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# Ventral mesoderm induction and patterning by bone morphogenetic protein heterodimers in *Xenopus* embryos

Shin-ichiro Nishimatsu, Gerald H. Thomsen*

*Department of Biochemistry and Cell Biology, Institute of Cell and Developmental Biology, State University of New York, Stony Brook, NY 11794-5215, USA*

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**Abstract**

Bone morphogenetic proteins (BMPs) perform diverse functions in vertebrate development. Here we demonstrate that the heterodimeric BMP-4/7 protein directly induces ventral mesoderm and blood in *Xenopus* animal caps, and BMP-2/7 heterodimers may function similarly. We also provide indirect evidence that BMP heterodimers function in embryos, using assays with dominant-negative BMP ligands. Homodimeric BMP-2 and BMP-4 proteins do not induce mesoderm, but they ventralize mesoderm induction by activin. In contrast, BMP-7 protein interferes with mesoderm induction by activin, but BMP-7 stimulates ventral mesoderm induction by the heterodimer, BMP-4/7. This novel property of BMP-7 distinguishes it from other BMPs. BMP-7 may therefore function in early embryogenesis to antagonize activin signals and potentiate BMP signals. We propose that BMP heterodimers convey signals for ventral mesoderm induction and patterning in *Xenopus* development. © 1998 Elsevier Science Ireland Ltd. All rights reserved

*Keywords:* Bone morphogenetic protein-2; Bone morphogenetic protein-4; Bone morphogenetic protein-7; Bone morphogenetic protein-4/7; TGF-$\beta$; Blood; Dominant-negative; Development; Dorsalization; Embryogenesis; Erythropoiesis; Growth factor; Heterodimer; Induction; Mesoderm; Pattern formation; Ventralization; *Xenopus laevis*

## 1. Introduction

In amphibian embryos, mesoderm is induced within the equatorial region of the blastula by intercellular signaling between the animal hemisphere (prospective ectoderm) and the vegetal hemisphere (prospective endoderm). This primary signal for mesoderm induction is provided by maternal substances (mRNA or proteins) stored in the egg, that encode or directly correspond to, soluble factors secreted by the vegetal cells. After mesoderm induction, patterning signals within the nascent mesoderm pattern it across the dorsal–ventral axis (for reviews, see Harland, 1994; Kessler and Melton, 1994; Klein and Melton, 1994; Slack, 1994; Hogan, 1996). A variety of mesoderm-inducing factors have been identified and they include members of the transforming growth factor-$\beta$ (TGF-$\beta$), and fringe growth factor (FGF) families (Wu et al., 1996 and reviews cited above). Factors that pattern the mesoderm include the ventralizing factors BMP-4 and Xwnt8, and dorsalizing substances such as noggin, chordin and follistatin, that antagonize BMP ligands, and frzb, an antagonist of Xwnt8 (reviewed by Hemmati-Brivanlou and Melton, 1997; Moon et al., 1997; Sasai and De Robertis, 1997; Thomsen, 1997).

Activins, Vg1, and various BMPs are members of the TGF-$\beta$ family, and proteins and mRNAs for these factors are present in *Xenopus* eggs, blastula and later-stage embryos. Vg1 mRNA, in particular, is localized to the vegetal hemisphere of the egg, and it is preferentially translated in cells cleaved from that region (Rebagliati et al., 1985; Tannahill and Melton, 1989). mRNAs for BMP-2, BMP-4, and BMP-7 are present, but not localized to any significant degree in the egg and blastula (Dale et al., 1993; Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995). The TGF-$\beta$ factors have distinct mesoderm-inducing properties: activins and Vg1 can induce a spectrum of ventral–dorsal mesoderm in a dose–dependent manner (Green et al., 1992; Kessler and Melton, 1995), and both factors can induce

* Corresponding author. Tel.: +1 516 6328536; fax: +1 516 6328575; e-mail: jthomsen@mcbsgi.bio.sunysb.edu

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secondary dorsal–anterior axial structures when overexpressed in the ventral marginal zone of the embryo (Thomsen et al., 1990; Dale et al., 1993; Thomsen and Melton, 1993). In contrast, BMP-2, BMP-4 and BMP-7 have been shown to induce ventral mesoderm in *Xenopus* animal caps when microinjected as mRNAs, however direct exposure of animal caps to purified BMP homodimer proteins does not result in any significant degree of mesoderm induction (Dale et al., 1992; Jones et al., 1992; Clement et al., 1995; Hemmati-Brivanlou and Thomsen, 1995; Wilson and Hemmati-Brivanlou, 1995).

Although BMP homodimers are ineffective mesoderm-inducers, BMP-4 protein has been shown to ventralize the effects of a dorsal mesoderm-inducing dose of activin in animal caps, and ectopic expression of BMP-4 within the dorsal marginal zone of *Xenopus* embryos causes ventralization (Dale et al., 1992; Jones et al., 1992; Jones et al., 1996; Hemmati-Brivanlou and Thomsen, 1995). Confirmation that BMPs function in ventral mesoderm patterning has been provided by loss-of-function assays with ectopically-expressed dominant-negative BMP receptors, dominant-negative ligands, and antisense BMP RNA (Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994; Hawley et al., 1995; Ishikawa et al., 1995; Steinbeisser et al., 1995). The ventralizing effects of BMP-4 can be antagonized by the zygotic dorsalizing factors noggin, follistatin and chordin, which bind to and inactivate BMP proteins (Holley et al., 1996; Piccolo et al., 1996; Zimmerman et al., 1996; Fainsod et al., 1997). In the ectoderm, BMP-4 regulates the decision between epidermal and neural cell fates, and BMP-4 might play a role in endoderm formation (reviewed by Hemmati-Brivanlou and Melton, 1997; Thomsen, 1997; Sasai and De Robertis, 1997). These patterning properties of BMP-4 are consistent with its zygotic expression domains, which include the non-neural ectoderm and ventral–posterior mesoderm during gastrulation (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995).

Of the three maternal and early embryonic BMPs in *Xenopus*, BMP-4 has been most widely studied, while the functions of BMP-2 and BMP-7 have not been well characterized. The potential influence of BMP heterodimers in embryonic development has also received little attention. Ligands in the TGF-$\beta$ growth-factor family are active as dimers composed of identical or different subunits, and the subunit composition can dramatically affect ligand activity. In particular, BMP heterodimers may differ in activity from BMP homodimers. In mammalian systems, a subunit of BMP-7 (or OP-1) can dimerize with subunits of either BMP-2 or BMP-4, to form BMP-2/7 or BMP-4/7 (Sampath et al., 1990; Hazama et al., 1995). In bone-induction assays, BMP heterodimers are more potent inducers than BMP-2, BMP-4 and BMP-7 homodimers (Aono et al., 1995).

In the present study we have focused attention on the question of whether BMP heterodimers might be involved in mesoderm induction and patterning in *Xenopus* development. We have used purified BMP proteins and dominant-negative BMP ligands to test the possibility that BMP heterodimers function in mesoderm induction and patterning. We report here that a heterodimeric BMP-4/7 protein, and possibly BMP-2/7, directly induces mesoderm in *Xenopus* animal caps, but homodimeric BMPs, or mixtures of homodimers, do not induce mesoderm. We find that BMP-2 and BMP-4 proteins ventralize animal cap responses to the dorsal mesoderm inducer, activin, and that BMP-7 (OP-1) interferes with mesoderm induction by activin. BMP-7, however, potentiates mesoderm induction by BMP-4/7. We provide evidence that BMP heterodimers may function in ventral mesoderm induction and patterning by ectopic expression of dominant-negative BMP-2, BMP-4, and BMP-7 ligands in embryos and animal caps. Our findings suggest that BMP heterodimers can function both as direct mesoderm inducers and as ventralizing agents in the embryo, while BMP homodimers function not as primary mesoderm inducers, but as mesodermal patterning agents. We propose that spatial control over BMP ligand dimerization regulates whether BMPs function as heterodimers that induce mesoderm, or as homodimers that pattern mesoderm.

