixed in MEMFA for 1 hour, and then stored in 100% ethanol.

**In situ hybridization**

Detection of SCL and αT3 globin expression by in situ hybridization was performed as described previously (Harland, 1991).

**RESULTS**

**Neither Xvent-1, Xvent-2 nor Xwnt-8 induce ventral blood islands**

Overexpression and dominant negative studies have shown that the homeobox-containing factors Xvent-1 and Xvent-2 play essential roles in early embryonic patterning of Xenopus (Gawantka et al., 1995; Onichtchouk et al., 1996, 1998). Overexpression of the wild-type proteins leads to expanded expression of genes that are expressed in the posterior marginal zone such as Xwnt-8 and Xpo, while dominant negative Xvent-1 or Xvent-2 constructs result in expanded expression of organizer/notochord-specific genes such as goosecoid and Xnot. It was proposed that the activities of Xvent-1 and Xvent-2 were primarily ventralizing, and that the combined actions of endogenous Xvent-1 and Xvent-2 in ventral mesoderm would lead to blood formation (Onichtchouk et al., 1998). However, the blood-inducing activity of Xvent-1 and Xvent-2 in intact embryos has not been directly tested, and Xvent-2 does not induce globin expression in activin-treated animal caps (Ladher et al., 1996).

In order to reassess the ventralizing activities of Xvent-1 and Xvent-2, as well as Xwnt-8, which is also expressed in the gastrula posterior marginal zone (Christian and Moon, 1993), embryos were injected with Xvent-1 or Xvent-2 RNA, or pCSKA-Xwnt-8, and assayed for αT3 globin expression by in situ hybridization at stage 37/38. The results show that the expression of this ventral mesoderm marker is actually decreased by Xvent-1 (Fig. 2B), Xvent-2 RNA (Fig. 2C) or pCSKA-Xwnt-8 (Fig. 2D) injection in comparison to GFP RNA-injected control embryos (Fig. 2A). The phenotype of Xvent-1 or Xvent-2 RNA- or pCSKA-Xwnt-8-injected embryos is consistent with previous reports and is characterized by the loss of anterior structures. To examine the activity of Xvent-1, Xvent-2 and Xwnt-8 in greater detail, the expression of αT3 globin, as well as a number of other genes that mark the anterior/posterior and dorsoventral axes at stages 30/31 and 37/38, were assessed by northern blotting. Consistent with the results from the in situ hybridization, we observed a dramatic reduction in the expression of αT3 globin at stage 30/31 in embryos injected with Xvent-1 or Xvent-2 RNA or with a combination of the two (Fig. 3A). Xwnt-8 expressed from the actin promoter construct caused a similar decrease in αT3 globin expression (Fig. 3A). Results from two independent experiments were quantified by densitometry and are shown in
overexpression of Xwnt-8 using the cytoplasmic actin promoter also led to higher expression of Xpo (Fig. 3A). At stage 30/31, the induction of Xpo ranged from 2.7-fold for Xvent-1 RNA-injected embryos, to 8.2 fold for embryos injected with Xvent-1 and Xvent-2 RNAs (Fig. 4A). On the contrary, the expression of the anteriorly expressed gene otx2 was generally decreased by Xvent-1, Xvent-2 or Xwnt-8, although there was some variability in the results (Figs 3A, 4B). Taken together, these results are consistent with Xvent-1, Xvent-2 and Xwnt-8 having posteriorizing activity rather than ventralizing, which is in agreement with their posterior domain of expression.