## 2. Results

### *2.1. Mesoderm induction by heterodimeric BMP-4/7 but not homodimeric BMPs*

Studies on bone induction have shown that the activity and potency of BMPs can differ, and that heterodimeric BMPs exhibit strong bone-inducing activity (Aono et al., 1995; Hazama et al., 1995). These observations prompted us to perform a side-by-side evaluation of various recombinant homodimer and heterodimer BMP proteins in mesoderm-induction assays. *Xenopus* blastula-stage animal caps were exposed to various concentrations of homodimeric BMP-7 (OP-1) or BMP-4, and mesoderm induction was scored by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis using various mesodermal markers. Fig. 1A shows that BMP-4 and BMP-7 (OP-1) proteins applied to animal caps beginning at stage 8 did not induce mesoderm at doses up to 1.0 $\mu$g/ml. We also tested whether a mixture of homodimeric BMP-4 and BMP-7 (OP-1) proteins induce mesoderm, since *Xenopus* embryos simultaneously express several BMPs at blastula and later stages (e.g. Nishimatsu et al., 1992; Ueno et al., 1992). The results of those tests, however, were negative (Fig. 1A). Similarly, BMP-2 alone, or in combination with BMP-4 or BMP-7, did not induce mesoderm (data not shown). We tested a preparation of purified recombinant BMP-4/7 heterodimer in the same animal cap assays and found that the general mesoderm marker, brachyury (XBra), and the ventral–posterior mesodermal markers Xwnt-8, Xhox3, and Xcad3, were induced at a dose of 100 ng/ml (Fig. 1A). Induction of

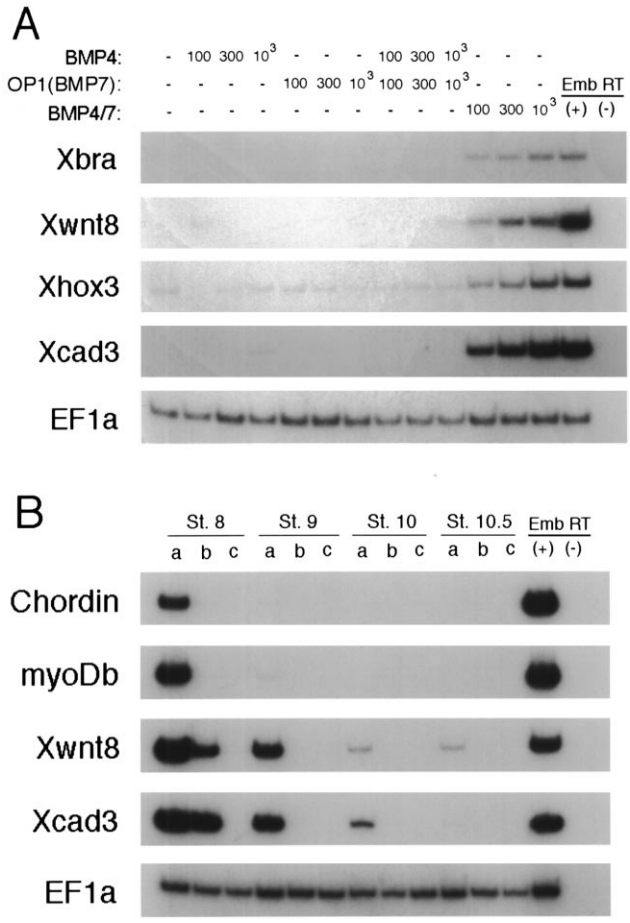



Fig. 1. Heterodimeric BMP-4/7 protein induces mesoderm. (A) Animal caps were explanted at stage 8 and cultured in low calcium modified ringers (LCMR) alone (negative control) or LCMR containing recombinant BMP-4, mammalian OP-1 (BMP-7), or BMP-4/7 proteins at the concentrations (ng/ml) indicated above each lane (see Section 4 for additional details on conditions). Treated caps were harvested at stage 15 and analyzed by RT-PCR for expression of the indicated mesodermal markers. Brachyury (Xbra) is a pan-mesodermal maker; Xwnt8 is a ventro-lateral marker; Xhox3 and Xcad3 mark ventro-posterior mesoderm; EF1a is a control for RNA recovery. The results show that the BMP-4/7 heterodimer induced ventral mesoderm, but homodimeric BMP-4 or OP-1 (BMP-7), or combinations of both, did not. Emb RT(+ and –) are positive and negative control, respectively, for cDNA synthesis genomic DNA contamination. (B) Temporal response of animal caps to BMP-4/7. Animal caps were excised at stage 8, 9 or 10 of development and incubated in LCMR containing either activin (final concentration 5 ng/ml, lanes a) or BMP-4/7 (500 ng/ml, lanes b). Caps treated at stage 10.5 were first excised into LCMR at stage 10 and proteins were added to the culture medium at stage 10.5. Control caps (lanes c) were cultured in LCMR only. All animal caps were harvested at stage 18 and analyzed by RT-PCR for the expression of mesoderm markers. Caps treated with BMP-4/7 at stage 8 expressed ventral\tqposterior markers, Xwnt8 and Xcad3, but cap responsiveness to BMP-4/7 was lost by stage 9. BMP-4/7 did not induce markers of the Spemann organizer (chordin) or dorso-lateral mesoderm (myoDb) at any stage of treatment or protein concentration (not shown). Animal caps treated with 5 ng/ml of activin through the onset of gastrulation at stage 10 formed mesoderm, but the type of mesoderm that was induced depended on the stage of treatment. Chordin and myoDb were induced by activin treatment prior to stage 9, but not at later times. Instead, the mesoderm induced by activin treatment at later stages was progressively more ventral- posterior. By stage 10.5 animal cap cells did not respond to either BMP-4/7 or activin.

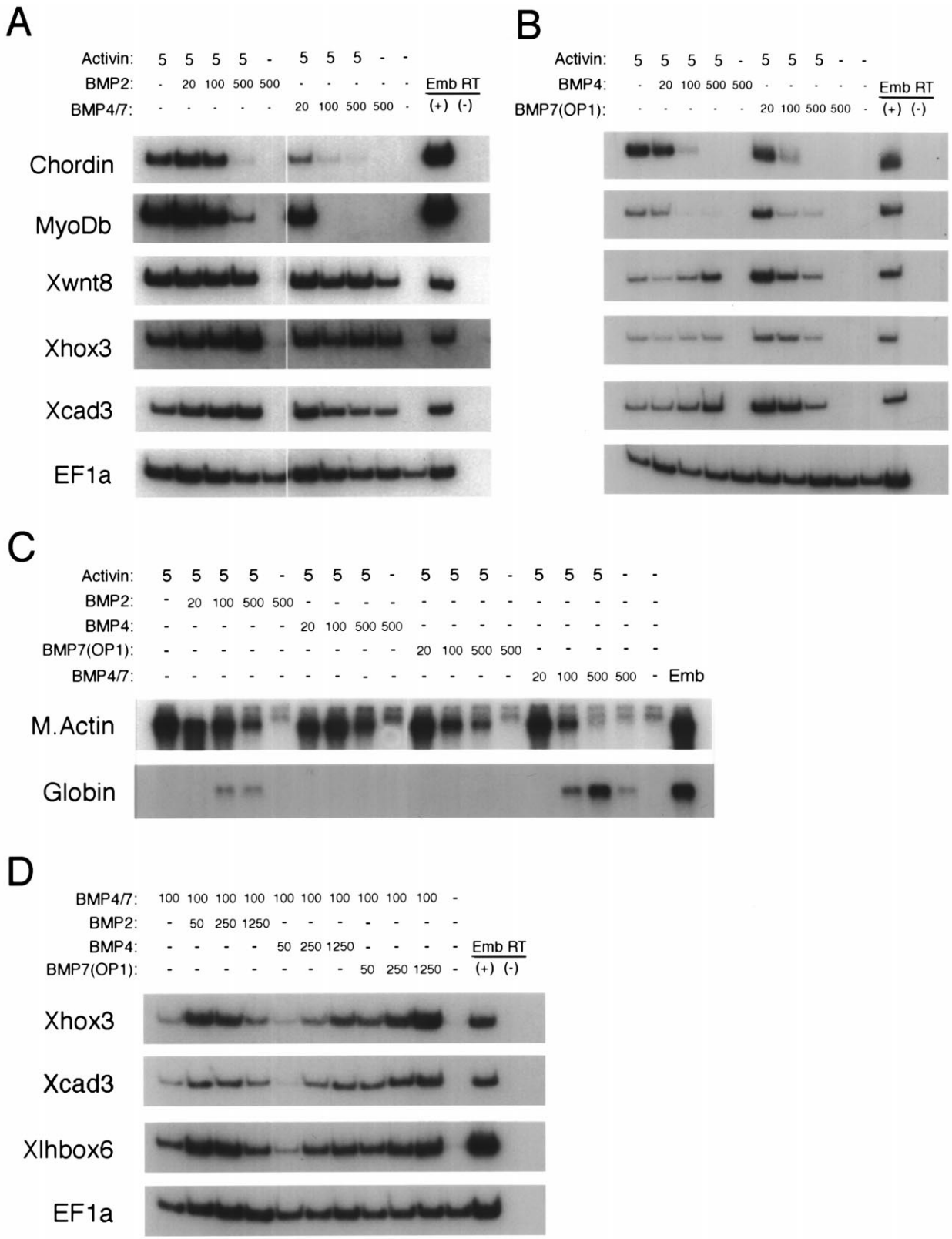
these mesodermal markers was boosted as a function of BMP-4/7 dose, but dorsal mesoderm was not induced at 100 ng/ml (Fig. 1B) or 1 $\mu$g/ml (data not shown). These results demonstrate that heterodimeric BMP-4/7 can directly induce ventral mesoderm, but BMP homodimers alone or in combination do not induce mesoderm. Our findings agree with results reported by Suzuki et al. (1997) who have also shown that BMP-4/7 protein can induce early ventral mesodermal markers in animal caps.