We also examined the expression of somite markers myoD and muscle actin in Xvent-1, Xvent-2, mixed Xvent-1 and Xvent-2 RNAs or pCSKA-Xwnt-8-injected embryos. We observed that at stage 30/31 the expression of muscle actin is reduced relative to controls (Figs 3A, 4E). This would appear to be consistent with the reduction in muscle actin expression by Xvent-1 or Xvent-2 RNA injection observed at stage 19 in dorsal marginal zones (Gawantka et al., 1995; Onichtchouk et al., 1996). However, by stage 37/38 the expression level of muscle actin was elevated to levels similar to those of controls (Figs 3B, 4G), suggesting that the action of Xvent-1 and Xvent-2 may be retarding somite development, rather than inhibiting it. The results with myoD expression at stage 30/31 appear at first to be at odds with the muscle actin results. In contrast to the results for muscle actin transcription, Xvent-1, Xvent-2, Xvent-1 plus Xvent-2 RNAs, or pCSKA-Xwnt-8 injection increased myoD expression (Figs 3A, 4D). However, myoD is expressed in the somites at stages 30 to 32 in a graded manner, with highest levels observed in the posterior (Dorsch et al., 1997; Frank and Harland, 1991). Again, these results point to the activity of Xvent-1 and Xvent-2 retarding, not inhibiting, somite development.

As demonstrated in Figs 2, 3 and 4, injection of Xvent-1 or Xvent-2 RNA or pCSKA-Xwnt-8 causes reduction in αT3 globin expression. Because αT3 globin is expressed very late in the differentiation of the VBIs, we wanted to determine if other, earlier expressed genes in the pathway of blood development were disrupted by these same treatments. The basic helix-loop-helix factor SCL, which is the earliest known definitive marker of the VBIs, can be detected as early as stage 15 (Mead et al., 1998). Both in situ hybridization and northern analysis showed that overexpression of Xvent-2 or Xwnt-8 decreases SCL expression compared to GFP RNA-injected control embryos (Fig. 5A, B). In contrast, Xvent-1 RNA-injected embryos showed a range of SCL expression levels (Fig. 5A-C). Of 18 embryos assayed by in situ hybridization, five showed elevated SCL levels (Fig. 5A; Xvent-1, left embryo), four showed decreased expression (Fig. 5A; Xvent-1, right embryo), while the remaining nine had levels close to controls (Fig. 5A; Xvent-1, center embryo). While Fig. 5A shows the variability in levels of SCL expression between individual embryos injected with Xvent-1 RNA, the results from northern analysis using RNA pooled from 7 to 10 individuals shows the variability between independent experiments (Fig. 5C). In four experiments, two showed reduced SCL expression and two showed elevated expression.

The reason for the variability in the expression of SCL at stage 24 in response to Xvent-1 overexpression is not clear, although the results from Fig. 4 indicate that the precise timing

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**Fig. 3.** Xvent-1, Xvent-2 and Xwnt-8 have a posteriorizing, not ventralizing, effect. Eggs were microinjected with 1.0 ng of Xvent-1 RNA, 4.0 ng of Xvent-2 RNA, 1.0 ng of Xvent-1 plus 4.0 ng of Xvent-2 RNAs, or 100 pg of pCSKA-Xwnt-8, and cultured until uninjected control embryos reached stage 30/31 (A), or 37/38 (B). RNA was isolated and analyzed by northern blotting for the expression of Xpo, myoD, otx2, αT3 globin and muscle actin. EF1α serves as a loading control.

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**Fig. 4.** The values for all of the densitometry readings were adjusted for the RNA loading control (EF1α).

While the Xvents and Xwnt-8 dramatically reduced αT3 globin expression levels at stage 30/31, by stage 37/38 the expression of globin in the injected embryos had increased considerably, although it was still lower than control embryos (Figs 3B, 4F). Thus, the expression of αT3 globin in Xvent-1 or Xvent-2 RNA or pCSKA-Xwnt-8-injected embryos is not only decreased, but also retarded.

As a marker for posterior tissue we used Xpo expression. Xpo is first expressed at gastrula stage throughout the marginal zone except for the Spemann Organizer. At neurula stage, its expression is restricted to posterior mesoderm and ectoderm (Amaya et al., 1993; Sato and Sargent, 1991) and remains posteriorly restricted up through stage 30/31 as assayed by in situ hybridization (our data, not shown). We observed that Xpo expression was increased at stage 30/31 by the injection of Xvent-1, Xvent-2 or mixed Xvent-1 and Xvent-2 RNAs, in comparison to uninjected embryos (Fig. 3A). In addition,
of measurement is crucial. Nevertheless, there was no obvious trend either towards inhibition or stimulation of SCL expression by Xvent-1 RNA injection, while by stage 30/31 Xvent-1-injected embryos show a consistent reduction in αT3 globin expression, as do Xvent-2 RNA- or pCSKA-Xwnt-8-injected embryos. The response to Xvent-1 overexpression clearly differs from those of Xvent-2 or Xwnt-8 overexpression and suggests that Xvent-1 overexpression has no net effect on VBI induction and may inhibit later differentiation.