It has been generally established that *Xenopus* animal cap explants are competent to respond to enodgenous mesoderm inducers (secreted by vegetal cells), or exogenous mesoderm inducing factors applied in vitro, from early blastula to early gastrulation (stage 10; Gurdon et al., 1985; Green and Smith, 1990; Green et al., 1990). We tested whether or not this precedent holds for mesoderm induction by BMP-4/7. Animal caps were excised at various stages and exposed to BMP-4/7 protein, harvested at stage 18 and scored for the expression of dorsal mesoderm (marked by chordin and MyoD) or ventral/posterior mesoderm (marked by Xwnt8 and Xcad3). Fig. 1B shows that ventral mesoderm was induced in animal caps by BMP-4/7 when it was applied beginning at stage 8, but mesoderm was not induced when BMP-4/7 was applied to caps at stage 9 or later. In comparison, activin induced mesoderm when exposed to animal caps at any stage until early gastrulation (stage 10.5; Fig. 1Ba), but the qualitative response of the caps to activin changed as the caps aged. Activin applied at stages 8 or 9 induced dorsal and ventral mesodermal markers, but when activin was applied at stages 10 or 10.5 it induced only ventral mesoderm. This time- and concentration-dependent loss of animal-cap sensitivity to soluble mesoderm inducers such as activin and FGF is well documented (Green et al., 1992; Kinoshita and Asashima, 1995). It is interesting that BMP-4/7 is only effective when exposed to animal caps at the mid-blastula, stage 8, but not later. This observation suggests that if BMP-4/7 functions as a mesoderm inducer in the embryo, it probably acts early by mobilization of maternally-stored BMP proteins or mRNAs translation, rather than through zygotic BMP gene activity. The fact that the heterodimeric BMP-4/7 induces mesoderm at blastula stages raises the important possibility that BMP heterodimers function as endogenous mesoderm inducers in embryos. Whether or not such heterodimers exist in *Xenopus* embryos and correspond to endogenous, vegetally-derived, mesoderm-inducing factors remains to be established.

### *2.2. BMP homodimers have distinct effects on mesoderm induction by activin and BMP-4/7*

Homodimeric BMP proteins did not induce mesoderm in our animal cap assays, but BMP-4 homodimer protein has been shown to ventralize mesoderm induced by activin in animal caps (Dale et al., 1992; Jones et al., 1992). Ectopic, zygotic expression of BMP-4 also ventralizes dorsal meso-

derm in the *Xenopus* embryo (Jones et al., 1996). These observations provide evidence that BMP proteins can at least modify, or pattern, the response of cells to activin or activin-like signals. We tested whether such patterning activity is also characteristic of other BMP proteins by simultaneously treating animal caps with a particular BMP protein and a dose of activin sufficient to induce dorsal mesoderm. Fig. 2A–C demonstrates the effects of BMP



homo- and heterodimers on mesoderm induction in the presence of activin. The BMP-2, BMP-4 and BMP-7(OP-1) homodimers each inhibited activin-mediated induction of early dorsal mesoderm markers (chordin and myoD), and the tadpole (stage 38) dorsal mesodermal marker of muscle (actin). Note that BMP-2 and BMP-4 triggered a modest increase in the expression level of ventral mesodermal markers (Xhox3, Xcad3 and Xwnt8), above levels induced by activin alone, and BMP-2 cotreatment also induced globin at the tadpole stage (Fig. 2C). These effects are completely consistent with the ventralization, although we are uncertain as to why globin was not induced by activin plus BMP-4. Fig. 2A,B are RT-PCR assays and Fig. 2C is a Northern blot.

Cotreatment of animal caps with BMP-7 interfered with the expression of early and late mesodermal markers induced by activin (Fig. 2B,C). Induction of all early ventral and dorsal mesodermal markers is suppressed by BMP-7. Late-stage expression of muscle is also suppressed by BMP-7, but this is not accompanied by globin induction. This contrasts the ventralizing effects of BMP-2 and BMP-4, which in the presence of activin boost induction of ventral mesodermal markers such as Xwnt8 and Xcad3, and globin (in the case of BMP-2). Therefore BMP-7 does not have a ventralizing action on activin-treated animal caps, but instead BMP-7 appears to act as a general antagonist of mesoderm induction by activin. This is a rather novel property of BMP-7.

We also noted that treatment of animal caps with BMP-4/7, together with activin, also inhibited dorsal mesoderm induction (Fig. 2A,C), and BMP-4/7 was about five times more potent in this respect than the BMP homodimers. BMP-4/7 did not boost or suppress early expression of ventral/posterior mesodermal markers relative to levels induced by activin alone. Of greater significance, however, is our observation that BMP-4/7 treatment alone induced red blood cell-specific globin expression in animal caps,. Furthermore, animal caps treated with a combination of activin and BMP-4/7 elicited a very strong blood induction response (Fig. 2C). Activin alone did not induce blood to any detectable level. Fig. 2A,C also reconfirmed that a high dose (500 ng/ml) of BMP-4/7 induced only ventral mesoderm, not dorsal mesoderm, and that homodimeric BMPs did not induce mesoderm.

We have shown that homo- and heterodimeric BMPs modify the response of animal caps to activin, but how might BMP homodimers affect mesoderm induction by the BMP-4/7 heterodimer? We tested this question by treating animal caps with a combination of BMP-4/7 and either BMP-2, BMP-4, or BMP-7 (mammalian OP-1) homodimers. Fig. 2D shows that treatment of animal caps with BMP-4/7 and any of the three BMP homodimers boosted the induction of ventral–posterior mesodermal markers above levels induced by BMP-4/7 alone. The effects of BMP-7 (OP-1) were particularly noteworthy, because at doses up to 1.2 $\mu$g/ml it did not reduce mesoderm induction by BMP-4/7, which is in contrast to its antagonistic action on mesoderm induction by activin (Fig. 2B). In fact, BMP-7 (OP-1) enhanced ventral mesoderm induction by BMP-4/7. This suggests that in early *Xenopus* development BMP-7 may function in two capacities: one antagonizes activin signaling and the other stimulates responses to BMP signals.

### *2.3. Expression of endogenous BMP genes in animal caps*

There is some conflict regarding whether BMPs are bona fide mesoderm inducers in *Xenopus*. Our results in Fig. 1 have shown that mesoderm is not readily induced in animal caps by direct treatment with purified BMP homodimer proteins. On the other hand, experiments have shown that ventral mesoderm can be induced by injection of BMP-2 or BMP-4 mRNAs into animal caps (Dale et al., 1992; Jones et al., 1992; Clement et al., 1995; Hemmati-Brivanlou and Thomsen, 1995). These observations have been rather difficult to reconcile, but one possible explanation for the apparent contradictions might be that BMP proteins encoded by microinjected mRNAs form heterodimers with BMPs synthesized from endogenous mRNAs in animal cap cells. To help evaluate this possibility we checked the expression

Fig. 2. BMP proteins alter the fate of mesoderm induced by activin or BMP-4/7. (A,B) Homodimer proteins of BMP-2, BMP-4, and OP1 (mammalian BMP-7) suppressed dorsal mesoderm induction by activin. Animal caps were explanted at stage 8 and treated singly or with combinations of activin and BMP proteins at the concentrations (ng/ml) indicated above each lane. Caps were harvested at stage 18 and mesoderm induction was scored by RT-PCR using the early mesoderm markers described in Fig. 1, as well as Xhox3 (a ventral–posterior marker). Activin alone induced a full range of dorsal and ventral/posterior mesodermal markers, but co-application of each BMP protein suppressed chordin and MyoDb expression as a function of BMP dose. BMP-4/7 was the most effective inhibitor. The induction of early ventral–posterior markers (Xwnt8, Xhox3 and Xcad3) by co-incubation of caps with activin and either BMP-2, BMP-4 or BMP-4/7 was relatively unchanged or slightly elevated relative to activin treatment alone. Co-incubation of caps with activin and OP-1 (BMP-7), however, reduced the expression of all mesodermal markers induced by activin. Conditions were as described in Fig. 1. (C) Animal caps were treated with activin and BMP proteins as in panels A and B, harvested at swimming tadpole stages 37–38, then scored for expression of muscle actin or a-T1 globin by a Northern blot. Activin alone induced muscle-specific actin, but co-application of any BMP proteins suppressed muscle actin expression. Alpha T1 globin, a marker of erythroid blood, was not induced by activin treatment, but globin expression was induced by the BMP-4/7 heterodimer. Globin was also induced by a combination of activin and either BMP-2 or BMP-4/7. In particular the highest level of globin induction observed was triggered by a mixture of activin and BMP-4/7. In the blot the strong, lower band corresponds to muscle-specific actin RNA. The top two bands correspond to cytoplasmic actin mRNAs, which cross hybridize with the muscle actin probe. The cytoplasmic actins serve as positive controls for RNA loading. (D) BMP homodimers enhance the ventral mesoderm inducing activity of BMP-4/7. Animal caps were treated beginning at stage 8 with BMP-4/7 alone, or a combination of BMP-4/7 and a BMP homodimer, as indicated. Note that the combination of BMP-2, BMP-4 or BMP-7 together with BMP-4/7 boosted ventral mesoderm induction above levels induced by BMP-4/7 treatment alone. It is noteworthy that BMP-7 did not antagonize mesoderm induction by BMP-4/7, as observed with activin (Fig. 2A). Explants were isolated, cultured and scored by RT-PCR as described in panels A–C.