The posterior marginal zone may be prepatterned along the animal-vegetal axis

It is well established that dissected posterior marginal zone will not give rise to muscle unless exposed to the Spemann Organizer (Lettice and Slack, 1993), or to a factor that mimics the activity of the Spemann Organizer such as Noggin (Smith et al., 1993). These results would appear to be consistent with the three-signal hypothesis. Lineage tracing of 32-cell-stage embryos has shown that the blastomeres located in the posteriormost marginal zone, B4 and C4, give rise to posterior somites, and posterior VBIs and somites, respectively (Lane and Smith, 1999). The three-signal model would predict that while the fates of these two blastomeres are different, they transiently pass through a period in the late blastula embryo during which they have equivalent states of specification (Harland and Gerhart, 1997; Heasman, 1997). In order to examine the state of specification of the posterior marginal zone at the late blastula stage, we examined the capacity of B4 and C4 descendants to form blood when dissected from the embryo at late blastula stage, and thus removed from the influence of the Spemann Organizer. To perform this experiment, embryos were grown to the 32-cell stage, at which time both B4 blastomeres were injected with a mixture of green fluorescent protein (GFP) and lacZ RNAs. At stage 9, posterior marginal zones were dissected using a fluorescent stereomicroscope to visualize the GFP fluorescence. The dissections were centered in the marginal zone (Fig. 6A) such that the animal half of the explant contained the fluorescing B4 descendants, while the vegetal half contained the descendants of C4 (and possibly D4). The explants were grown to stage 32 under the pressure of glass cover slips to minimize folding of the explants as they healed. At stage 32, the explants were stained with X-gal to mark the B4 descendants, and αT3 globin expression was detected by in situ hybridization (Fig. 6B). While there was spreading of the B4 descendants through the explants, perhaps due to ectoderm cells migrating around and covering the explants, the X-gal staining was centered and extended down from the animal pole. Using this landmark, we observed that the αT3 globin expression was restricted to the maternal most region of the explants (Fig. 6B). Thus the B4 and C4 blastomeres, which in the blastula and early gastrula embryo are furthest from the Spemann Organizer, do not appear to have had equivalent states of specification when dissected, and only a portion of the posterior marginal zone is specified as presumptive VBI. In agreement with the lineage tracing in whole embryos (Lane and Smith, 1999; Mills et al., 1999), this region is offset towards the vegetal pole of the marginal zone. This result suggests that significant patterning of the posterior marginal zone may occur before and/or independently of the dorsalizing activity of the Spemann Organizer.

Spatial and temporal induction of the ventral blood islands

Studies with dominant negative BMP-4 receptors have shown that BMP signaling is essential for blood development (Graff et al., 1994; Maeno et al., 1996). The origin of VBI cells from near the Spemann Organizer, a rich source of BMP-inhibiting signals, would appear to be contradictory. It is possible that the induction of the VBIs by BMPs does not occur until late gastrulation or neurula stages, at which point all presumptive VBIs have migrated away from the Spemann Organizer derivatives and toward the ventral midline. In fact, SCL, which is the earliest known definitive marker of the VBIs, is not expressed until stage 15 (Mead et al., 1998).

In order to reassess the spatial and temporal induction of the VBIs by BMPs, we first examined how overexpression of Noggin, a BMP antagonist, would alter VBI induction when injected into various blastomeres of 32-cell-stage embryos. Injection of 500 pg of noggin RNA into blastomeres that give rise to the Spemann Organizer [B1 or C1 (Vodicika and Gerhart, 1995)] yielded a surprising result. Neither B1 nor C1 noggin RNA-injected embryos showed any reduction in globin expression, as determined by in situ hybridization and northern blotting (Figs 7A, 8A,B,D,E). Also, the overexpression of noggin in the B1 or C1 blastomeres resulted in no apparent phenotypic change in the embryos, despite the fact that this dose of noggin RNA is sufficient to rescue UV-treated embryos or to induce neural tissue when injected into animal caps. Additionally, coinjecting a lineage tracer (lacZ RNA) shows that the noggin RNA injections did not alter the lineages of those blastomeres: B1 gave rise to notochord while C1 became head mesoderm (Fig. 8B,E, compared to Fig. 8A,D, respectively).

C4 descendants make up most of the posterior marginal zone at stage 10 and, according to the models mentioned above, we would expect that noggin RNA injection into C4 blastomeres would lead to decreased globin expression. While the injected noggin RNA did result in partial secondary axes, the expression of αT3 globin was similar to control injected embryos (Figs 7A, 8J). Additionally, the noggin RNA injection changed the fate of C4 progeny from primarily posterior mesoderm (Fig. 8I) to axial mesoderm extending from the tail through the secondary axes (Fig. 8J).

In contrast to the results of noggin overexpression in B1, C1 and C4, injection of noggin RNA into A4 blastomeres, which give rise to trunk epidermis (Fig. 8N), dramatically reduced globin expression, despite the tadpoles being otherwise phenotypically normal (Figs 7A, 8O). The same result was obtained when one A4 blastomere was injected with pCSKA-noggin plasmid (Fig. 7A), which drives the expression of noggin at stage 10 (Smith et al., 1993). While A4 blastomeres do have a minor contribution to the VBIs (Lane and Smith, 1999), these results point to the descendants of the A4 blastomeres as being important sources of inductive factors in the VBI formation, and are in agreement with previous results demonstrating that animal cap tissue has blood-inducing activity (Maeno et al., 1994). Chordin, another BMP antagonist, was less effective than Noggin at inhibiting αT3 globin expression in similar experiments (Figs 7B, 8C,F,K,P), although, as with noggin, only A4 injection decreased expression.

Because the leading edge mesoderm and A4 descendants do
not become juxtaposed until late in gastrulation, the chordin and noggin overexpression results provide important clues to the temporal induction of the VBIs (see Discussion). However, they do not distinguish whether the source of BMP activity inhibited by these factors is derived from the A4 descendants as opposed to the underlying leading edge mesoderm itself. In order to examine this issue, we injected either C1, C4 or A4 blastomeres with Cm-XBMP-7 RNA. Cm-XBMP-7 is a dominant negative version of Xenopus BMP-7 (Hawley et al., 1995). Cm-XBMP-7 lacks the proteolytic processing site required for the synthesis of active dimers and will cell-autonomously result in the synthesis of inactive Cm-XBMP-7/BMP-7 homodimers, and probably Cm-XBMP-7/BMP-2 and Cm-XBMP-7/BMP-4 heterodimers as well (see Discussion) (Nishimatsu and Thomsen, 1998). The result shows that Cm-XBMP-7 is most effective at inhibiting aT3 globin expression when injected into A4 (Figs 7C, 8G,L,Q).

Fig. 4. Quantitative analysis of the northern blots. Relative expression levels of Xpo, myoD, otx2, muscle actin and aT3 globin in response to injection of Xvent-1 RNA (Xvent-1), Xvent-2 RNA (Xvent-2), Xvent-1 plus Xvent-2 RNAs (1+2), or pCSKA-Xwnt-8 (Xwnt-8) are shown. Optical densities of the bands from the northern blots were calculated using NIH Image software. The values, after adjusting for total RNA loading by EF1α hybridization intensity, are shown for two independent experiments. (A) Xpo, (B) otx2, (C) aT3 globin, (D) myoD and (E) muscle actin at stage 30/31. Expressions of (F) aT3 globin and (G) muscle actin at stage 37/38 are also shown.