profile of endogenous BMP mRNAs as animal caps were aged in vitro from blastula stage 8.5 through neurulation (stage 18). RNAse protection assays on the cap RNA were performed with probes against the *Xenopus* BMP-2, BMP-4 and BMP-7 genes described in Nishimatsu et al. (1992), and the results are displayed in Fig. 3A. The bands in this gel were quantified relative to synthetic BMP mRNA standards, and Fig. 3B shows a plot of the average levels of each BMP mRNA per animal cap explant. At stage 8.5, BMP-2 and BMP-7 were about 2-fold more abundant than BMP-4. An average animal cap had 0.7 ng total RNA, of which there was 0.41 pg of BMP-2, 0.19 pg of BMP-4, and 0.35 pg BMP-7. As the caps aged BMP-2 mRNA levels fell to undetectable levels by gastrulation, stage 10, which is when BMP-7 levels peaked at 1 pg per cap. BMP-4 mRNA levels peaked shortly thereafter at 0.8 pg per cap at the end of gastrulation, stage 13.

In general, the expression profiles of BMP-4 and BMP-7 in animal caps are similar to their expression dynamics in the whole embryo (Nishimatsu et al., 1992; Hemmati-Brivanlou and Thomsen, 1995). BMP-7 mRNA is significantly more abundant than BMP-2 and BMP-4 mRNA levels in isolated animal caps when caps are competent to respond to mesoderm inducers (between stages 8 to 10.5). The high levels of BMP-7 observed in isolated caps at stages 9 -10 may provide an important clue why microinjection of synthetic BMP-2 and BMP-4 mRNAs into animal caps results in ventral mesoderm induction. Since BMP-7 transcripts are very abundant in animal caps, we suggest that when exogenous BMP-2 or BMP-4 mRNAs are microinjected into animal caps heterodimers would be likely to form between endogenous BMP-7 and BMP proteins translated from the microinjected BMP mRNAs. This would result in the secretion of BMP-2/7 or BMP-4/7 heterodimers by the injected animal cap cells, inducing ventral mesoderm in neighboring cells.

### *2.4. Interactions between different BMPs in animal caps*

To help substantiate the heterodimer model we just proposed, we tested whether co-expression of BMP-2 and BMP-7 in animal caps (by co-injection of mRNA) induces mesoderm more efficiently than injection of either message alone. In this assay, depicted by the diagram in Fig. 4, animal caps were injected with sub-mesoderm inducing levels of BMP-2 or BMP-7 mRNA alone (400 pg) or together (200 pg each), and pairs of the injected caps were combined in a sandwich after cutting at stage 8 (refer to the diagram in Fig. 4). Caps were combined to control for the possibility that BMP-2 and BMP-7 homodimers might be produced by the injected cap cells and synergize to induce mesoderm. This possibility is low, however, since mesoderm was not induced in animal caps by mixtures of homodimeric BMP proteins (Fig. 1A). The combined pairs of injected animal caps were harvested at stage 22 and analyzed for mesoderm induction by RT-PCR. The results in Fig. 4 show that a single injection of BMP-2 or BMP-7 mRNA into animal caps did not induce mesoderm, regardless of whether each cap in a pairing was injected with the same or different BMP mRNAs. These results suggest that if each cap was secreting a homodimer BMP encoded by the injected mRNA, simultaneous exposure of cap cells to both BMP-2 and BMP-7 did not induce mesoderm, This is consistent with the results of our protein treatment experiments in Fig. 1. However, when BMP-2 and BMP-7 mRNAs were injected into the same animal cap, synergistic induction of

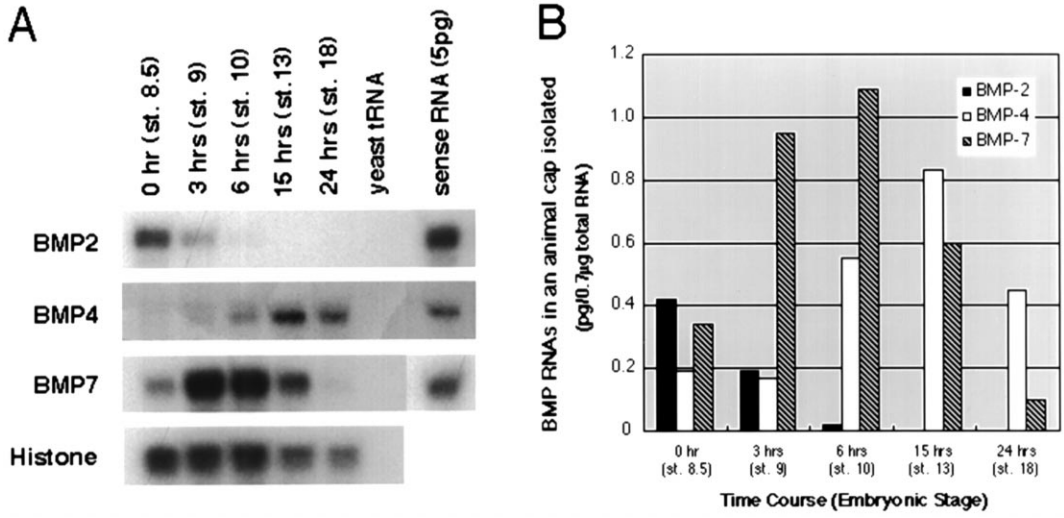



Fig. 3. Expression of endogenous BMP mRNAs in isolated animal caps. Animal caps were explanted into 0.5× MMR at stage 8.5, cultured at 18°C and harvested when sibling embryos reached stage 9, 10, 13 and 18. (A) Gel electrophoresis of RNAse protection fragments obtained with cap RNA and 5 pg of a sense strand BMP RNA standard. Time and stage of cap harvest is indicated above each lane. (B) The bands in panel A were quantified and the absolute and relative levels of each BMP mRNA are plotted. An average cap corresponded to 0.7 ng total RNA. Note that BMP-7 messenger RNA is more abundant than BMP-2 and BMP-4 at the mid-late blastula stages of competence to respond to mesoderm induction signals.

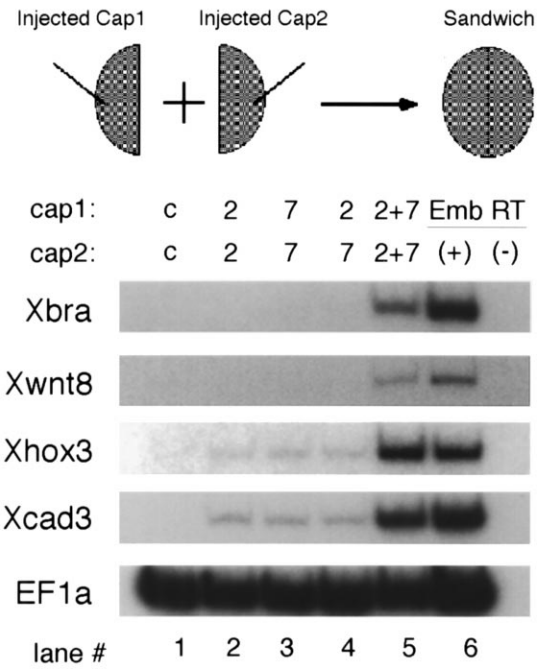



Fig. 4. Synergistic mesoderm induction by co-expression of BMP-2 and BMP-7 mRNA in animal caps. To test for synergy between BMP-2 and BMP-7, pairs of animal caps were injected singly with BMP-2 or BMP-7 mRNA, or with both BMP mRNAs. The dose of each injected mRNA was made limiting for mesoderm induction (see Section 4). Pairs of animal caps injected with the same (lanes 2 and 3) or different (lane 4) mRNAs were combined into sandwiches at stage 8.5, as shown in the diagram. Combined caps were cultured until stage 22 and RNA was harvested for analysis of mesoderm induction by RT-PCR. Single injection of a particular mRNA would favor production of the corresponding BMP homodimer, so combinations of caps injected with different mRNAs is meant to test the effects of expressing different BMPs in the same tissue. Single injection of either BMP-2 (lane 2) or BMP-7 (lane 3) did not appreciably induce mesoderm, even when the re-combined caps were injected with a different BMP (lane 4). In contrast, injection of BMP-2 and BMP-7 mRNA into the same cells triggered significant, synergistic, induction of mesoderm (lane 5). This result suggests that the co-injected cells produce BMP-2/7 heterodimers, which induce mesoderm. Mesodermal marker genes were analyzed as in Figs. 1 and 2.

ventral mesoderm was observed. The most likely explanation for this result is that BMP-2 and BMP-7 proteins synthesized from the injected mRNAs form a BMP-2/7 heterodimer, and this heterodimer induces mesoderm.