Fig. 5. SCL expression in Xvent-1 RNA, Xvent-2 RNA or pCSKA-Xwnt-8-injected embryos. Fertilized eggs were microinjected with 1.0 ng of GFP RNA, 1.0 ng of Xvent-1 RNA, 4.0 ng of Xvent-2 RNA or 100 pg of pCSKA-Xwnt-8, and the injected embryos were assayed for SCL expression at stage 24 by (A) in situ hybridization, and (B) northern analysis. (A) Xvent-1-injected embryos showed the greatest degree of variation and examples of elevated (left), control-like (center) and reduced (right) levels of expression are shown. (C) For Xvent-1 injection, optical densities of bands from northern blots were calculated using NIH image software, and the values are shown for four independent experiments after adjusting for total RNA loading by EF1α hybridization intensity.
and explants. Both B4 blastomeres were injected with 50 pg each (A) Procedure for labeling and isolation of posterior marginal zone marginal zone explants were stained for control embryos reached stage 32. (B) Representative posterior progeny. The dissected explants were cultured under coverslips until GFP expression to direct dissection of an explant containing B4 posterior marginal zones were dissected under fluorescence, using which are expressed in the gastrula posterior marginal zone and are attributed with ventralizing activity (Gawantka et al., 1995; Ladher et al., 1996; Onichtchouk et al., 1996, 1998; Schmidt et al., 1996), and Xwnt-8, which is also expressed in the gastrula posterior marginal zone (Christian and Moon, 1993). While Xvent-1 and Xvent-2 overexpression can expand the expression of genes centered around the gastrula posterior marginal zone, such as Xpo and Xwnt-8, overexpression of Xvent-1, Xvent-2 or Xwnt-8 fails to lead to an expansion of the definitive ventral mesoderm marker globin, and also fails to decrease the expression of the dorsal mesoderm marker muscle actin. While there is a transient decrease in muscle actin expression, by stage 37/38 tadpoles overexpressing these factors express muscle actin at control levels. In fact, studies with dominant negative Xwnt-8 show that Xwnt-8 signaling is essential for somite induction (Hoppler et al., 1996).

When assessed at tailbud and tadpole stages, the activities of Xvent-1, Xvent-2 and Xwnt-8 can best be described as posteriorizing. As has been well documented previously (Christian and Moon, 1993; Gawantka et al., 1995; Onichtchouk et al., 1996), the overexpression of any one of these three factors leads to a reduction in head structures. As expected, we observed a reduction in otx2 and an expansion in Xpo expression upon Xvent-1, Xvent-2 or Xwnt-8 overexpression. Because the expression of these three genes is centered around the posterior marginal zone, this posteriorizing activity is not unexpected. Overexpression of Xvent-1, Xvent-2 or Xwnt-8 retards the expression of αT3 globin and muscle actin. This may also be indicative of posteriorizing activity by these molecules. The retardation is probably a manifestation of the delayed gastrulation in the injected embryos (Gawantka et al., 1995). In the injected embryos, gastrulation initiates around the delayed gastrulation in the injected embryos (Gawantka et al., 1995). These activities of Xvent-1, Xvent-2 and Xwnt-8 are essential for somite induction (Hoppler et al., 1996).
Ectodermal BMPs may be essential for VBI induction

Our results suggest that ectodermal cells may be an important source of BMPs in VBI induction. We observed that overexpression of Noggin or Chordin, potent inhibitors of BMPs (Piccolo et al., 1996; Zimmerman et al., 1996), or Cm-XBMP-7, a dominant negative version of *Xenopus* BMP-7 (Hawley et al., 1995), is most effective at inhibiting blood induction when ectopically produced in the epidermis (A4 descendants) of the 32-cell-stage embryo, rather than in the mesoderm (B1, C1 or C4 descendants). Specifically, since Cm-XBMP-7 is known to act cell autonomously to block BMP signaling, the result from the A4 Cm-XBMP-7 injection strongly suggests that the ectodermal cells are a source of VBI-inducing factors. This is consistent with the previous results showing that the animal pole explants stimulate blood differentiation in marginal zone explants (Maeno et al., 1994).