We have previously reported that microinjected BMP-2 mRNA induces ventral mesoderm in animal caps, much like BMP-4 mRNA injection (Dale et al., 1992; Jones et al., 1992; Clement et al., 1995; Hemmati-Brivanlou and Thomsen, 1995). Our hypothesis is that mesoderm induction caused by injection of a single BMP mRNA results from the formation of heterodimers between endogenous BMP-7 and proteins encoded by the microinjected BMP-2 or BMP-4 mRNA. To further test whether or not different BMPs interact in animal caps, we used dominant-negative mutant BMPs to challenge mesoderm induction by injected BMP-2 mRNA. Ligands in the TGF-b superfamily are processed from a larger precursor by proteolytic cleavage within the precursor's C-terminus (Hogan, 1996; Meno et al., 1996). Mutations at the cleavage recognition sites of activins and BMPs have been shown to function as dominant-negative inhibitors in *Xenopus* or fish embryos by causing interference with the assembly and cleavage of mature, dimerized ligands (Wittbrodt and Rosa, 1994; Hawley et al., 1995).

The dominant-negative *Xenopus* BMPs used in our tests were constructed by altering potential cleavage recognition sites as shown in Fig. 5A. BMP-2 and BMP-4 harbor two potential ligand processing sites that are conserved between vertebrate BMPs and their ortholog, Drosophila DPP. Cleavage recognition site 1 corresponds to the authentic processing site for mammalian BMP-2 and BMP-4, and Drosophila DPP (Panganiban et al., 1990; Wang et al., 1990). Site 2 also conforms to an RXXR consensus, and a site at this position is utilized for processing both human and *Xenopus* BMP-7 (Jones et al., 1994; Aono et al., 1995). Whether site 2 is used for the processing of DPP, BMP-2 or BMP-4 has not been established, but we found that when we mutated BMP-4 at cleavage site 1 (as in Hawley et al., 1995) it still retained weak ventralizing activity (not

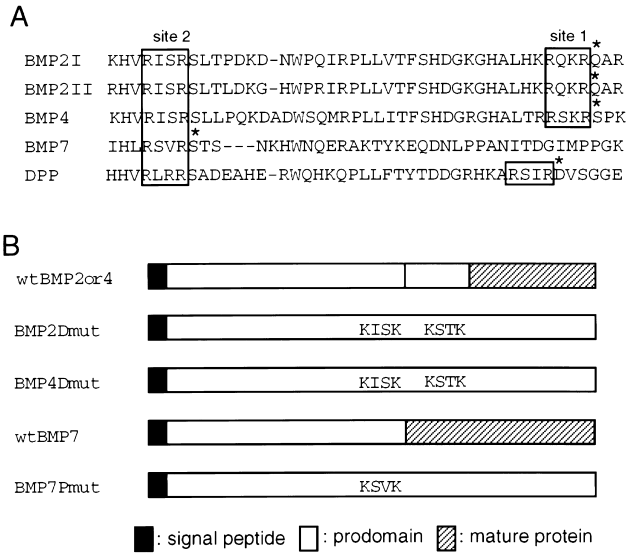



Fig. 5. BMP ligand processing sites and the BMP processing mutants. (A) Amino acid sequences at the processing sites of *Xenopus* BMPs and Drosophila DPP. Cleavage recognition sites for BMPs conform to the general consensus tetrapeptide RXXR. Amino acid residues located immediately C-terminal to each cleavage site are indicated by asterisks. *Xenopus* BMP4 expressed in baculovirus has been shown to be cleaved at site 1, preceding the serine residue (Aono et al., 1995). The BMP-2 cleavage site at position 1 is predicted from mammalian studies (Wang et al., 1990). Drosophila DPP, an ortholog of vertebrate BMP-2 and BMP-4, is processed at site 1, whereas *Xenopus* BMP-7 is cleaved further upstream at site 2, preceding the serine residue. A consensus cleavage sequence at site 2 also exists in DPP and *Xenopus* BMP-2 and BMP-4. It is not known whether these sites are actually used in vivo. Two BMP-2 cDNAs cloned from *Xenopus* (Nishimatsu et al., 1992) are shown in the comparison, and gene number II was used in this study. (B) Schematic representation of wildtype and mutant BMP proteins. The black box is the secretion signal sequence of the precursor, the open box is the pro domain, and the hatched region is the mature BMP ligand. The location of cleavage sites 1 and 2 in the wildtype (wt) BMPs are indicated by vertical lines in the schematic. The sequences of mutated cleavage sites are displayed at their relative positions in each BMP mutant.

shown). We therefore made double mutations of BMP-4 (BMP4Dmut) and BMP-2 (BMP-2Dmut) at sites 1 and 2, which completely inactived these BMPs in embryonic ventralization assays (not shown). BMP-7 does not harbor a cleavage site at position 1, so it was altered only at site 2 to create mutant BMP-7 (BMP7Pmut). We will refer to these constructs simply as 'mutant BMP-2, -4, or -7'.

We tested whether the BMP cleavage site mutants depicted in Fig. 5 interfered with mesoderm induction by BMP-2. In these assays we co-injected animal caps with mRNAs encoding mutant BMPs and a mesoderm-inducing dose of wildtype BMP-2 mRNA. A block in mesoderm induction would be expected to occur if the wildtype BMP-2 precursor forms a heterodimer with the co-expressed, mutated BMP protein, and that heterodimer is not processed correctly. Note that in these assays we used BMP-2 mRNA that was synthesized from the CS-2 plasmid (Turner and Weintraub, 1994). In our experience BMP-2 mRNA transcribed from this vector is greater than 100-fold more potent in mesoderm induction assays than BMP-2 synthesized from the pSP64T vector (compare the results of Fig. 4 with those described below). As a negative control we challenged mesoderm induction by injected activin mRNA, because activins are very distinct from BMPs at the protein sequence and functional level, and activin does not form heterodimers with BMPs (Wittbrodt and Rosa, 1994). Fig. 6A shows that injection of BMP-2 mRNA alone into animal caps induced the general mesodermal marker Xbra and the ventral–posterior mesodermal marker Xcad3. We note that induction of Xcad was more sensitive to BMP-2 than induction of Xbra. Mesoderm induction by injected wildtype BMP-2 mRNA was inhibited, however, when mRNA encoding any of the BMP cleavage site mutants were co-injected together. Interestingly, mutant BMP-2 was a more effective inhibitor of mesoderm induction than the other mutant, with almost full inhibition observed at a dose equal to that of wildtype BMP-2 (20 pg). The mutant forms of BMP-4 and BMP-7, on the other hand, required about a 5-fold higher dose than wildtype BMP-2 to interfere with mesoderm induction. These results suggest that when ectopic wild type BMP-2 is injected into caps its translated protein dimerizes with endogenous BMP-7, resulting in mesoderm induction by production of BMP-2/7 ligands. However when the mutant BMP mRNAs are co-injected with BMP-2 mRNA the mutant BMP proteins form heterodimers with co-expressed BMP-2, and the production of abnormal heterodimers results in a loss of activity. Note that the same explanation for interference by the mutants would pertain even if mesoderm induction by microinjected BMP-2 mRNA occurs through formation of homodimeric BMP-2 ligands.

To test the specificity of the mutant BMP ligands, mesoderm induction triggered by activin was challenged by co-injection of animal caps with mRNAs encoding activin and the BMP mutants. Fig. 6B shows that none of the mutated BMPs inhibited dorsal or ventral mesoderm induction by activin at mRNA doses 5-fold higher than the level of injected activin mRNA (10 pg). In fact, the mutant forms of BMP-2 and BMP-4 did not inhibit mesoderm induction at doses up to 125-fold greater (1250 pg) than activin, although the induction of ventral markers was partially inhibited at the highest dose of mutant BMP-7. Thus the effects of the BMP mutants are reasonably specific to BMP-2. Our interpretation of these results is that all three BMPs in this study can form dimers with BMP-2 but not activin.