The results with ectopic Noggin and Chordin provide clues to the timing of VBI induction. Significantly, the descendants of A4 are not juxtaposed to the presumptive VBIs in the leading edge mesoderm until late gastrulation (Keller, 1991; Lane and Smith, 1999). Although it is possible that A4-derived Noggin could be acting on the marginal zone at blastula or late gastrula stage, it has been shown that injection of noggin RNA into marginal zone blastomeres efficiently rescues axis formation in UV-treated embryos, while animal pole injection does not (Smith and Harland, 1992). This result would argue that, despite the ability of Noggin to diffuse within the embryo (Jones and Smith, 1998), long-distance signaling of Noggin between the animal pole and the marginal zone is unlikely, and that close contact of the tissues is required. Thus, induction of the VBIs may not initiate until late in gastrulation. In fact, noggin expression in the A4 blastomere driven by the cytoskeletal actin promoter, which initiates at stage 10, is even more effective at inhibiting globin expression than is noggin RNA injection into 32-cell-stage embryos.

If indeed VBI induction does not begin until late gastrulation and requires contact with ectodermal cells, the ability of cells originating near the Spemann Organizer to populate the VBIs may not be problematic. The presence of the presumptive VBI mesoderm adjacent to the Spemann Organizer during late blastula and early gastrula stages initially appears paradoxical. The induction of the VBIs requires BMP signaling, yet the Spemann Organizer is a rich source of BMP-inhibiting factors. Our results show that neither overexpression of noggin nor chordin in the Spemann Organizer, nor their mis-expression in the posterior marginal zone, has an impact on the amount of differentiated VBI. However, the VBIs are not refractory to the

Fig. 7. Effects of inhibitors of the BMP signaling on αT3 globin expression in whole embryos. 32-cell-stage *Xenopus* embryos were injected in a single B1, C1, C4 or A4 blastomere with 500 pgs of noggin and GFP RNAs, 500 pgs of chordin and GFP RNAs or 500 pg of GFP RNA alone as a control, and also injected in the single A4 blastomere with 50 pg of pCSKA-noggin and 500 pg of GFP RNA. Other embryos were injected in both C1, C4 or A4 blastomeres with 500 pgs of Cm-XBMP-7 and GFP RNAs or 500 pgs of dnBMP-R and GFP RNAs. The injected embryos were allowed to develop to stage 37/38, at which time RNAs were isolated and analyzed by northern blotting for αT3 globin expression. EF1α serves as a loading control. (A) noggin-injected embryos; (B) chordin-injected embryos; (C) Cm-XBMP-7-injected embryos; (D) dnBMP-R-injected embryos.
BMP-inhibiting activities; *noggin* or *chordin* overexpression in the ectoderm will reduce the level of *globin* expression. Therefore, it is most likely that the presumptive VBIs derived from C1, which either no longer express *noggin* and *chordin* after stage 10, or never did, migrate away from the Organizer during gastrulation and escape the range of Organizer-derived BMP inhibitors to become blood. The presumptive VBI cells are either unresponsive to the activity of the Spemann Organizer early in gastrulation, or insufficient quantities of secreted Spemann Organizer factors have accumulated before the cells have migrated away.

**Identity of epidermis-derived BMP activity is not certain**

For the experiments presented here, we chose to use the dominant negative BMP-7 because this construct has only inhibitory activity, as opposed to similar constructs of BMP-4, which appear to have weak stimulatory activity, probably due to the presence of alternative cleavage sites (Nishimatsu and Thomsen, 1998). The dominant negative *Xenopus* BMP-7 protein likely has broad inhibitory activity, as suggested by the ability of either BMP-2, BMP-4 or BMP-7 to rescue the phenotype caused by Cm-XBMP-7 (Nishimatsu and Thomsen, 1998). Similarly, the dominant negative BMP-4 receptor has broad inhibitory activity (Suzuki et al., 1994). Thus, we cannot identify the particular molecules responsible for the epidermis-derived BMP activity, although several reports indicate a role for heterodimers, particularly BMP-4/BMP-7, in *Xenopus* mesoderm induction and patterning (Nishimatsu and Thomsen, 1998; Suzuki et al., 1997).