### *2.5. BMP cross-talk in ventral mesodermal patterning by BMP-7*

Our animal cap assays have shown, both directly and indirectly, that mesoderm can be induced by a BMP heterodimers containing a BMP-7 subunit. The results with dominant-negative ligands in mesoderm induction assays, just described, also make it likely that different BMPs can interact in vivo to form heterodimers. We next tested whether such interactions can influence ventral mesodermal patterning. We focused attention on BMP-7 since it can form heterodimers with either BMP-2 or BMP-4 in other systems

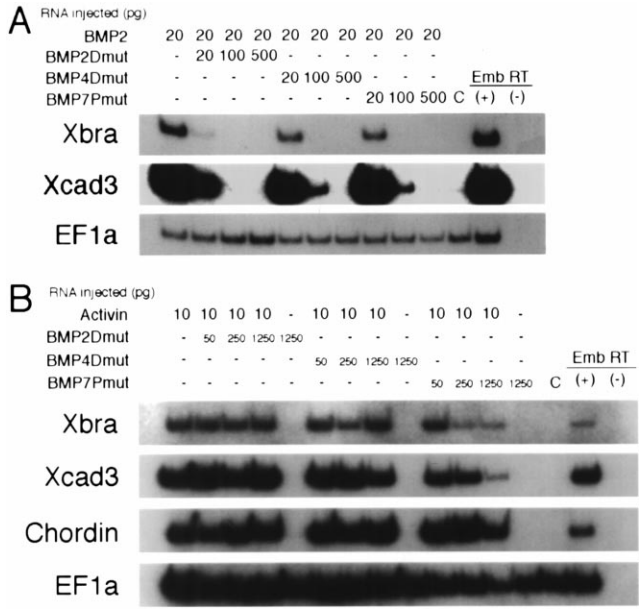



Fig. 6. Mutant BMPs inhibit mesoderm induction by wild type BMP-2, but not activin. To test the specificity of ligand interference by mutant BMPs, mesoderm induction elicited by injected mRNA encoding wild type BMP-2 (panel A) and activin (panel B) was challenged by co-injection of mutant BMP-2 (BMP2Dmut), mutant BMP-4 (BMP4Dmut) or mutant BMP-7 (BMP7Pmut) mRNAs. Animal caps were cut at stage 8.5, harvested at stage 11, and scored for mesoderm induction by RT-PCR. (A) Mesoderm was induced by injection of 20 pg of BMP-2 mRNA (synthesized from CS2+; see Methods), but co-injection of a 5-fold excess of each mutant BMP was sufficient to block BMP-2 activity. Note the loss of Xbra and Xcad3 expression. (B) Mesoderm induced by injected activin mRNA was not inhibited by a 125-fold excess of mutant BMP-2 or mutant BMP-4. High doses of mutant BMP- 7 slightly inhibited mesoderm induction by activin. Mesodermal gene expression was analyzed by RT-PCR as in previous figures.

(Sampath et al., 1990; Aono et al., 1995; Hazama et al., 1995). Also, a cleavage site mutant of BMP-7 can dorsalize ventral mesoderm (Hawley et al., 1995), implicating BMP-7 or a heterodimer containing BMP-7 in ventral mesodermal patterning.

To determine whether BMP-2 and BMP-4 can interact with BMP-7 in the ventral marginal zone (VMZ) of *Xenopus* embryos, we tested whether wild type BMP-2, BMP-4 or BMP-7 could rescue the dorsalizing effects of our mutated BMP-7 molecule. Fig. 7 shows the results of a VMZ explant assay in which we injected mRNA for our mutant BMP-7 into two ventral blastomeres at the 4 cell stage, followed by explantation of the VMZ at stage 10 and subsequent culture in vitro. Fig. 7B shows that VMZs injected with mutant BMP-7 elongated and expressed melanocytes, characteristic of dorsalization. Similar effects were also observed when VMZs were injected with mutant BMP-2 mRNAs (not shown). VMZs injected with wildtype BMP-7 mRNAs (Fig. 7A), or control vector RNA (Fig. 7C), developed in a typically wildtype manner. At the molecular level, a Northern blot on the explants shown in panels A–C demonstrated that control VMZs expressed $\alpha$-T1 globin, as expected, but VMZs injected with mutant BMP-7 (100 pg) lacked globin expression and instead expressed muscle actin (Fig. 7D), indicating dorsalization. The dorsalizing effects of mutant BMP-7 were challenged by injection of mutant BMP-7 mRNA together with either wild type BMP-2, BMP-4, or BMP-7 mRNAs. Northern blot analysis of these explants (Fig. 7E) demonstrated that co-expression of any wild type BMP rescued the phenotype. Muscle actin gene expression was eliminated and $\alpha$-T1 globin expression was restored in a dose-dependent manner by co-injection of any wildtype BMP. Interestingly, BMP-2 was the most potent BMP in this rescue. It completely rescued the mutant phenotype at a 10-fold lower dose (10 pg) than the amount of mutant BMP-7 mRNA (100 pg) that induced dorsalization. Wildtype BMP-4 and BMP-7 rescued the mutant BMP-7 phenotype at doses equal to or higher than the level of injected mutant BMP-7 mRNA.

This VMZ rescue experiment demonstrates that each of the three wildtype BMPs can complement the dorsalizing effects of mutant BMP-7. Rescue of mutant BMP-7 by wildtype BMPs also shows that the mutant acts as a dominant-negative inhibitor of BMP ligand functions. We propose that mutant BMP-7 competes, at the step of ligand dimer assembly, with endogenous BMPs present in the ven-

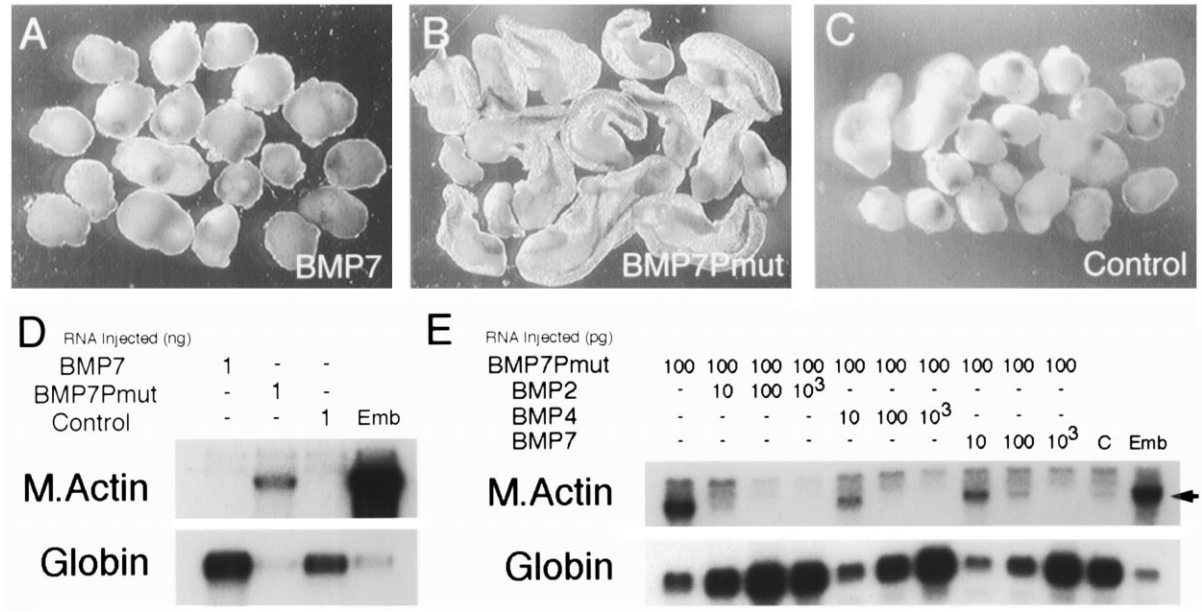



Fig. 7. Wildtype BMPs rescue VMZ dorsalization by a dominant-negative BMP-7 ligand. (A–C) Two ventral blastomeres were injected at the 4 cell stage with mRNA (1 ng) encoding wild type BMP-7, mutant BMP-7 (BMP7Pmut), or vector sequences (control). At stage 10.5 ventral marginal zones (VMZ) were explanted and cultured until they were harvested at stage 39. Panels A, B, C display photographs of the VMZs just before harvest. VMZs injected with BMP-7 mRNA (panel A; $n = 19$) or control mRNA (panel C; $n = 18$) were characteristically cylindrical, but VMZs injected with mutant BMP7 (panel B; $n = 17$) elongated into trunk-like structures with fins, and formed melanocytes associated with neural differentiation, all characteristics of dorsalization. (D) A Northern blot on RNA from the explants shown in panels A - C. Injection of 1 ng of wild type BMP-7 in the VMZ boosted red blood formation (indicated by elevated globin expression relative to control VMZs). In contrast, expression of the mutant BMP-7 (BMP7mut) in VMZ explants caused muscle differentiation (indicated by muscle actin expression) and a loss of blood, characteristic of dorsalization. (E) Mutant BMP-7 also dorsalizes VMZ explants at a dose of 100 pg, which we used for rescue experiments depicted in this panel. The results of a Northern blot on VMZs injected with mutant BMP-7 alone, or in combination with different doses of wildtype BMP mRNAs, are shown. The blot was sequentially probed with muscle actin (M. Actin) and $\alpha$-T1 globin and the results show that dorsalization caused by mutant BMP-7 can be rescued by co-injection of mRNAs for wild type BMP-2, BMP-4 or BMP-7. This rescue is revealed by the loss of muscle actin expression and the increase in globin expression as a function of wt BMP mRNA dose (10 pg, 100 pg, and 1000 pg of each BMP). Note that BMP-2 was more effective than BMP-4 or BMP-7 in this rescue. The phenotypes of the rescued VMZs (not shown) were similar in appearance to the controls in panel C. The muscle- specific actin band is indicated by an arrow.