**A prepattern in the posterior marginal zone?**

Previous experiments have shown that excised posterior marginal zones express *globin* (Kelley et al., 1994), and that they can be induced to express *muscle actin* if combined with a Spemann Organizer or exposed to Noggin or other BMP inhibitors (Lettice and Slack, 1993; Smith et al., 1993). Our result has shown that the *globin* expression in these explants is restricted to the vegetal pole, despite the fact that BMPs are widely expressed in the posterior marginal zone and ectoderm (Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995). These results argue against the hypothesis that all
mesoderm in the posterior marginal zone initially passes through a state in which it is specified as blood before being exposed to the Spemann Organizer. Additionally, these results indicate that patterning of the posterior marginal zone into a globin-expressing mesoderm and non-globin-expressing mesoderm can occur independently of the Spemann Organizer. We hypothesize that the marginal zone may already be patterned along the animal/vegetal axis by the late blastula stage, the stage when the explants were dissected. The leading edge mesoderm, although perhaps not yet specified as VBI, may be prepatterned to respond to ectoderm-derived BMPs during gastrulation to differentiate as blood. One possibility is that presumptive VBI cells originating near the Spemann Organizer may, due to the prepattern, be able to respond to BMP signaling to differentiate as blood but be unable to respond to the inhibition of BMPs to differentiate as dorsal mesoderm. In accordance with this hypothesis, suppression of VBI differentiation by A4 injection of noggin or chordin RNA does not lead to an expansion of dorsal (muscle actin) or lateral (pronephros) mesoderm (data not shown). In fact the phenotype of these embryos was indistinguishable from controls, with the exception of decreased globin expression. Thus, the cells that make up the ventral-most mesoderm of these embryos, while not differentiating as blood when BMP signaling is inhibited, may not be competent to make dorsal mesoderm. These results suggest a two-step process in VBI formation. In the first step, the prepattern establishes a territory at the vegetal limit of the blastula marginal zone that is competent to respond to inducers to differentiate as blood. In the second step, the morphogenetic movements of gastrulation bring the presumptive VBI-expressing cells into contact with ectoderm which induces blood differentiation by the BMP signaling pathway.

Might somitic mesoderm also be specified in a two-step process? The mesoderm in the posterior marginal zone explants that did not express globin was situated toward the animal pole of the explant, and thus contains cells that would have contributed to posterior somites had the tissue not been explanted. Although we cannot be certain of the differentiated state of these cells, they are neither somite nor VBI. Perhaps they are an intermediate type of mesoderm such as lateral plate mesoderm, for which no molecular markers are known in Xenopus. While it is known that these cells can and do respond to the Spemann Organizer to make somites, is prepatternning at the blastula stage required for them to respond differently than presumptive VBIs? One clue that a dorsal identity may be specified in these cells by the blastula stage is the observation that myoD is widely expressed around the marginal zone at the onset of gastrulation in a Spemann Organizer-independent manner (Harvey, 1992; Frank and Harland, 1991). However, these cells will lose myoD expression unless induced by the Spemann Organizer. Antibody staining has shown that at stage 10.5, there is no detectable myoD protein in the leading edge mesoderm, although staining extends from the posterior marginal zone laterally to the Spemann Organizer (Harvey, 1992). Because stage 10.5 posterior marginal zone explants do not autonomously make muscle (Smith et al., 1993) and thus have not yet come under the influence of the Spemann Organizer, the animal/vegetal pattern of myoD protein in the marginal zone would appear to be established independently of the Spemann Organizer. This raises the possibility that myoD expression, which starts at late blastula stage, may initiate in a Spemann organizer-independent prepattern that delineates the future somitic mesoderm.

In summary, we have made three principal observations regarding VBI induction and dorsoventral mesoderm patterning in Xenopus. First, we have found that, while Xvent-1, Xvent-2 and Xwnt-8 are frequently cited as markers and/or inducers of ventral mesoderm, including VBI, their activity can best be described as posteriorizing. The revised Xenopus fate map depicts that the expression domains of these genes is centered on the posterior pole of the blastula and gastrula embryo, and would appear to be in agreement with the results presented here. The second observation is that induction of the ventral blood islands may not occur until late in gastrulation, and that ectoderm-derived BMPs may be essential for proper induction. Finally, our posterior marginal zone explant cultures suggest that the marginal zone does not exist in a binary state, and that patterning can occur independently of the Spemann Organizer.

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Xenopus ventral blood island induction