tral marginal zone (particularly BMP-4, which is highly expressed there). Dorsalization then results from the loss of BMP signals in the VMZ, but the ventralized phenotype is restored when exogenous wildtype BMP mRNAs are co-expressed in the VMZ along with mutant BMP-7. Rescue of the dorsalized VMZ phenotype may be accomplished by synthesis of BMP homo- or heterodimers. Rescue of the dorsalized VMZ phenotype by wildtype BMPs mirrors the results of our animal cap assays in Fig. 6, which showed that any mutant BMP can interfere with mesoderm induction by BMP-2. Our VMZ and animal cap assays provide indirect, but rather substantial evidence that BMPs can interact with one another through heterodimerization to affect mesoderm induction and patterning,. Direct examination of BMP proteins will be required to establish conclusively whether or not heterodimers form between mutant and wildtype BMPs.

## 3. Discussion

### *3.1. BMP heterodimers as mesoderm inducers*

We have demonstrated in this study that a BMP-4/7 heterodimeric protein directly induces ventral mesoderm in animal caps when applied beginning at the mid-blastula stage 8.5. Our experimental findings agree with results from Suzuki et al. (1997) who also observed induction of early mesodermal markers in animal caps treated with BMP-4/7 protein. Our experiments make an important observation that a hematopoeitic differentiation program can also be induced by BMP-4/7. Our results have also demonstrated that ventral mesoderm is induced in a synergistic manner by co-injection of BMP-2 and BMP-7 mRNA in animal caps. This synergy is observed only when BMP-2 and BMP-7 are co-expressed in the same cells, which suggests that BMP-2/7 heterodimers are produced in the co-injected cells, and that their production triggers mesoderm induction. The alternative possibility that homodimers of BMP-2 and BMP-7 act together to induce mesoderm seems unlikely because we have shown that treatment of animal caps with a mixture of BMP-7 (OP-1) and either BMP-2 or BMP-4 proteins does not induce mesoderm (Fig. 2B and data not shown). Furthermore, we never observed mesoderm induction by homodimeric BMP-2, BMP-4, or -BMP-7 at any dose.

The potential for BMP-2/7 heterodimers to form in vivo is also supported by our mutant interference assays in animal caps which demonstrated that mesoderm induction by BMP-2 could be blocked by co-expression of cleavage site mutants of BMP-2, BMP-4, or BMP-7. *Xenopus* BMP-2 ligand is more than 90% identical to *Xenopus* BMP-4, and therefore heterodimers formed between BMP-2 and BMP-7 might be expected to function similarly to BMP-4/7. The capacity for BMP-4/7 (and perhaps BMP-2/7) to induce mesoderm in animal caps makes it plausible that BMP heterodimers function as primary mesoderm inducing factors produced by vegetal cells in the *Xenopus* blastula. This proposition, however, awaits direct experimental confirmation.

### *3.2. BMPs trigger hematopoiesis*

Several lines of evidence have implicated BMPs in vertebrate erythropoiesis (reviewed by Zon, 1995). Prospective blood cells in the *Xenopus* embryo are derived from tissues originating from at least two sources. One is the ventral marginal zone of the early gastrula (Dale and Slack, 1987; Moody, 1987; Vodicka and Gerhart, 1995), where BMP-4 is highly expressed (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995), and the other has been recently shown to correspond to a population of cells originating near the Spemann organizer (Tracey et al., 1998), which encounter tissues expressing BMP-4 and BMP-7 during neurulation (Hemmati-Brivanlou and Thomsen, 1995). Loss of function experiments have also shown that blocking BMP signals dorsalizes ventral mesoderm and eliminates blood formation (Fig. 7 of this study; and Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994; Schmidt et al., 1995; Steinbeisser et al., 1995; Hawley et al., 1995). More directly, injection of *Xenopus* animal caps with mRNAs encoding BMP-2 and BMP-4, or the BMP signal transducer, Smad1, induces globin expression (Jones et al., 1992; Maeno et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Graff et al., 1996; Thomsen, 1996). Injection of BMP-2 or BMP-4 mRNA also induces benzidine positive erythrocytes in animal caps (Hemmati-Brivanlou and Thomsen, 1995). Furthermore, injection of BMP-2, BMP-4 or BMP-7 mRNA (or a BMP-4 zygotic expression plasmid) into the dorsal marginal zone of *Xenopus* embryos causes ventralization, accompanied by ectopic expression of globin (Dale et al., 1992; Jones et al., 1992; Hemmati-Brivanlou and Thomsen, 1995; Suzuki et al., 1997).

Our present study has demonstrated that a purified BMP-4/7 heterodimer protein alone induces blood differentiation in animal caps. Blood can also be induced by a mixture of activin and either BMP-2 or BMP-4/7, and the combination of activin and BMP-4/7 is a very potent inducer of blood. These results are particularly important because they are the first to demonstrate that a purified growth factor, BMP-4/7 is sufficient to initiate de novo hematopoietic differentiation in *Xenopus* embryonic tissue. Neither activin, soluble Vg1, FGF nor other proteins that can induce mesoderm in *Xenopus* are alone sufficient to induce blood (Kessler and Melton, 1994).

Our findings lead us to suggest that two inductive pathways may specify ventral mesoderm and blood in *Xenopus*. In one pathway, BMP-4/7 (and perhaps BMP-2/7) protein directly induces blood and other ventral mesoderm independent of other mesoderm inducing signals. This pathway would act during primary mesoderm induction at blastula stages through mobilization of maternally-encoded BMP factors. In the second pathway, an activin-like signal in-

duces primary mesoderm which would then be patterned by zygotic BMP signals. It is also possible that BMP and activin-like signals act together during primary mesoderm induction or its subsequent patterning to specify hematopoietic differentiation. In any case, BMP signals could be supplied by ligands composed of BMP-2, BMP-4, or BMP-7 subunits since these are expressed maternally and zygotically in the *Xenopus* eggs and embryos.

### *3.3. Ventral patterning by BMPs and activin inhibition by BMP-7*

It is well documented that mesoderm is induced in the *Xenopus* embryo at blastula stages, and that mesoderm is patterned across the dorsal–ventral axis during late blastula and gastrula stages (for reviews refer to Sasai and De Robertis, 1997; Hemmati-Brivanlou and Melton, 1997; Thomsen, 1997; Moon et al., 1997). The patterning action of dorsal mesoderm dominates over endogenous ventralizing activities present in the ventral marginal zone, as illustrated by the ability of the Spemann organizer to induce an ectopic dorsal axis when transplanted into the ventral marginal zone. Presently, BMP-4 and Xwnt8 are the best candidates for zygotic ventralizing agents resident in the VMZ, and they are considered to act subsequent to primary mesoderm induction by the vegetal cells (Clement et al., 1995; Hemmati-Brivanlou and Thomsen, 1995; Jones et al., 1996; Suzuki et al., 1997; and refer to review by Moon et al., 1997).

The results we have reported here are summarized in Table 1, and they extend the range of effects that BMPs have been shown to have on mesodermal patterning. One aspect is our demonstration that BMP-2 and BMP-4/7 can ventralize mesoderm induced by activin in animal caps, as has been observed for BMP-4 (Jones et al., 1992). Furthermore, our dominant-negative BMP ligand interference experiments support, albeit indirectly, the potential for BMP heterodimers to act in ventral mesodermal patterning. At minimum we have demonstrated that some level of functional 'cross-talk' or redundancy exists among BMP ligands in the *Xenopus* embryo. Direct evidence that BMP heterodimers actually exist in the embryo is required to substantiate this possibility. Some of the most novel patterning effects we have observed involve BMP-7, which can interfere with mesoderm induction by activin yet stimulate mesoderm induction by BMP-4/7. Thus BMP-7 may function in the embryo to inhibit dorsal mesoderm induction by activin-like signals, while simultaneously stimulating ventral mesoderm induction by BMP-4/7 and perhaps other BMP signals. At the mechanistic level, mammalian BMP-7 (OP-1) has been shown to bind activin receptors (Yamashita et al., 1995), so perhaps the inhibition of activin effects by BMP-7 results from direct competition between BMP-7 and activin ligands for receptor binding and activation. Whatever the mechanism, the actions of BMP-7 could provide important dorsal–ventral patterning influences in the embryo. The spatial location of BMP-7 gene expression in the embryo is, however, rather uniform through neurulation (Hawley et al., 1995 and our data not shown). BMP-7 activity is likely to be spatially regulated at the translational or post-translational level to control where its ligands act (discussed below).

### *3.4. Spatial regulation of BMP-7 activity in embryos*

BMP transcripts are not localized in the blastula, and in the gastrula only BMP-4 displays localized expression in the ventral-lateral marginal zone. BMP-2 and BMP-7 remain uniformly expressed through neurulation. Mechanisms must exist, therefore, to limit the actions of BMPs to particular regions of the developing embryo such as the ventrolateral mesoderm and ectoderm, and perhaps endoderm (Graff, 1997; Sasai and De Robertis, 1997; Thomsen, 1997). BMP activities are regulated, in part, by spatial acti-

Table 1
Effect of BMPs on mesoderm induction and patterning in animal cap cells

| Applied BMP proteins | | Ventral mesoderm induction at stage 8 | Ventral mesoderm patterning with mesoderm-inducing factor | | Dorsal mesoderm formation by activin |
|---|---|---|---|---|---|
| | | | Activin | BMP4/7 | |
| | BMP2 | – | ↑ | ↑ | ↓ |
| S | BMP4 | – | ↑ | ↑ | ↓ |
| | BMP7 (OP1) | – | ↓ | ↑ | ↓ |
| S | BMP4/7 | + | ↑ ↑ | na | ↓ ↓ |
| | BMP2/7 | (+) | nt | na | nt |

nt, not tested; na, not applicable; (+), mesoderm-inducing activity by BMP2/7 is from the result of an mRNA injection.

vation of BMP-4 transcription in the ventro-lateral mesoderm and ventral ectoderm during gastrulation, and by BMP antagonists secreted by the Spemann organizer. Other aspects of regulation most likely involve post-transcriptional steps to ensure delivery of inductive signals from BMP homo- and heterodimers at the appropriate places and times in the embryo. For example, if BMP-4/7 heterodimers function as primary mesoderm inducers in the blastula, then their synthesis, assembly, and/or secretion are anticipated to be spatially controlled so that only ventral vegetal cells deliver mesoderm inducing signals to the marginal zone. Likewise, synthesis or delivery of BMP ligands might be spatially regulated during gastrulation in order to pattern the ventral marginal zone and epidermis. We have attempted to detect processed BMP-7 ligand subunits and heterodimers of BMP-7 in embryos with an anti-BMP-7 antibody, but have been unsuccessful (data not shown). A more thorough analysis of native BMP ligand formation in embryos will be required to substantiate whether endogenous BMP heterodimers exist in embryos and whether there is spatial regulation of ligand production. Other regulatory mechanisms governing translation, ligand assembly, processing or secretion may control temporal-spatial aspects of BMP activity in the embryo. These regulatory mechanisms remain important topics for future investigation.

## 4. Experimental procedures

### *4.1. Preparation and microinjection of synthetic mRNA*

Wild type BMP cDNAs and all BMP mutants were subcloned into the CS2(+) plasmid (Turner and Weintraub, 1994), and linearized with PvuII. *Xenopus* activin bB cDNA was linearized with *Xba*I. The empty CS2(+) vector was digested with PvuII and used to transcribe control mRNA. Capped mRNA was synthesized by SP6 in vitro transcription (Krieg and Melton, 1984) with the SP6 mMessage Machine kit (Ambion Inc., Austin TX). Microinjection of mRNA into the ventral marginal zone of embryos was performed as described (Hemmati-Brivanlou and Thomsen, 1995). Note that BMP-2 mRNA synthesized from the CS2(+) vector was more potent at mesoderm induction than BMP-2 mRNA synthesized from the pSP64T vector reported in Hemmati-Brivanlou and Thomsen (1995).

### *4.2. Embryo and explant assays*

*Xenopus* laevis eggs were fertilized in vitro with testes minced in 0.1× Marc's modified Ringer's solution (MMR), and embryos were staged according to Nieuwkoop and Faber (1967). Animal cap and VMZ explants were cultured in 0.5X MMR until harvest as described (Hemmati-Brivanlou and Thomsen, 1995). For treatment with proteins, animal caps were explanted into 0.5× LCMR and 0.5× CMFR (Lamb et al., 1993; Piccolo et al., 1996) then incubated with purified BMP proteins in 1 × LCMR containing 10 mg/ml gentamicin, and 100 mg/ml bovine serum albumin (BSA). Under these conditions the animal cap inner sensorial layer remains open and exposed to culture medium longer than in typical animal cap assays performed in 0.5× MMR (Lamb et al., 1993). The procedure does not, however, cause animal cap cells to disaggregate. All embryological assays were repeated at least two times. Purified recombinant proteins were generous gifts from the following sources: BMP-2 and BMP-4 (Genetics Institute, Cambridge, MA), OP-1 (BMP-7; Creative Biomolecules, Hopkinton, MA), BMP-4/7 (Takeda Pharmaceutical, Osaka, Japan), and activin (Ajinomoto, Kawasaki, Japan).

### *4.3. RNA analysis*

Total RNA was extracted from five embryos, or at least 15 explants, and RT-PCR was performed as described (Hemmati-Brivanlou and Thomsen, 1995; Horb and Thomsen, 1997). PCR reactions were cycled 21 times to detect EF1a and 25 times for other markers. Sequences of the primers used for PCR were obtained from the *Xenopus* Molecular Maker Resource homepage (http//:vize222.zo.utexas.edu) except for the following ones provided here: chordin (accession no. L35764), upstream 5′-TTT CGC AAC AGG AGC ACA GAC-3′ (base 3439–3459), downstream; 5′-TAC CGC ACC CAC TCA = AAA TAC-3′ (base 3722–3702); Xcad3 (accession no. U02034) upstream, 5′-TGT GTT CTG TAC AGA GCG-3′ (base 692–709), downstream, 5′-TCA CAG AGC GAT GGT TGC-3′ (base 995–978). Northern blots were performed according to standard procedures (Sambrook et al., 1990) using nylon membranes (Amersham). Blots were hybridized overnight at 60°C in modified Church buffer (500 mM sodium phosphate (pH 7.2), 5% dextran, 7% SDS, and 100 mg/ml yeast tRNA). Probes were $^{32}$P-labeled with a random primed labeling kit (Boehringer Mannheim). Filters were washed twice in 0.1× SSC/0.1% SDS at 60°C for 30 min. RNAse protection assays were performed as previously described (Hemmati-Brivanlou and Thomsen, 1995) in which 7 mg of total RNA, equivalent to ten animal caps, was analyzed with the BMP and histone probes. The BMP-7 protection probe was constructed by subcloning the *Bg*III/*Hin*dIII fragment of *Xenopus* BMP-7 (Nishimatsu et al., 1992) into pGem3Z, which was linearized with *Xba*I and transcribed with SP6 to produce an RNAse protection probe. Ten milligrams of yeast tRNA with or without 5 pg of synthetic, sense RNA were used as positive and negative controls in the protections. BMP mRNA levels were quantified by measuring the level of radioactivity in the protected probe bands cut from the gel.

### *4.4. Site directed mutagenesis*

Site directed mutagenesis was performed with the Transformer Site-Directed Mutagenesis Kit (Clonetech, CA)

according to the manufacturer's instruction. *Xenopus* cDNA clones for XBMP-2#2, XBMP-4 and XBMP-7 (Nishimatsu et al., 1992) were subcloned into pUC19 for mutagenesis. Lysine codons were substituted for arginine codons at the BMP ligand cleavage sites because these alterations inhibit cleavage by serine proteases such as furin (Watanabe et al., 1993), which recognize the BMP cleavage site consensus. The sequences of the mutagenic primers were as follows; BMP-2 site 1, 5′-GGA CAT GCT CTT CAC AAA AAA AGT ACT AAG CAA GCT AGG CAC AAA C-3′; BMP-2 site 2, 5′-CAC A = AA TGT GCC CAA GAA GCA TGT GAA GAT CTC TAA GTC TTT AAC CCT GGA T-3′; BMP-4 site 1, 5′-CAT GCA CTG ACT AGG AAG TCA ACA AAG AGT CCA AAA CAG CAG AG-3′; BMP-4 site 2, 5′-CAG GGG AAG CAT GTA AAG ATC TCT AAA TCT TTA TTA CCT CAA AAG-3′; BMP-7 site 2, 5′-CAG ATA TCC ATC TCA AGA GTG TTA AGT CTA CTA GCA ATA AG-3′. Mutations were confirmed by DNA sequencing, and the altered cDNAs were subcloned into pCS2+ for mRNA synthesis in vitro as described above.

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